CHAPTER 13

THE USE OF INTEGRATED AND INTELLIGENT TESTING STRATEGIES IN THE PREDICTION OF TOXIC HAZARD AND IN RISK ASSESSMENT

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Abstract: There is increasing concern that insurmountable differences between humans and laboratory animals limit the relevance and reliability for hazard identification and risk assessment purposes of animal data produced by traditional toxicity test procedures. A way forward is offered by the emerging new technologies, which can be directly applied to human material or even to human beings themselves. This promises to revolutionise the evaluation of the safety of chemicals and chemical products of various kinds and, in particular, pharmaceuticals. The available and developing technologies are summarised and it is emphasised that they will need to be used selectively, in integrated and intelligent testing strategies, which, in addition to being scientifically sound, must be manageable and affordable. Examples are given of proposed testing strategies for general chemicals, cosmetic ingredients, candidate pharmaceuticals, inhaled substances, nanoparticles and neurotoxicity.

INTRODUCTION: RECOGNISING THE PROBLEMS

A variety of stakeholders, including industrialists, scientists, consumers, patients, workers and politicians are faced with the need to evaluate the benefits of chemicals and chemical products of many kinds, in the light of the hazards they may represent and the risks which may result from exposure to them. This has resulted in a vast and complex array of laws, regulations, guidelines and practices and many of the mandated test procedures in place require the use of laboratory animals. This is a cause of concern, not only because of increased public awareness of animal welfare issues, but also because insurmountable differences between humans and laboratory animals exist. These differences exacerbate

interspecies extrapolation, because animals and humans can differ substantially in their responses to the same chemical. In cases where the animal model is oversensitive, such problems can inhibit the authorisation of new chemicals and their incorporation into useful new products, or can limit the discovery, development and approval of new treatments against disease. On the other hand, where the animal model is too insensitive, this can lead to failure to detect adverse effects at the right time in product development and approval for marketing and use. Another serious problem is that the regulations and the ways in which tests they specify are required and in which the data they produce are applied, are now so very complicated that only those directly involved, as manufacturers or regulators, can hope to understand them. Members of the general public are not alone in failing to recognise what is meant by "safe".

The problems which currently cause concern involve many different kinds of products—we cannot consider all of them, so we will mainly confine our discussion to two of the most important of them, namely, chemicals and pharmaceuticals, while also taking into account the other chapters in this book. Our emphasis will be principally on the evaluation of toxic hazard and the prediction of risk to humans, which should be focused on the following: (a) the evidence that a given chemical or drug can cause adverse effects; (b) the frequency of incidence of those effects in a given population; (c) the degrees of severity of the effects; (d) variations in susceptibility to and in the expression of the effects within the population and between populations; and (e) the epigenetic factors which can modulate them.

CHEMICALS

Chemicals are regulated with regard to their manufacture, marketing and transport and their use in many thousands of different products, including cosmetics, household products, medical devices and pesticides. Their entry into the body is not usually intended, but can occur as a result of accidental, environmental or occupational exposure. The population exposed to a given chemical usually cannot readily be identified and levels of exposure must be predicted, as they are usually unknown and are relatively uncontrollable.

There are a vast number of regulatory requirements, which are laid down and applied at national (e.g., Japan, USA), regional (e.g., EU) and international (e.g., UN) levels. It is important to recognise the differences between requirements concerned with the timing of testing, what tests should be done, how they should be conducted and how the results obtained should be reported (e.g., in submissions to the appropriate authorities) and acted upon (e.g., via classification and labelling, restrictions on use, the issuing of hazard warnings and the setting of acceptable daily intake [ADI] levels).

Nowadays, the issue of chemicals regulation is dominated and complicated by the EU REACH (**R**egistration, **E**valuation, **A**uthorisation and Restriction of **CH**emical Substances) system, which came into force in June 2007 and is backed by EU legislation agreed in 2006 and managed by the European Chemicals Agency (ECHA) in Helsinki, Finland.[1] The ECHA operates in collaboration with the Competent Authorities in the Member States, e.g., the Health and Safety Executive in the UK.[2] In the USA, chemicals are regulated by the Environmental Protection Agency (EPA), according to the *Toxic Substances Control Act 1976*,[3] while in Japan, they are regulated by the Ministry of the Environment, according to the *Chemical Substances Control Law 1973*, as amended

in 2009.[4] Over the last few years, many other countries have been in the process of strengthening their own requirements.

The regulations are focused principally on the prediction of hazard and the methods used have traditionally been dominated by the use of laboratory animal models. Testing is conducted according to internationally-agreed guidelines, primarily the OECD Health Effects Test Guidelines (TGs).[5] Data produced according to these TGs (primarily for the manufacturers of chemicals) are then incorporated into the submissions made by the manufacturers and downstream users of chemicals to the national and international regulatory authorities responsible for the protection of human health and of the environment in general from the effects of exposure to hazardous chemicals. This should involve assessments of the likely routes, types and scales of exposure, but all too often, there is a routine, all-embracing check-list approach, as favoured by regulators, which can lead to unnecessary testing. The complicated processes involved in approving and updating the TGs are a matter of concern.[6]

Given the progressive globalisation of manufacturing and marketing, differences between regulations and how they are applied can lead to serious difficulties for companies and governments. A number of steps have been taken in attempts to resolve this problem. For example, the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) is an internationally-agreed system to replace the various different classification and labelling standards used in different countries.[7] This is most encouraging, but serious differences remain, e.g., between the EU REACH and US TCSA systems.[8]

The implications of the original REACH proposals caused great alarm to both chemical manufacturers and their downstream user customers. In addition, animal welfare organisations and scientists spoke out against the proposed check-list approach to hazard prediction based on tonnage, which would fail to take sufficient account of the nature of the chemicals themselves or of likely human exposure to them. There have been many political and administrative developments in relation to the REACH system proposals since they were first put forward in 2001 and there is now a vast and burgeoning literature on the subject, including thousands of pages of guidance from the ECHA.

