Toxicity testing: creating a revolution based on new technologies

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Biotechnology is evolving at a tremendous rate. Although drug discovery is now heavily focused on high throughput and miniaturized screening, the application of these advances to the toxicological assessment of chemicals and chemical products has been slow. Nevertheless, the impending surge in demands for the regulatory toxicity testing of chemicals provides the impetus for the incorporation of novel methodologies into hazard identification and risk assessment. Here, we review the current and likely future value of these new technologies in relation to toxicological evaluation and the protection of human health.

Aricle Outline

Introduction

Several different emerging technologies have created ample opportunities for a more modern approach to toxicology, to replace the traditional 'black box' animal-based paradigms of hazard identification and risk assessment by providing mechanistic details of events at the cellular and molecular levels. The current *in vivo* tests on laboratory animals are performed according to outdated guidelines, and interspecies extrapolation is unavoidable [1]. The risk assessment of the future will increasingly rely on integrated and intelligent testing strategies involving *in silico* and sophisticated *in vitro* procedures, a consideration of relevant

mechanistic information from molecular studies, and the use of biomarkers of susceptibility, exposure and effect of direct relevance to human health. Animal testing will either be supplanted entirely or will become a last resort.

Without such a shift from check-list *in vivo* testing to the use of alternative methods, the proposed application of the new EU Registration, Evaluation and Authorization of Chemicals (REACH; see also the Glossary) policy to the assessment of tens of thousands of chemicals will be unworkable [2]. However, the advantages offered by the new technologies – in terms of industrial efficiency and animal welfare, as well as human health – will only be gained if sufficient effort is invested in the basic science of toxicology, if applied industrial toxicology is sufficiently adaptable to change, and if regulatory authorities will accept new risk assessment procedures.

The available and emerging biotechnologies

In vitro systems

In vitro systems range from relatively simple subcellular fractions, tissue slices or perfused organ preparations, through primary cultures and cell lines – grown either as monolayer cultures or suspension cultures and as mono-cultures or co-cultures – to 3D organotypic cultures, which include reconstructed tissue models (Table 1).

Table 1.

The advantages and disadvantages of the various types of tissue culture systems used in *in vitro* toxicology

System	Advantages	Disadvantages
Primary cultures	Obtainable from various target tissues; can retain *in vivo* tissue-specific characteristics	Short *in vitro* lifespan; progressively lose *in vivo* properties; prone to contamination
Monolayers and mono-cultures	Can be grown to confluency and subcultured; can be used as barrier models; used to quantify cell proliferation/growth; suitable for genetic manipulation	Limited interactions between cells; absence of other cell types, nervous, immune and endocrine systems
Co-cultures	Involve more than one cell type, so resemble *in vivo* situation more closely (e.g. blood–brain barrier)	Some cell combinations are incompatible with each other in culture; complicated/conflicting cell culture requirements
Continuous cell lines	Readily available and reproducible source of cells; avoids repeated cell isolation from animals or humans	Tend to lose *in vivo* differentiation and take on new properties induced by culture conditions; enter senescence and decline after a certain number of population doublings
Genetically engineered cell lines	Generated by transforming cells with foreign DNA; DNA can confer cell line stability; DNA might encode structural or functional proteins; used to create polymorphic cell line batteries	Techniques are specialized; methods do not always lead to permanent changes; limited potential for altering cellular features
Immortalized	Generated from human/animal cells by introducing	The immortalization techniques are

System	Advantages	Disadvantages
cell lines	oncogenes/telomere-controlling DNA; cells have cell line longevity but can retain tissue-type specific features	specialized; there is not always permanent immortalization
Stem cells	Cells are able retain their stem cell capacity and to differentiate into many cell types	Limitations on cell types that can be generated; some animal species/strain limitations; ethical problems when using human embryonic stem cells
Tissue slices	Represents complexity of the organ; cellular contacts retained; useful for inter-species comparisons; many organs from same donor can be obtained; histological and biochemical tests possible; slices from different organs can be co-cultured; regional effects in same organ are particularly useful for metabolism studies	Difficult to produce reproducibly; exposure and activity of cells in slices can vary; limited *in vitro* lifespan
Organotypic cultures	Multilayered and spatially differentiated; exhibit cellular communication; good retention of *in vivo* physiology; can be generated from primary/immortalized cells; proprietary models available	Correct culture conditions can be difficult to define; batch variation of propriety models; limited *in vitro* lifespan
Perfused cultures	Applicable to a variety of the systems above; perfusion restores media and removes metabolites; allows cells to grow for extended periods; high cell densities possible; long-term repeat exposure/recovery possible; can be used for whole organs (e.g. kidney)	Technically complex; high risk of contamination; only a small number of samples can be set up; limited *in vitro* lifespan
Reconstructed tissue cultures	Components can be controlled and varied according to purpose	Technically complex
Whole organs	Organ functions modelled closely; different cell types with cellular interactions; particularly useful for embryotoxicity studies	Can be difficult to culture; limited culture life; must be freshly isolated; tend to require complex perfusion systems