One major problem with the REACH system is the requirement to identify Substances of Very High Concern (SVHC), i.e., CMR chemicals (those which are carcinogenic, mutagenic and/or reprotoxic), or are PBT chemicals (those which are persistent, bioaccumulative and toxic), or are identified as SVHCs on a case-by-case basis from scientific evidence.[9] Attempts to identify potential human carcinogens are very unsatisfactory, since they rely on the lifetime, two-species rodent bioassay, which is fundamentally flawed, since it cannot be trusted to accurately identify rodent carcinogens, let alone human carcinogens.[10] It is estimated that at least 60% of the animal testing required by the REACH system will be for reproductive toxicology and there is pressure from some traditional toxicologists and regulators to have this testing based on the two-generation test in rats, sometimes with additional testing in a second species, such as the rabbit. However, whether such an approach can be justified on scientific grounds is highly questionable, as it is unlikely that it would be successful in identifying the very small number of human teratogens likely to be found among the 10,000 or so "existing" chemicals which are of potential CMR concern.[11] The development of human-oriented strategies for identifying reproductive toxins and chemical carcinogens, involving in vitro and in silico procedures, should therefore be a matter of the highest priority.

These issues were discussed in detail by Hartung, who said that the difficulties introduced by the REACH system were so very great that they could lead to the more-active

search for non-animal tests and testing strategies and the application of evidence-based toxicology approaches.[12] We have long been calling for a revolution in toxicity testing based on the intelligent use of new technologies.[13] That is also the theme of the 2007 report of the US National Academy of Sciences on behalf of the EPA, entitled *Toxicity Testing in the 21st Century: A Vision and a Strategy*, which spells out a much more intelligent approach than that currently being followed in Europe. It emphasises the need to benefit from experience in the pharmaceutical industry and to benefit from the emerging fields on systems biology and bioinformatics.[14]

PHARMACEUTICALS

Unlike most chemicals and chemical products, medicines are designed to be deliberately taken into the body, there to exert powerful effects on the body's cells, organs and systems, in order to assist in the diagnosis, treatment or prevention of disease. Given the complexity of the body and its control systems, it is not surprising that they can also induce adverse and serious side-effects. The principal aim in drug discovery and development is to identify compounds which will evoke the maximum desired therapeutic response according to dosing regimens which induce only minimal and manageable adverse effects.

In the EU, medicines can be licensed in two ways—via the national control agency (the Medicines and Healthcare products Regulatory Agency [MHRA] in the UK[15]) or via the EU authority, the European Medicines Agency (EMA), based in London, UK.[16] Licensing via the EU is also accepted in some non-EU European countries. The equivalent authority in the USA is the Food and Drug Administration (FDA),[17] and, in Japan, it is the Ministry of Health, Labour and Welfare (MHLW).[18]

On the whole, whereas definitive guidelines are laid down by the regulatory authorities for chemicals and many other chemical products, pharmaceutical companies tend to be able to discuss a promising new compound with the appropriate medicines control agency or agencies, before all the testing has been completed. In addition, since its inception in 1990, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has been bringing together the regulatory authorities and pharmaceutical industries of Europe, Japan and the USA, to discuss scientific and technical aspects of drug registration.[19] The ICH is concerned with the design, conduct, safety and reporting of clinical trials and has produced a comprehensive set of safety guidelines to uncover potential risks such as carcinogenicity, genotoxicity and reprotoxicity.[20] The process does not always work smoothly, as, for example, there appear to be regional differences in the value of using nonclinical data in assessing the risk of delayed ventricular repolarisation (QT interval prolongation), which has been one of the most important causes of drug withdrawal in recent years.[21]

The pharmaceutical industry is currently in a state of crisis, because of the increased costs of drug discovery and development and the fact that, despite the increased numbers of candidate compounds in the development pipeline, the rate of entry to the market has steadily decreased, while the rate of postmarketing withdrawal, because of lack of efficacy or unpredicted adverse effects, has increased.

A variety of causes have contributed to this unsatisfactory situation. The new pharmacological targets to be tackled are more difficult than those tackled in the past and

there is insufficient understanding, not only of diseases themselves or of the mechanisms of the pharmacological and toxicological responses and effects involved, but also of the influence of human polymorphisms and the effects of a variety of contributory epigenetic factors. There has been a growing recognition that differences among patients can affect not only the efficacy or safety of a drug, but even the results of clinical trials (since a sizeable cohort of nonresponders or idiosyncratic responders can throw doubt on the efficacy of a drug which is effective for the majority of the individuals in the trial).

A further complicating factor is that the currently-available nonclinical tests and testing strategies, which are heavily dependent on laboratory animal tests, have failed to correctly predict adverse clinical effects, including the main causes of late drug withdrawal, namely, damage to the cardiovascular system, the liver and the respiratory system.[22] This is partly because the animal models used in various testing strategies are not sufficiently closely-related to what is being modelled and therefore cannot be expected to provide a sufficiently relevant or reliable basis for making important decisions.

As a result of this, the need to fundamentally reappraise the value of animal studies as an essential and required background to human studies is increasingly being emphasised. The US Food and Drug Administration (FDA) put it like this in 2004, in *Challenge and Opportunity on the Critical Path to New Medicinal Products*:

> *Despite some efforts to develop better methods, most of the tools used for toxicology and human safety testing are decades old. Although traditional animal toxicology has a good track record for ensuring the safety of clinical trial volunteers, it is laborious, time-consuming, requires large quantities of product and may fail to predict the specific safety problem that ultimately halts development. Clinical testing, even if extensive, often fails to detect important safety problems, either because they are uncommon or because the tested population was not representative of the eventual recipients. Conversely, some models create worrisome signals that may, in fact, not be predictive of a human safety problem.[23]*

This remarkable FDA initiative presents a great challenge and an enormous opportunity, which should be welcomed and responded to by all concerned, in the interests of good science and human benefit, as well as animal welfare. Those who have a tendency to want to protect the status quo at all costs, should note that phrases such as "animal toxicology … may fail to predict the specific safety problem that ultimately halts [drug] development" and, elsewhere in the document, "currently available animal models … have limited predictive value in many disease states" have also been used by the FDA and not by animal rights protagonists alone.