Interactions between multiple cell types can be assessed readily with organotypic preparations. These systems range from whole perfused organs via tissue slices [3], tissue isolates and organ fragment cultures to reconstituted tissue equivalents. However, many organotypic preparations can have limited *in vitro* longevities, which limits their use to short-term studies. By contrast, a wide variety of cell types – ranging from stem cells via undifferentiated fibroblast-like or epithelial-like cells to highly differentiated tissue-specific cells – can be isolated from many tissues and species, cultured over extended periods of time and/or cryopreserved for future use [4]. The use of human tissues and cells has the obvious advantage that the need for interspecies extrapolation can be avoided [5]. However, this approach involves complex ethical, legal, logistical and safety issues, and it is difficult to acquire freshly-isolated samples of important tissues, such as liver and kidney [6].

Cell-based assays can provide essential information about the potential effects of chemicals on specific cell properties, and provide a more relevant and more manageable basis for

molecular and mechanistic studies than can the conventional laboratory animal models. However, when using simplistic cell-based systems to assess toxicity, it is important to recognize that cells are finely balanced homeostatic machines that respond to external stimuli through complex pathways. Therefore, because toxicity could be the result of a multitude of cellular events, including changes in cell morphology, differentiation, proliferation, function, excitability and/or communication, such systems might require refinement before they are adequate for risk assessment purposes. For example, cell culture systems often lack essential systemic contributors to overall absorption, distribution, metabolism and excretion (ADME), and the complex interactions and effects of the immune, endocrine and nervous systems. One way to circumvent problems with metabolic competence is by adding subcellular or cellular metabolizing systems and assessing the production of known metabolites [7]. This is particularly important when considering the elimination of lipophilic compounds, which is crucially reliant on phase I metabolism that can generate toxic intermediates from innocuous parent chemicals. The most important enzymes involved in such metabolism are the cytochrome P450-mixed function monooxygenases (CYPs), which have wide substrate specificity, and vary in nature and activity according to tissue and species. Metabolic cellular systems can be divided into three main categories: (i) metabolically-competent indicator cells (e.g. hepatocytes); (ii) co-culture systems comprising non-competent indicator cells (e.g. fibroblasts) and metabolically-competent cells (e.g. hepatocytes); and (iii) genetically-engineered cell lines that can simultaneously act as both indicators of selected metabolic pathways and of toxicity.

Co-culture systems are playing an increasingly important role in toxicity testing. This is largely because they enable important intercellular networks to be recreated *in vitro* [8]. For example, in models of the blood–brain barrier, brain microcapillary endothelial cells can be cultured with astrocytes, glial cells or neurones 9 and 10. Similarly, Sertoli cells can be cultured with spermatocytes, Leydig cells or peritubular cells for reproductive toxicity testing [11]. The development of more-complex *in vitro* models has been greatly facilitated in recent years by tissue engineering [12]. Indeed, it is now possible to grow stratified layers of epidermal cells, with each layer exhibiting morphological and functional differentiation. This has given rise to several commercially-available organotypic and reconstructed *in vitro* culture models, including EPISKIN™ (http://www.loreal.com) and EpiDerm™, and its fibroblast-supported version, Full Thickness EpiDerm™ (http://www.mattek.com) [13]. To date, test protocols for skin corrosivity, based on EPISKIN™ and EpiDerm™, have been validated scientifically and accepted as EU and Organization for Economic Cooperation and Development (OECD) test guidelines. Organotypic models of the eye have also been developed for predicting ocular irritation [14] and 'whole-brain' spheroid culture systems have been used in neurotoxicology [15]. Multilayered models of the tracheobronchial tract are also available, and enable squamous metaplasia [16], mucin production and mucociliary clearance to be analyzed for predicting respiratory toxicity [17]. Of particular relevance to the development of many cell-based organotypic models is the use of micro-porous substrates, which have led to physiologically more-relevant culture conditions for studies on transcellular transport and on cell/tissue interactions.