Meanwhile, in Europe, the Innovative Medicines Initiative (IMI) has been established, as a joint undertaking between the European Union and the European Federation of Pharmaceutical Industries and Associations (EFPIA), with the aim of improving the drug development process by supporting more-efficient drug discovery leading to the development of better and safer medicines for patients.[24] The IMI supports collaborative research projects and builds networks of industrial and academic experts in Europe. The focus is on the development and use of in silico and in vitro methods and omics and imaging approaches, so that the new technologies can be used for the benefit of patients, as a result of dynamic interactions between what takes place in the laboratory and in the clinic.

OTHER PRODUCTS

The following other kinds of products are important in different ways, as they raise issues which are different from those encountered with chemicals and pharmaceuticals.

Cosmetics

In the EU, the term **cosmetic** covers substances or preparations intended to be placed in external contact with parts of the human body or with the teeth or mucous membranes of the oral cavity, to clean or perfume them, change their appearance, correct body odours and/or protect them and keep them in good condition. It excludes products with pharmacological effects, but new types of preparation are now appearing, which could be called cosmeceuticals, which, while principally intended to be used for cosmetic purposes, do have biological effects on body tissues.

The crucial question is whether the uptake of a cosmetic ingredient could lead to a level in the body which approached the thresholds of toxicological concern (TTCs) for various possible toxic effects and the concept of a margin of uptake (MoU), i.e., the difference between likely uptake into the body and a level which could be a cause for concern (i.e., above a TTC), is worth serious consideration.[25] The goal is to ban the animal testing of cosmetic ingredients to be manufactured or marketed in the EU, but its achievement has been repeatedly delayed by political and scientific considerations.[26,27]

Medical Devices

The term **medical device** covers a very wide range of products, including implants, which, like cosmetics, are intended to be brought into contact with the human body, but are not designed to affect the body's cells, tissues and systems in ways comparable with those for which medicines are designed and used. They tend to be biologically inert, but their biocompatibility needs to be evaluated. In a sense, the question is not what the medical device will do to the body's cells and tissues, but what the body's cells and tissues will do to a medical device. This is exemplified by dental implants, which are usually made of pure titanium and are screwed into the jaw bone, whereupon osteocytes migrate to and adhere to or enter the surface of the implant, before laying down new bone by a process known as the osseointegration of the implant. This is another area where more should be done to obviate the need for animal tests.[28]

Pesticides

Pesticides are substances or mixtures of substances intended to repel, control or destroy living organisms regarded as pests. They can be classified in various ways, but most usefully, according to target organism. The regulations require that pesticides and their individual ingredients must be tested for safety, but, despite extensive testing in animals, many pesticides have been withdrawn because they caused acute or delayed health effects and/or accumulated in the environment and found their way into water or food. The best-known example of this is DDT, an organochlorine insecticide, which was widely used as an agricultural pesticide from the 1940s and in the fight against malaria in the 1950s. Although it helped millions of people to avoid malaria, concern about its range of toxic effects in humans gradually increased and it was progressively banned

from use, first in Hungary in 1968, then in Germany and the USA in 1972 and in the UK in 1984. It continues to be used in some countries, e.g., India and North Korea. Serious problems have also arisen as a result of accidents, such as that in Bhopal in 1984. Pesticides represent a class of useful chemicals which must always be handled with great care and their application must be kept under continuous review.

Biological Products

The US Public Health Service (PHS) Act defines a biological product as a "virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product, applicable to the prevention, treatment, or cure of a disease or condition of human beings." This includes somatic cells and tissues and gene therapy products, including recombinant therapeutic proteins. They can be isolated from natural sources, but increasingly are produced by biotechnology procedures.

The efficacy and safety testing of biologicals involves procedures and regulations which are different from those which apply to chemical-based pharmaceuticals. Nevertheless, the issues raised in the remarkable FDA initiative referred to above,[23] present no less a challenge and an opportunity in relation to biologicals. These issues were brought sharply into focus by the very serious situation which arose in March 2006, when eight healthy male volunteers took part in a Phase I trial on an anti-inflammatory monoclonal antibody, TGN1412.[29] Six of the volunteers rapidly developed multi-organ failure, one of whom will suffer long-term disability. Preclinical studies had been conducted with a number of animal models, including monkeys, but no effects were seen which discouraged the manufacturer from proceeding to seek permission to conduct the Phase I trial, or the regulatory authority from granting its permission. The problem appears to have stemmed from the fact that the antibody was fully humanised, i.e., it was an antibody with human-specific properties, derived by protein engineering from an antibody produced in a nonhuman species. It could be said that the assumption that animal models could be used to establish the safety of such a product is a supreme example of the danger of the high fidelity fallacy. It seems likely that this tragic event will lead to fundamental changes in the way that preclinical and clinical studies on new medicines will be conducted in the future, especially as more and more "humanised" biological products are likely to be developed.[30]

Nanomaterials

Nanotechnology involves the creation and use of materials with a length or diameter of 1 nm, which are most-commonly carbon-based or metal-based. Nanomaterials have a variety of potential applications in industry and in medicine as tools in diagnosis, monitoring or drug delivery. The extent to which they may pose risks to human health is not yet known, but it is unlikely that animal tests designed for the application of high doses of macroparticles, will have any meaningful role to play.[31] The focus is therefore likely to be on in vitro test systems, as discussed in this book by Schrand et al,[32] by using procedures which will themselves take advantage of other technological developments, including ultrahigh resolution light microscopy and various physical and biochemical techniques, to characterise the nanomaterials themselves and investigate their effects on cells. These are early days, but it can be expected that the knowledge about the mechanisms of nanomaterial toxicity which is gained, will also offer insights into the mechanisms involved in the toxicity of larger particles.

THE PROMISE OF THE NEW TECHNOLOGIES

The FDA's *Critical Path* document goes on to say that:

> *There are currently significant needs, but also significant opportunities, for developing tools that can more reliably and more efficiently determine the safety of a new medical product. ... Proteomic and toxicogenomic approaches may ultimately provide sensitive and predictive safety assessment techniques; however, their application to safety assessment is in early stages and needs to be expanded. Targeted research aimed at specific toxicity problems should be undertaken. ... As biomedical knowledge increases and bioinformatics capability likewise grows, there is hope that greater predictive power may be obtained from in silico (computer modelling) analyses such as predictive toxicology. Some believe that extensive use of in silico technologies could reduce the overall cost of drug development by as much as 50%. ... FDA's files constitute the world's largest repository of in vitro and animal results that are linked with actual human outcomes data. Further data mining efforts that effectively protect proprietary data could form the basis for useful predictive safety models.[23]*

What are these developing tools and how could they best be used to provide better and safer chemicals and products of many different kinds? Happily, many of them are based on human material or human experience, so the scientific limitations of nonhuman models because of species differences and the ethical questions they raise, can be avoided.

The use of computers will be essential in virtually every aspect of the further development and use of the new technologies, as a means of collecting, storing, organising and analysing data and detecting associations and correlations which deserve further attention. Therefore, while recognising the interdependence of most, if not all, the new technologies, we will use in silico as a term to distinguish certain computer-based approaches from approaches described by older terms, e.g., in vivo, ex vivo and in vivo. In the same way, while informatics applies broadly to the application of all the other technologies, it has more-precise uses in bioinformatics and chemoinformatics.

Descriptions of the available and developing technologies which can contribute to the replacement of animal procedures, could be based on various classifications and subdivisions (Table 1). There is a vast and rapidly-expanding literature on this subject and all we can do here is to give a few examples, including some recent and comprehensive reviews.[33-35]

1. **The use of existing knowledge.** The consideration of any novel compound or preparation should always begin with a search of the literature and the consultation of in-house data banks and other, more-widely available, resources.
2. **In chemico analysis and chemical interactions.** The physicochemical properties of molecules are significant and measurable, including stability under various conditions, volatility and acidity/alkalinity. For example, compounds with a low or high pH can cause severe and direct damage to the eye or the skin because of this, irrespective of the properties of the eye or skin themselves, so it is not necessary to test them with living material, in vivo or in vitro. The covalent binding of xenobiotic molecules, including drugs, to biological macromolecules, such as proteins, can affect their uptake into the body, their passage across membranes and their half-lives in the blood or body tissues.

Table 1. New technologies and approaches which can contribute to drug discovery and development

Use of existing knowledge
In chemico analysis and chemical interactions
In silico structure–activity methods
In vivo studies on lower organisms
In vitro methods (Table 2)
High-throughput screening
High-content screening
Omics approaches
PBPK models
Virtual tissue models
Human volunteer and clinical studies
Virtual patient populations
Biomarkers
Clinical imaging
Informatics
Systems biology
Integrated testing strategies

3. **In silico methods.** There are many computer-based analytical approaches, from (quantitative) structure–activity relationships ([Q]SARs) to category formation and read-across, which are combined with database compilation and the integration of information from different sources. The use of in silico methods in drug discovery was discussed in detail by Ekins et al, who stressed the importance of integrating computational and experimental data.[36-37] In silico methods are increasingly used in toxicology, and Combes has discussed how they are developed, with an emphasis on the importance of the applicability domain concept, the need for their validation and their use in intelligent testing strategies relevant to the REACH system.[38] A comprehensive consideration of principles and applications in in silico toxicology has recently been published, which deals, inter alia, with the development of QSAR and other models and the procedures for assessing their quality and applicability for the prediction of toxic hazard.[39]

4. **In vivo studies on lower organisms.** Many types of organism undoubtedly have value for studies at the basic research level, including plants such as onion (*Allium cepa*) and garlic (*Allium sativum*), bacteria such as *Escherichia coli*, fungi such as yeast (*Saccharomyces cerevisiae*), coelenterates such as hydra (*Hydra magnipapillata*), nematode roundworms such as *Caenorhabditis elegans*, insects such as *Drosophila melanogaster*, lower vertebrates, including fish such as the zebrafish (*Danio rerio*) and amphibians such as the South African clawed toad (*Xenopus laevis*). These organisms are being extensively used in research on cell and molecular biology, cell death, ageing, developmental biology, immunobiology and neurobiology. However, although some pharmacotoxicological test systems involving lower organisms and aimed at predicting effects in humans have been proposed, they are unlikely to provide satisfactory solutions, because the differences between these organisms and humans are too great for tackling other than certain highly specific questions (e.g., to find out what is involved in DNA damage and repair).

5. **In vitro systems.** As is clear, not least from other chapters in this book, a wide variety of in vitro systems are now available, which range in complexity from the use of isolated cell fractions over a few hours to the long-term culture of cells and tissues in multi-organ bioreactors (Table 2). By definition, "culture" is applied to cell, tissue or organ preparations which can be maintained in vitro in a nutrient medium for more than 24 hours. Further dramatic developments can be expected. The trend is toward greater sophistication, greater humanisation and greater miniaturisation, which leads to greater physiological and pharmacotoxicological relevance, but also to higher and higher costs in terms of time and human and economic resources.[40-44]

Stacey[45] has described developments in terms of culture vessels and the culture environment and the availability of stem cell lines and immortalised primary cell culture and recombinant cell lines, with an emphasis, based on his own experience and responsibilities, on the importance of Good Cell Culture Practice. Stummann and Bremer[46] have discussed progress in human embryonic stem cell (hESC) technology and the development of methods for screening for embryotoxicity, cardiotoxicity and hepatoxicity. Also of great importance are the emerging procedures for producing pluripotent stem cells from adult somatic cells, i.e., what are known as induced pluripotent stem cells (iPSCs). The use of iPSCs avoids the ethical issues related to obtaining hESCs and, in the future, it may be possible to use iPSCs from an individual patient to provide replacement tissues for that same patient.