Cell cultures that can be maintained for extended periods of time *in vitro* obviate the need to isolate and maintain fresh tissue samples for each experiment. However, such serially or continuously cultured cells tend to lose the tissue-specific morphology and functions that could, in turn, affect the expression of key transporters, receptors and metabolic enzymes. Methods, including those based on the transfer of oncogenic sequences into cell lines and/or increased telomerase activity, can prevent senescence and the loss of differentiation status

(cell immortalization), such that long-term studies are becoming possible 18 and 19. Moreover, it is possible to: (i) create genetically engineered cell lines that stably express specific proteins and display the desired characteristics; and (ii) guide differentiation of stem cells into specific cell types (see http://www.stemcellresearch.org).

The 'omics' technologies

The advent of the 'omics' technologies has added further impetus to the development of alternatives to *in vivo* toxicity testing. Genomic (or transcriptomic), proteomic and metabonomic (or more correctly, perhaps, metabolism profiling) approaches 20 and 21 are based on the premise that physiological, pharmacological and toxicological events ultimately change the protein compositions and activities of cells and, hence, their structural and functional characteristics. These technologies promise to transform traditional toxicology by providing a means of deciphering mechanisms of toxicity, and by providing biomarkers for individual variations in susceptibility to toxicants and for use as early indicators of toxic exposure and effect. This will be achieved, following exposure to a test compound, by comparing gene expression profiles at either the transcriptional (genomic) level or the translational (proteomic) level with expression profiles specific to exposure to known toxicants.

Differential transcription can be measured by microanalysis, in which extracted RNA is subjected to reverse transcription to obtain labelled cDNA or to RNA polymerase amplification to generate labelled cRNA. These species are hybridized to microarrayed oligonucleotides, then scanned under laser light to record between 4000 and 50 000 measurements of gene expression. The design of highly specific panels of oligonucleotides, although reliant on as yet limited transcriptomic information, permits dose-dependent and tissue-dependent temporal and spatial patterns to be monitored. Online Mendelian Inheritance in Man™ (OMIM; http://www.ncbi.nlm.nih.gov), Biocarta™ (http://www.biocarta.com) and SuperArray™ (http://www.superarray.com) are examples of bioinformatics databases that provide genetic and biochemical information on specific metabolic pathways.

Proteomics is the analysis of functionally, structurally or anatomically related proteins (protein clusters) to determine physiologically or pathologically significant patterns of protein expression. It is largely based on the 2D electrophoretic resolution of proteins according to size and charge, followed by proteolytic cleavage, peptide mass determination and peptide identification. Currently, proteomics is less suitable for high throughput microarray analysis but its usefulness is being augmented by several other methods, including on-line detection by mass spectrometry [22]. Again, data outputs from proteomics are meaningless without proper calibration, which can be achieved with the assistance of analytical resources, such as Proteomic Investigation Strategy for Mammals (PRISM [23]), which permits large-scale expression profiling, and employs algorithms such as STATQUEST to facilitate the identification and categorization of proteins into groups by gene ontology.

Proteomics suffers from two important limitations: (i) the process of sub-fractionation of tissue or cell samples is complicated and prone to contamination problems, and (ii) protein expression patterns vary greatly between samples in response to circadian cycles, age, sex and disease. The second problem can be addressed only by analyzing proteins from *in vitro* batteries that model individual variations in response, by sampling multiple time-points, and by using isotopic labelling protocols, which permit comparative analysis by sample overlay.

Similar data processing problems currently limit the utility of metabolic profiling. However, as high throughput handling and bioinformatics systems improve, we can expect to see the emergence of a wealth of information relevant to risk assessment.

Current and future applications in toxicity testing

The overall strategy

The intelligent, selective, integrated and strategic application of the emerging technologies will revolutionize the gathering of information on which the toxic potentials of chemicals and products are evaluated, and on which human risk assessments are based, because they will provide for a mechanistic, case-by-case approach.

One such strategy, which is appropriate for application in relation to the EU REACH policy, has been put forward by FRAME ([2], adapted for this paper in Figure 1). However, it must be recognized that further technological developments are necessary, so that problems with the currently available systems can be overcome. It will also be necessary for specific tests and testing strategies to be validated (i.e. independently evaluated for relevance and reliability for particular purposes).


(98K)

Figure 1. An integrated toxicity testing strategy involving maximum use of advanced *in vitro* methods (adapted from Ref [2]). For the risk assessment of chemicals to be workable under the REACH chemical policy, the check-list approach must be replaced be a case-by-case risk assessment strategy, which takes into account all the available information for a chemical and predictions of the hazard it poses to humans and the environment. Hazard data from initial screens can then be used to refine additional tests, and to make risk assessment scientifically sound, cost effective and considerate of animal welfare. Abbreviation: D, decision to discard chemical.