In relation to drug discovery and development, the use of iPSCs opens up the possibility of producing normal and damaged differentiated cells of various types from individual humans, both before and after drug treatment. This would permit the performance of detailed studies on the desired and/or adverse effects of the drug, as a means of studying genetic predisposition, dose–effect relationships and the effects of epigenetic variables, such as treatment with other drugs, life-style and occupational factors, and infections.[47-51]

Table 2. In vitro systems which can contribute to the replacement of animal experimentation

Cell fractions (including postmitochondrial supernatant [S9], cytosolic [S100] and microsomal fractions for biotransformation studies)
Primary cell monolayer or suspension cultures
Continuous cell lines
Immortalised cell lines
Stem cells
Genetically-engineered cells
Co-cultures
Organotypic cultures
Precision-cut slices
Perfused cultures
Reconstituted tissue equivalents
Engineered tissues
Dynamic bioreactors
Multi-organ systems
Cell-/organ-/human-on-a-chip

The development of dynamic bioreactors, described by Marx,[40] which began with liver bioreactors, has now been extended to the creation of the human artificial lymph node and organ-on-a-chip and lab-on-a-chip systems, where micro-technologies and nano-technologies are leading to multi-organ systems and micro total analysis systems (µTASs) and, given time, the human-on-chip. Wen et al[41] have also considered the use of microfabrication and chip-based technology to provide for medium to high throughput screening, for which biochips and microplate-based assays are already available for cytotoxicity, cardiotoxicity, neurotoxicity, embryotoxicity, absorption and metabolism. The use of tissue engineering strategies, as described by Shakesheff and Rose,[43] is contributing to these developments, by offering tissue scaffolds and microenvironments which recreate the conditions under which cells form functional tissues. Their examples include the skin, liver, nerves, cardiovascular tissues, skeletal muscle, the gastrointestinal tract, the cornea and the airway epithelium.

The features of in vitro systems emphasised by Sbrana and Ahluwalia are somewhat different. They say that:

> Researchers have only just begun to appreciate that the intricate interconnectivity between cells and cellular networks as well as with the external environment is far more important to cellular orchestration than are single molecular events inside the cell. For example many questions regarding cell, tissue, organ and system response to drugs, environmental toxins, stress and nutrients cannot possibly be answered by concentrating on the minutiae of what goes on in the deepest recesses of single cells. New models are required to investigate cellular cross-talk between different cell types and to construct complex in vitro models to properly study tissue, organ and system interaction without resorting to animal experiments.[41]

They then describe how tissue and organ models can be developed by using the multi-well plate scale Quasi-Vivo® system and discuss how they can be used in drug toxicity studies. This system is based on the Multi Compartmental Bioreactor (MCB) and cell ratios and medium passage times are scaled to provide more-meaningful physiological relationships, avoiding some of the problems encountered when microfluidics, microfabrication and miniaturisation are pushed too far. Various cell types have now been incorporated, including hepatocytes, lung epithelial cells, intestinal epithelial cells and endothelial cells.

6. **High-throughput screening.** Made possible by advances in robotics and computer technology, high-throughput screening (HTS) involves the rapid testing of huge numbers of compounds for selected activities or interactions with specific proteins, receptors or other cell components. Methods including drop-based microfluidics can now permit 100 million reactions to take place in 10 hours.[44] In drug discovery, selection on the basis of HTS can be followed by lead optimisation, which can involve the synthesis of new analogues with improved potency, reduced off-target activity and properties indicative of manageable in vivo pharmacokinetics, as well as in silico analyses.

7. **High-content screening.** Originally developed as a drug discovery method, high-content screening (HCS) permits the evaluation of multiple biochemical and morphological parameters in cells.[52,53] For example, fluorescent tags or fluorescent antibodies can be used to detect proteins of interest via the parallel

use of spatially or temporally resolved methods to provide multiple sources of quantitative information suitable for integrated analyses. Changes in cells in response to potential drugs or toxicants can be detected with high resolution in automated systems. HCS can be used, for example, to look at test item–cell surface interactions, signal transduction cascades and effects on the cytoskeleton and can be linked through phenotypic/visual screening to various omics and other approaches relevant to target identification and responses, and to genetic polymorphism. Although slower than HTS, HCS provides much more information.

8. **Omics approaches.** The sequencing of the human genome and the development of a wide range of methods for application in molecular and cellular genetics has opened up dramatic new possibilities for increasing our understanding of human diseases and devising effective and relatively safe ways for managing them.[54] Hence, *pharmacogenetics*, a rather descriptive approach to differences between individuals in terms of disease and their responses to drugs, has been supplanted by *pharmacogenomics*, a dynamic approach to obtaining and using genetic information at the population level as a basis for drug design and development, then as a basis for the management of drug therapy by choice of drug and selection of dosing regimen, followed by monitoring of positive and adverse effects in the individual patient. The omics approaches themselves afford a wide range of tools with special uses, but which must be used in integrated and intelligent strategies. There seem to be an ever-expanding number of omics, including:

- *cellomics* (about phenotype and functions at the cellular level);
- *cytomics* (distinguishable from cellomics by its application at the single cell level);
- *epigenomics* (about the parts of the genome, other than the DNA code, which modulate the operation of the genome);
- *interactomics* (about interactions and their consequences among proteins and other molecules within a cell);
- *metabolomics* (about the chemical processes involving metabolites), which is related to:
- *metabonomics* (about quantitative, dynamic and multiparametric metabolic responses);
- *pharmacogenomics* (a generic term, as referred to above);
- *phenomics* (about the functional biochemical and physiological characterisation of cells, tissues and organisms in response to genetic changes and environmental influences);
- *proteomics* (about the proteome, the entire complement of proteins and about their individual production, modification and functions);
- *toxicogenomics* (about responses to toxic substances); and
- *transcriptomics* (about all the types of RNA, including mRNA, rRNA and tRNA, as applied to the total set of transcripts or a specific subset).

The omics approaches are particularly important as ways of linking information obtained from in silico and in vitro systems to clinical situations and especially to bioinformatics and to the identification of much-needed biomarkers of susceptibility, response and effect in, for example, new drug development, as in the case of drug-induced liver injury described by Casciano.[54]

9. **Physiologically-based pharmacokinetic (PBPK) models.** In vitro tests, in silico modelling and the *omics*-based systems tend to concentrate primarily on potential pharmacodynamic and toxicodynamic events. However, if drugs are to be developed and chemicals and chemical products are to be used and patients, workers and consumers are to benefit and be protected, the information provided by these approaches is of little value, unless major biokinetic processes and, in particular, absorption, distribution, metabolism and elimination (ADME), are fully taken into account. These questions are now approached through the use of biokinetic models, especially PBPK models, a term which is used to cover toxicokinetic models as well.[55] PBPK model development takes account of ADME on the basis of interrelationships among the critical determinants of these processes, such as tissue volumes, flow rates, rates of absorption, diffusion across membranes, tissue:blood partitioning and rates and affinities for biochemical reactions. PBPK models are designed to provide a representation of the intact organism, including routes of entry or uptake (via gastrointestinal tract, skin, lungs or blood), distribution (via bloodstream), target organs (e.g., liver, kidneys, brain) and storage sites (e.g., adipose tissue). Account can be taken of the differences between rapidly-perfused tissues (liver, kidneys, brain) and slowly-perfused tissues (muscle, skin). Equations can be derived for describing features such as tissue influx and efflux, hepatic metabolism and renal clearance. The data on which the model is developed are obtained from the literature, from physicochemical considerations, from in silico approaches and in vitro tests and from experiments on animals and, where ethically acceptable, from studies on humans or human preparations and tissues obtained from organ donors. PBPK models can be used to guide dose selection, as well as to signal responses to be looked for in later stages of the drug development process. By using PBPK modelling, the internal concentration at the target(s) can be determined for any given chemical, route of administration and dosing regimen, and for any species. This information can then be used to assess the effects of "in vivo equivalent" concentrations of chemicals in in vitro tissue culture, ideally by using human cells of the target tissues(s).

    Lipscomb et al[55] have described how PBPK models are developed, evaluated and applied in toxicity testing and health risk assessment, emphasising the importance of using a systems approach to provide much-needed improvements in understanding of the exposure–dose–response relationship. Thomas[56] assessed the challenge of using physiologically-based simulation modelling to reduce and replace the use of laboratory animal in the discovery of new pharmaceuticals. The failure of animal studies to predict adverse effects in humans is one of the reasons for the postmarketing withdrawal of drugs, but 60–80% of the animals used in drug discovery and development are used in lead identification and lead optimisation. He concluded that PBPK models are versatile simulation models, which could be ideal replacements for animal studies for predicting ADME in humans, resulting in improvements in the prediction of human pharmacokinetics.

10. **Virtual tissue modelling.** The first mathematical model of the working heart was produced by Denis Noble in 1960.[57] A number of other models are now available, in which the virtual tissues are biophysically and anatomically detailed and provide quantitatively predictive models of the physiological and pathophysiological behaviour of the tissues within the isolated organ. For

example, Holden has described a model of the heart involving the reconstruction of the electrical activities of cardiac tissues by producing computer models in the form of virtual tissues and also a model of uterine tissues that may identify possible mechanisms involved in premature labour.[58]

11. **Human volunteer and clinical studies.** The ethical and strictly-controlled use of human volunteers and patients can provide samples of body fluids from individuals with and without particular diseases, as well as subjects to be involved in clinical trials. The samples are essential for the application of the omics approaches and in vitro systems, but problems with the safe and ethical provision of cells and tissues for use in the in vitro approaches summarised in Table 1 can be a limiting factor for progress with this type of replacement alternative.

12. **Virtual patient populations.** This novel approach can be used to conduct virtual clinical trials for efficiently screening drug candidates and for evaluating the prospect that they could be brought to the market successfully.[59]

13. **Biomarkers.** As used in pharmacotoxicology, biomarkers are objectively measured and evaluated indicators of normal biological processes, pathogenic processes and (desired) responses to, or (adverse) effects of, deliberate, incidental or accidental exposures to chemicals, drugs and other chemical products or to pathogens and biological products, such as vaccines. It is useful to distinguish between disease-related biomarkers and drug-related biomarkers, which can also be subdivided into various categories, such as biomarkers of susceptibility, of exposure, of drug efficacy, of toxicity, or of patient response, or as diagnostic biomarkers. Another distinction is between imaging biomarkers and molecular biomarkers. They should be specific to particular circumstances and processes, reliably measurable and fit for use for a defined purpose. They can provide vital and exploitable links between all the elements in the new pharmacotoxicological technologies, from computer-aided drug design, via in silico modelling for efficacy and toxicity, through the use of the omics and medium throughput and high throughput screening, along with the use of in vitro systems at various levels of sophistication, to PBPK and PBPD modelling and eventually to the investigation of polymorphisms within the human population, as a basis for planning and monitoring therapies designed for the individual patient or protecting workers and consumers from damage caused by exposure to harmful chemicals and products. The principal way of discovering biomarkers is by the application of the omics approaches in the toolbox, linked with bioinformatics, but most importantly, also with clinical observations and analyses. Their development involves five main stages: target biomarker identification (relevant to a given disease or drug therapy); clinical characterisation (in people with/without the disease or using/not using the drug); retrospective repository studies (on samples relevant to the disease or the use of the drug); prospective screening studies (to predict the occurrence of the disease or the effects of using the drug); and clinical use (in control of the disease or management of the use of the drug). For example, Vasan[60] and Corrias et al[61] have discussed biomarkers in relation to cardiovascular disease and cardiotoxicity and the FDA has formally accepted seven renal safety biomarkers for use in the nonclinical and clinical stages of drug development.[62]

14. **Clinical imaging.** The application of clinical imaging techniques to patients is likely to be of increasing value, as they are non-invasive and can produce

multi-dimensional results, which can be both qualitative and quantitative, and which can used in association with information obtained by applying other technologies.[63] X-ray computed tomography (CT) has great promise, since, for example, it can produce a 3D image of the heart and its blood supply from a series of 2D images. Magnetic resonance imaging (MRI) can also be used to visualise internal structures and is especially useful in showing what is happening in the brain tissues. Optical coherence topography (OCT), now typically employing near infrared spectroscopy, can provide detailed images from within the retina; this can be used to assess axonal integrity in multiple sclerosis and to visualise lipid-rich plaques in coronary arteries. Positron emission tomography (PET) is another way of producing images of what is taking place within the body. Used with the glucose analogue, fludeoxyglucose, PET can be used to measure which cells in the body take up glucose and this can throw light on sites of inflammation and early tumour development. Thus, by applying clinical imaging techniques, the nature and progression of events can be followed at the molecular level, within the human body. In drug development, it is possible to develop PET imaging strategies to visualise drug interactions with the targets on cells.