The use of existing experimental and physicochemical data

Our strategy begins with the collection and consideration of all the available data about a chemical, including details of its physicochemical properties and any available knowledge about its toxicity. This can often be combined with the careful application of a read-across approach, whereby inferences are made about the likely toxicity of a group of structurally-related chemicals, for some of which toxicity profiles and (Q)SAR data are available. Sometimes, it is also possible to establish a maximum level of regulatory concern, whereby historical data are used to define a safe exposure threshold. Where the maximum likely exposure is predicted to be well below the threshold, a reverse risk assessment can be conducted, from which it might be concluded that no further testing is necessary.

The use of (Q)SAR and expert systems

It is often possible to use quantitative structure–activity relationships [(Q)SARs] and expert systems to interpret the existing information on a chemical. (Q)SAR modelling is based on a mathematical analysis of physicochemical information about a molecule and its structure, from which equations are established concerning the mechanism of action of the chemical and, therefore, of structurally related compounds [24]. The four main physicochemical parameters used are: (i) intrinsic reactivity; (ii) overall 3D shape; (iii) molecular volume; and (iv) lipid solubility (the ability to traverse membranes and partition into biological systems).

(Q)SARs have already been developed for several purposes (e.g. for predicting skin sensitization or mutagenic potential), but the application of this approach is frequently limited by a lack of reliable experimental data. Furthermore, (Q)SAR analysis is generally more applicable to chemicals that are closely related structurally, but a large number of structurally diverse chemicals need to be screened for their potential toxicity to many targets. This problem is less significant in drug discovery, where the purpose is to select the best lead compounds from a congeneric series in relation to specific and known effects.

Expert systems (Table 2) are used to make predictions on the basis of prior information, and to mimic the ways in which groups of human experts solve problems [25]. They are intended to help users make decisions, and are either automated rule induction systems, aimed at discovering patterns within data, or knowledge-based systems, which use existing relevant information relating physico-chemical properties to specific toxicity endpoints, in the form of specific rules.

Table 2.

Computer-based expert systems currently available for toxicity predictions[a]

Name	Supplier	Website	Examples of endpoints predicted
CASE/MCASE/ CASETOX	MultiCASE Inc.	http://www.multicase.com	Carcinogenicity; terratogenicity; mutagenicity; acute toxicity; chronic toxicity
DEREK for Windows	LHASA Ltd	http://www.chem.leeds.ac.uk/luk	Teratogenicity; mutagenicity; respiratory sensitization; carcinogenicity; skin irritation; skin sensitisation
HazardExpert	CompuDrug Ltd	http://www.compudrug.com	Oncogenicity; mutagenicity; teratogenicity; immunotoxicity; neurotoxicity; membrane irritancy/sensitivity; bioavailability; bioaccumulation
TOPKAT	Accelrys Inc.	http://www.accelrys.com	Carcinogenicity; mutagenicity; developmental toxicity; skin sensitization; eye irritancy; biodegradability; acute toxicity; chronic toxicity
OncoLogic®	US EPA	http://www.epa.gov/oppt/cahp/actlocal/can.html	Carcinogenicity
COMPACT	University of Surrey	http://www.surrey.ac.uk/SBMS	Carcinogenicity via CYP1A and CYP2E-related metabolic activation

[a] Expert systems are computer programs that guide hazard assessment and that are able to predict the most relevant forms of toxicity (endpoints) based on the information available. The main expert systems currently used are listed, together with the endpoints they are able to predict.

Deduction of Risk from Existing Knowledge (DEREK) is an example of a knowledge-based system, which focuses on molecular substructures (or 'alerts') associated with known toxicological endpoints. The prediction of skin sensitization is one endpoint that has been refined extensively 26 and 27. A two-step process is used to predict the sensitizing potential of a test chemical, because this effect depends on the reactivity of the chemical (or its metabolites) with skin proteins and its ability to penetrate the skin to reach its site of action.

Computer Automated Structure Evaluation (CASE) is an example of an automated rule induction system [28]. A molecule of interest is divided into chemical fragments of 2–10 heavy atoms, and a statistical distribution is performed to determine which fragments are biophores or biophobes (i.e. whether they are associated with specific activity or no activity, respectively). Predictions of carcinogenicity and mutagenicity are endpoints that have been extensively developed within CASE, and there are now many database modules available for these endpoints, including Ames mutagenicity and male/female rat/mouse carcinogenicity [29].