15. **Informatics.** In its broadest sense, *informatics* encompasses information science and information technology (IT) and is essential to the successful management of human activities of virtually all kinds, given the enormous amounts of information now available on all kinds of topics, not least through the Internet. At least three kinds of informatics technologies are involved in drug discovery, development and use: *cheminformatics*, the application of computer and IT techniques to problems in the field of chemistry; *health informatics*, where computer science and IT meet health care and which includes *biomedical informatics* and *clinical informatics*; and *bioinformatics*, the application of computer science, IT and statistics in molecular biology, especially in the management and analysis of data provided by the omics approaches. Bioinformatics involves the use of computer-intensive techniques to search through vast amounts of data on, for example, DNA sequences and protein sequences and structures, in order to increase understanding and facilitate problem solving, by identifying patterns and correlations and creating algorithms (mathematical formulae consistent with expressions of finite lists of well-defined instructions as a basis for guiding data processing and automated reasoning). This can provide, for example, a basis for the comparative analysis of genome content, of gene expression, of gene mutation, of gene regulation, of protein expression, of network modelling and of molecular design, in ways which facilitate the identification of applicability domains and prediction models in in silico modelling and in vitro testing.[64]

16. **Systems biology.** Discussions on drug development frequently mention a systems biology approach, i.e., a multidisciplinary approach to considering the interactions between the components of a biological system and combining this knowledge to increase understanding of the organism or of the phenomenon being considered.[65] In a way, this represents a holistic approach to combining the data provided by a variety of confirmatory or complementary reductionist approaches. Relatives of systems biology are *meta-analysis*, which combines parts of the results of several studies, and *evidence-based medicine* and *evidence-based toxicology*.[66]

THE DEVELOPMENT AND USE OF TESTING STRATEGIES

Given the great number and variety and the ongoing development and expansion of the new technologies, their employment in the development and evaluation of new chemicals, pharmaceutical and other products will involve their selective use according to integrated and intelligent strategies, which, no less than the methods to be employed, will themselves need to be subjected to independent and critical evaluation, not least in terms of their manageability and affordability.[67]

The process begins with a consideration of the comparative nature and value of the chemical or product which is to be evaluated. Will the aim be to select a small number of the most promising candidates from among a much larger number? Is the objective to determine how a new chemical, to be used in an industrial process, should be classified and labelled and what precautions should be used to control exposure to it? Is the purpose to ensure that a cosmetic ingredient is sufficiently safe for use under normal conditions of exposure? Is the intention to seek approval for the clinical testing of a new drug? What is to be done will depend on the test item and its proposed usage. The compounds subjected to HTS have very little individual value, but the value of a selected lead pharmaceutical compound steadily increases as it progresses through development toward the clinic.

What must also be made clear is that no test should be conducted for regulatory purposes, unless its relevance and reliability have been established for a particular purpose (i.e., unless it has been validated). Each test should give clear answers to a limited number of precise questions, appropriate to its applicability domain, with the outcome expressed in clear terms, according to prediction models, as a basis for making justifiable decisions. These stipulations apply equally to animal tests and non-animal tests.

Individual tests can be duplicative or confirmatory (i.e., the result of one test can be used with a comparable result from another test to strengthen the conclusion reached about a particular toxic hazard), or they can be additive or complementary (i.e., they can provide different kinds of information which, taken together, can support a particular conclusion, perhaps as part of a weight-of-evidence [WoE] approach). This is especially the case, where tests involve different mechanisms which produce the same toxicity endpoint. Sometimes, when other considerations have to be taken into account, such as the patent life of a new chemical entity, tests may be conducted in parallel rather than in sequence. Here, the time factor would affect the affordability of this kind of application of the testing strategy.