The further development and validation of *in silico* systems are necessary, if the undoubted value of this approach is to be fully exploited. It must also be recognized that the prediction of toxicity from chemical structure requires a multi-disciplinary approach covering detailed knowledge of chemistry, toxicology and statistics. It is not sufficient just to collect data and then attempt to analyze them with a battery of computational and statistical methods.

Biokinetic modelling

A fundamental problem when using hazard data for risk assessment is the need to relate the effects detected at the dose level applied to the test system (the external dose) with the effects that would be caused by the dose that actually reaches the target in humans (the internal dose). The internal target organ dose can be predicted by undertaking toxicokinetic studies, which must take ADME into account. Physiologically based pharmacokinetic (PBPK) modelling is an approach for predicting ADME *in vivo* by combining results from the literature and computational techniques [30], and by extrapolating data from *in vitro* studies between species (Table 3). Differential equations can be derived, which, when solved, give the information of relevance to humans. A better, biologically based, dose–response model of *in vivo* toxicity can then be developed from external dose data.

Table 3.

Programs for the prediction of biokinetic properties (i.e. of how a chemical is absorbed, distributed, metabolized and excreted)[a]

Name	Supplier	Website	Properties predicted
Cloe PK®	Cyprotex	http://www.cyprotex.com	Potential exposure; absorption from GI tract; plasma, tissue and organ concentrations; renal excretion; hepatic metabolism

Name	Supplier	Website	Properties predicted
iDEA pkEXPRESS™	LION bioscience	http://www.lionbioscience.com	Absorption from GI tract; systemic circulation; bioavailability; plasma concentration; elimination
Megen100	Health and Safety Laboratory	http://www.hsl.gov.uk/capabilities/pbpk.htm	Oral and i.v. absorption; concentration/time profiles for: plasma, major organs and tissues; hepatic metabolism
PK-Sim®	Bayer Technology Services	http://www.bayertechnology.com	Oral absorption; concentration/time profiles for: plasma and major organs; bioavailability; renal and biliary excretion

[a] One of the main problems of using hazard data for risk assessment is that the amount of chemical applied to a system might bear little relation to the effective dose (i.e. the dose that actual reaches its biological target and is able to cause a toxic effect). By using biokinetic modeling, together with data from *in vitro* studies, these parameters can be predicted and extrapolated to *in vivo* situations.

Significant advances are currently being made in biokinetic modelling, including the development of software programs and databases for the rapid generation of new models (http://www.hsl.gov.uk/capabilities/pbpk-jip.htm). These will improve the usefulness of the approach for evaluating large numbers of chemicals, and will assist with the interpretation of *in vitro* hazard predictions for risk assessment purposes.

The use of *in vitro* systems

It is sometimes possible for companies to make in-house decisions by evaluating information derived from computer-based models alone, but it will often be necessary to confirm or supplement such predictions experimentally by using *in vitro* and/or *in vivo* tests. Many *in vitro* systems are already used in-house, and some of these have been validated independently and accepted by regulatory authorities (Table 4 and Table 5).

Table 4.

In vitro and refined *in vivo* methods that have been validated and/or accepted for regulatory use[a]

Test/method for	System
Chemicals and products, e.g. pharmaceuticals, cosmetics, agrochemicals	
Acute neutropenia	Granulocyte/macrophage colony-forming unit test
Embryotoxicity	Embryonic stem cell test; whole-embryo culture test; micromass test
Genotoxicity	*Salmonella* mutagenicity assay; cytogenetic damage in cultured mammalian cells
Percutaneous absorption	isolated skin
Phototoxicity	3T3 NRU phototoxicity test
Skin corrosivity	Reconstituted skin equivalents (EPISKIN™,[b] Epiderm™,[c]);

Test/method for	System
	CORROSITEX™,[d]; rat transcutaneous electrical resistance (TER)
Skin sensitisation	Local lymph node assay (*in vivo*)
Acute lethal potency	Fixed Dose Procedure[e] (*in vivo*); Up and Down Procedure[e] (*in vivo*); Acute Toxic Class Method[e] (*in vivo*)
Biologicals, e.g. hormones, vaccines, antibodies and surgical fluids	
Monoclonal antibody production	Hollow fibre methods
Pyrogenicity	*Limulus* amoebocyte lysate (LAL) test; whole human blood test
Recombinant follicle stimulating hormone (recFSH) potency	Isoelectric focusing and capillary zone analysis
Vaccine potency	ToBi test (tetanus); ELISA tests (tetanus and erysipelas)

[a] It is not always possible to predict the hazard posed by a chemical from existing knowledge or from computer-based models alone. The *in vitro* methods and refined animal test procedures listed have been accepted into official test guideline programmes or recognized by the regulatory authorities as useful adjuncts to animal-based tests.
[b] http://www.loreal.com/.
[c] http://www.mattek.com/.
[d] http://www.corrositex.com/.
[e] to replace the LD50 test.

Table 5.

Non-animal methods currently being validated or being considered for validation[a]

Test for	Test system
Cell transformation	Syrian hamster embryo cells, Balb/c 3T3 cells
Chronic toxicity	Perifusion systems
Cytotoxicity	NRU 3T3 cell assay for acute lethal potential
Dermal irritancy	EPISKIN™ human skin model; Epiderm™ human skin model; pig ear (trans-epidermal water loss) test; mouse skin integrity function test (SIFT)
Ecotoxicity	Fish cell test
Embryotoxicity	Embryonic stem cell test with extracellular metabolism
Endocrine disruption	(Q)SAR models for androgen (AR) and oestrogen (ER) receptor recognition
Genotoxicity	*In vitro* micronucleus test
GI absorption	Caco-2 cell models
Reproductive toxicity	Leydig cell models
Skin sensitization	Dendritic cell models; (Q)SAR models; expert systems

[a] Although several non-animal alternatives have been accepted for regulatory use, there are many methods that are used for non-regulatory purposes or that have been only accepted as alternatives for specific toxicological endpoints. The methods listed have yet to be validated, but might soon become available for regulatory use.

There continue to be rapid developments in the types and complexities of *in vitro* systems for research purposes. Careful consideration should be given to how these technological advantages can be harnessed as the basis for manageable and cost-effective tests. Nevertheless, some key points can be highlighted.

Metabolism Metabolism is one of the main factors that influence the toxicity of a chemical, its transport and partitioning within the body, and its rate and route of elimination. Predicting the susceptibility of a chemical to metabolism and identifying the principal metabolites likely to be generated and the target organs involved are essential elements of toxicological evaluation. The conditions of exposure and the species concerned are important considerations. In addition to tissue-specific differences, there are many interspecies variations and intraspecies polymorphisms that affect phase 1 metabolism, including CYP isozyme distribution. Particular isozymes are associated with the metabolism and activation of specific chemical groups. For example, CYP1A1 is active in metabolizing polycyclic aromatic hydrocarbons, CYP2B1 and CYP1A2 preferentially metabolize aromatic amines, and CYP2E1 metabolizes low molecular weight chemicals, whereas CYP3A4 metabolizes larger molecules. CYP2D6 is especially important in human drug metabolism [31]. By contrast, if a chemical is primarily subjected to phase II metabolism, this will often, but by no means always, lead to detoxification via a variety of conjugation reactions, such as glucuronidation or glutathione binding.

Therefore, determining the susceptibility of a chemical to metabolism and establishing the nature and relative quantities of the main metabolites derived from a parent molecule, are indispensable prerequisites to the overall chemical hazard assessment process, and to maximizing the return from any computer-based prediction systems. Useful information can be obtained from *in vitro* studies by using subcellular metabolizing fractions or cellular metabolizing systems, such as hepatocyte primary cultures or hepatocyte-derived and genetically engineered cell lines (Table 6), as well as *in silico* prediction methods [32]. A better approach is to use established cell lines that have been genetically engineered to express various phase I and phase II enzymes, either singly or in combination. For example, the V79 Cell Battery™, comprises cells of a Chinese hamster lung cell line that have a stable diploid karyotype and no CYP background activity and which have been transfected with a diversity of rodent and human CYP activities [33]. This permits the contributions of specific isozymes to metabolism to be investigated, and species-specific differences to be detected. It is currently possible for one laboratory to screen around 200 chemicals per month (Johannes Doehmer, personal communication; http://www.genpharmtox.com).

Table 6.

Systems for incorporating metabolizing enzymes and for metabolism screening[a]

Type of system	System
'Test tube' analysis	Pure enzymes; enzyme inhibitors; co-factors for phase 1 and 2 (conjugating) enzymes

Type of system	System
Cell fractions and extracts	Post mitochondrial supernatant (S9); microsomes; cytosolic fractions; prostaglandin H synthase systems
Tissue slices	Liver, kidney
Cell cultures	Metabolically competent cells (e.g. hepatocytes); toxicity indicator cells; co-cultures of metabolizing and toxicity indicator cells; genetically engineered cell lines expressing single/multiple CYPs and phase 2 enzymes
Whole organisms	Gut microflora systems; host-mediated assays
Monitoring systems	Oxygen consumption and test substance concentration after incubation with CYP-active extracts; metabolite identification by mass spectrometry; testing of known or suspected metabolites

[a] Understanding how a chemical is metabolized within the body and what metabolites are produced is vital for the chemical hazard assessment process. *In vitro* studies involving purified components of metabolic pathways, cells and cellular fractions, as well as tissue and organotypic preparations (sometimes of human origin), can be used in conjunction with a variety of assays to identify key toxic metabolites, instead of using whole animals.

Screening systems for other endpoints have been developed that involve a variety of cell types and organisms, including the yeast two-hybrid system [34], and mammalian and human reporter cell lines. The reporter cell lines are engineered so that the expression of a protein is under the control of a transcriptional response element, the activity of which is dependent on upstream molecular recognition events. The activation of a specific cascade by a test chemical stimulates the expression of a fluorescent protein or the formation of a coloured or luminescent product. For example, a system in which apoaequorin expressed in mammalian cells reconstitutes to form active aequorin upon exposure to coelenterazine, exhibits calcium-dependent luminscence. This reporter system can be used to monitor toxin-induced changes in intracellular calcium [35].

Endocrine disruption Reporter gene systems are also available in mammalian cell transactivation systems used as screens for endocrine disruptors, with luciferase or chloramphenicol transferase as reporter genes. For example, cell lines co-transfected with a hormone response element–luciferase construct and α– and β-oestrogen receptor genes, were able to detect a dose-dependent induction of oestrogen receptor expression in response to picomolar concentrations of oestradiol [36]. However, there are many more oestrogen-responsive genes, and this has formed the basis of a microarray screen for biomarkers of endocrine disruption [37].

Human genetic polymorphisms The value of both standard animal and *in vitro* toxicity test systems is greatly limited by their inability to take account of human population differences in sensitivity to toxic insult, which itself results from human receptor and enzyme gene polymorphisms. As mentioned previously, one significant factor is the expression of different forms of phase I and II enzymes. There are at least 30 variants of human CYP2D6 – an isozyme involved in metabolizing almost one-third of the drugs currently on the market. By using genetically engineered cell lines expressing differing isoforms of the human CYP2D6, Krebsfaenger *et al.* [33] have constructed a panel of V79 cell lines that stably express variants

of the human CYP2D6 gene, based on their V79 Cell Battery, which can model individual differences in metabolism.

Modelling chronic repeat-dose exposure Until relatively recently, tissue culture systems had been used to model acute exposure only, partly because of the difficulty of maintaining cells in long-term culture. It is now possible to create cell-based systems into which *in vivo* rates of perfusion are assimilated; therefore, the bioaccumulation of metabolites, such as reactive oxygen species, or the deprivation of cofactors are avoided, and the metabolic kinetics more closely resemble the *in vivo* situation. Such perfusion systems might be based on single-cell cultures or co-cultures. For example, hepatocytes maintained as reconstructed collagen sandwich monolayers show extended viability and metabolic competence, so repeat-dose, long-term and reversible effects can be studied [38].

Long-term *in vitro* studies are needed, for example, to distinguish between mild and moderate skin irritants on the basis of rates of recovery. With two-compartment human skin models, confluent cells cultured on an inert filter can be washed after an initial exposure and analyzed for trans-membrane permeability by using a non-toxic indicator, before being rechallenged and reanalyzed at a later date.

Hollow fibre technology is being used increasingly to generate long-term cultures of high densities of cells with continuous replacement of the culture medium. This technology was originally developed for the commercial *in vitro* production of monoclonal antibodies, as a complete replacement for the ascites mouse procedure [39], but is now being adapted for use in evaluating the chronic, repeat-dose effects of chemicals 40 and 41.

Stem cells Stem cells from human umbilical cord blood (HUCB) are being used increasingly for a variety of purposes 42 and 43. For example, a neural-stem-cell-like subpopulation can be selected, which is devoid of haematopoietic and angiogenetic potential but which is capable of self-renewal *in vitro* for several months. With appropriate treatments, these cells can differentiate into populations that display neuronal or glial cell markers and respond to neurogenic signals. One HUCB-derived neuronal cell line has a stable karyotype, and can be cultivated in serum-free medium for about a year to form neurospheres with a self renewing inner-core mass of cells that express stem cell markers, and an outer layer of cells which display typical neural markers. The surface cells are well-suited to examining the toxic effects of xenobiotics on neuronal development.

The way ahead

Great opportunities are emerging for revolutionizing the current animal-dominated approach to regulatory toxicity testing. Advances in computer science and cellular and molecular biology promise to transform chemical hazard identification from a poorly-practised art into a modern science (Figure 1). It is particularly important that, while the new ‘omics’ approaches are vigorously pursued and bioinformatics programs are developed to handle the vast amounts of data that they will provide, genomics and proteomics are applied to cultured cells and tissues of human origin. Thus, mechanistically relevant information about the toxic potentials and potencies of chemicals can be obtained and applied, not only as a basis for risk assessment, but also to provide biomarkers of susceptibility, exposure and effect. If the necessary resources are provided, and if human ingenuity and intelligence are properly focused, the traditional check-list, laboratory animal approach to toxicity testing could soon be consigned to history.

References

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Glossary

ADME:
absorption, distribution, metabolism and excretion.

Biokinetics:
a useful generic term, which is increasingly employed to cover the ADME of both pharmaceutical compounds (pharmacokinetics) and toxic compounds (toxicokinetics).

Biomarkers:
Biological indicators of changes that occur in cells and tissues.

CASE:
Computer Automated Structure Evaluation.

Co-culture:
Two or more cell types cultured together.

CYP:
cytochrome P450-mixed function monooxygenase.

DEREK:

Deduction of Risk from Existing Knowledge.

Differentiated cells:

Cells that have become specialized to perform a specific role and that display distinct tissue-specific or organ-specific patterns of protein expression.

Gene expression:

A multi-step process that involves the transcription of DNA into messenger RNA (mRNA), the translation of mRNA into a protein sequence, and then the folding, post-translational modification and targeting of the resultant protein.

Genomic:

Pertaining to the total genetic make-up of a cell, tissue, organ or organism.

In silico:

Computer-based.

In vitro:

Literally means 'in glass', but used to indicate maintenance outside a living organism.

Intrinsic activity:

A measure of the biological effect produced per unit of a chemical.

Metabonomic:

Pertaining to the metabolism of a compound and the metabolites produced.

Microarray:

A 2D miniaturized system, which enables complex protein, DNA or RNA mixtures to be analyzed with regard to their interactions with specific probes immobilised on materials such as glass or silicon filters.

Mono-culture:

Culture of one cell type.

Mucin:

A protein with high carbohydrate content; the main component of extracellular mucus.

OECD:

Organization for Economic Co-operation and Development.

Oligonucleotides:

Short synthetic stretches of DNA.

OMIM:

Online Mendelian Inheritance in Man.

Oncogene:

Viral DNA or a gene present in normal cells, which can transform normal cells into cancerous cells.

Organotypic:

Resembling the complexity of an organ or tissue.

PBPK:

Physiologically-based pharmacokinetic.

Primary culture:

Cells that have been freshly isolated from tissues obtained by biopsy or autopsy, and maintained in artificial medium outside the body. When these cells divide and are subcultured, they become known as secondary cultures. Eventually, some cell types will die out, while others might survive to form a continuous cell line.

PRISM:

Proteomic Investigation Strategy for Mammals.

(Q)SAR:

Quantitative structure–activity relationship.

Proteomic:

Pertaining to the total protein content of a cell, tissue, organ or organism.

REACH:

Registration, Evaluation and Authorization of Chemicals.

Senescence:

A process by which most normal cells in continuous cell culture ultimately stop dividing and die out.

Spheroid culture:

Spherical, heterogeneous aggregates of proliferating and non-proliferating cells that might retain their differentiated functions.

Squamous metaplasia:

A flattening of cells indicative of the pre-cancerous state of cells.

Stem cells:

Cells found in embryos, in umbilical cord blood and in various adult tissues (e.g. bone-marrow, epidermis, intestinal epithelium), which have the potential to divide to produce more stem cells and cells that are committed to one or more specific differentiation pathways.

Telomerase:

An enzyme complex that maintains the ends of chromosomes.

Transactivation:

Where one gene product (or protein) causes a different gene to be activated and another gene product to be expressed.

Transcription:

The process by which DNA is copied by RNA polymerase to produce a complementary messenger RNA (mRNA) sequence.

Transcriptomic:

Pertaining to the transcriptome (i.e. the total amount of messenger RNA in a cell fraction, cell, tissue, organ or organism).

Translation:

The process that follows transcription and results in the production of the amino acid chain encoded by the original DNA sequence.

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