Integrated Testing Strategies

We have proposed several integrated testing schemes (ITS) for toxicity testing,[68] based on the recommendations made by the authors of the various chapters in the book, as well as on previously published ITS that were developed as part of a research project to generate testing strategies for prioritisation of chemicals for further evaluation in the EU REACH legislation.[69-77] These schemes are for: general chemicals (Fig. 1); cosmetic ingredients (Fig. 2); candidate pharmaceuticals (Fig. 3); inhaled substances (Fig. 4); and nanoparticles (Fig. 5). While some of the information required concerning potential hazard is common to all these chemicals or products, other considerations affect the order in which the tests should be conducted, mainly due to the relative importance of likely target organs and the toxicity endpoints concerned.
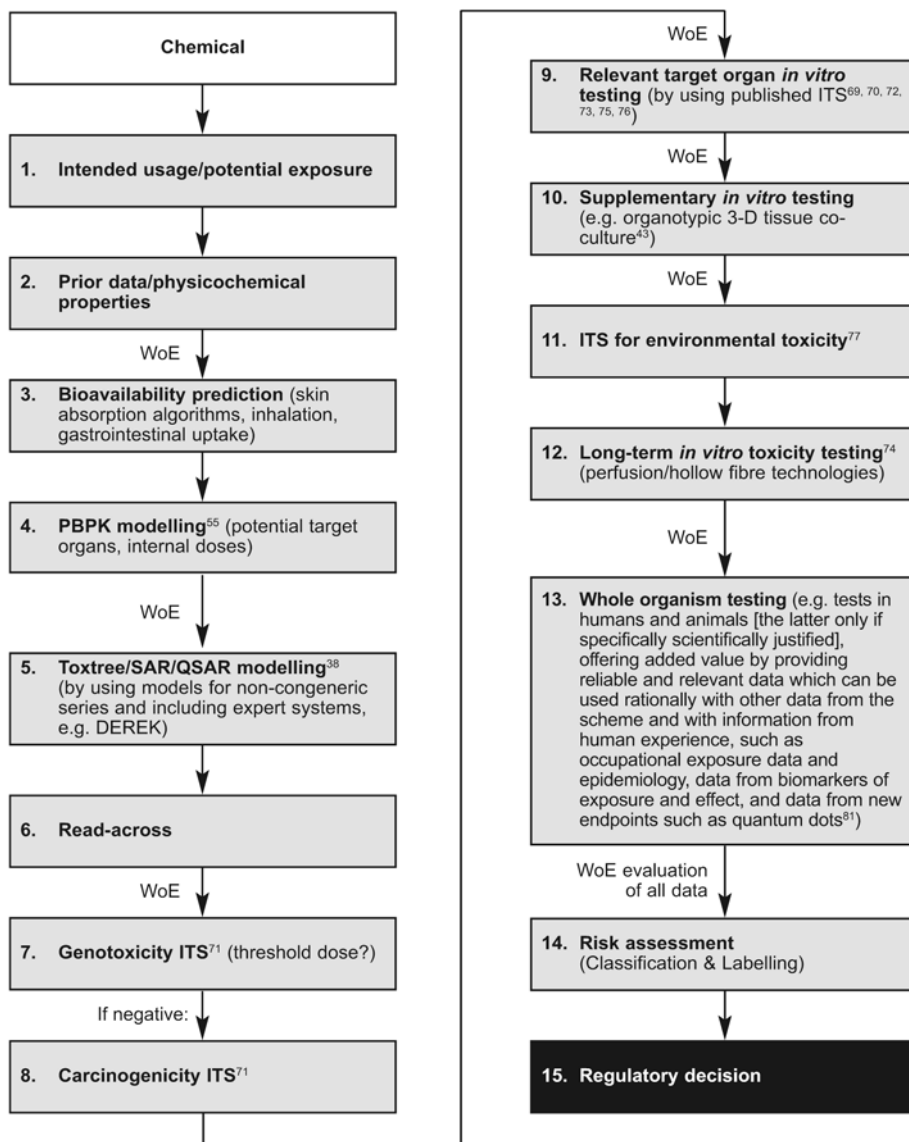
Figure 1. An ITS for general chemicals. This ITS scheme could be used, for example, for compliance with the EU REACH system and US HPV system, to provide the required comprehensive set of information for new chemicals and so-called 'missing' information for existing chemicals. It is based on proposals made during the FRAME/Liverpool John Moores University/Defra REACH project,[69–77] and some of the other chapters in this book, and incorporates some of the ITS proposed as part of that project. These individual ITS schemes, which can be downloaded free from www.frame.org.uk, include animal tests, but these should only be used as a last resort (as explained in box 13, above). The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68]
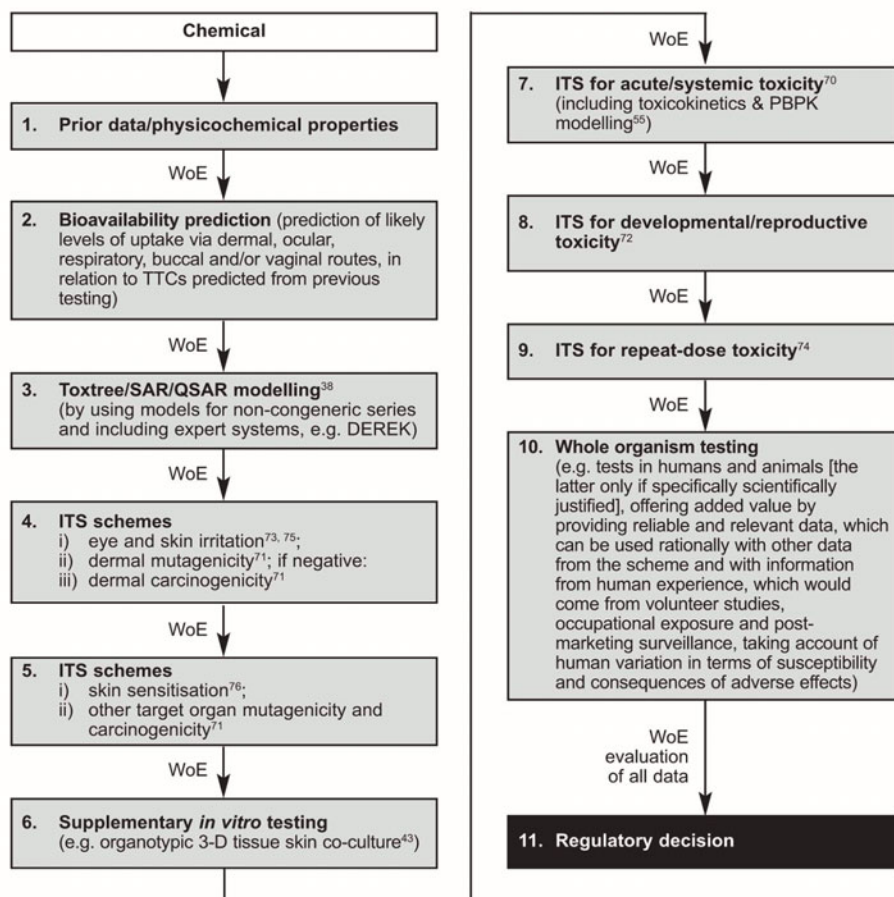
Figure 2. An ITS for cosmetic ingredients. This ITS scheme could be used, for example, for compliance with legislation such as the EU Cosmetics Directive. It is based on proposals made during the FRAME/ Liverpool John Moores University/Defra REACH project,[69–77] and some of the other chapters in this book, and incorporates some of the ITS proposed as part of that project. These individual ITS schemes, which can be downloaded free from www.frame.org.uk, include animal tests, but these should only be used as a last resort (as explained in box 10). Three important issues should be taken into consideration. Firstly, most cosmetic ingredients are also used in other products and, if they are produced on anything other than a very modest scale, they will have had to be tested in compliance with the regulations for industrial chemicals in general. Secondly, additional testing for chemicals to be used in cosmetics should only be required, if they are scientifically justified by the special nature of cosmetics and the ways in which they are used. In particular, testing according to boxes 5–9 in the scheme should not be required, if the internal systemic or target organ concentrations are not likely to approach TTCs (thresholds of toxicological concern), as predicted from previous testing.[25] Thirdly, for ingredients that are only used in cosmetics, as with cosmetics finished products, there is legislation in several countries banning the use of animals for testing. The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68]

Each ITS is characterised by a first step which involves an assessment of prior data which might or might not allow a decision to be made as to whether or not to continue testing, or to reject the chemical as being likely to be too hazardous. The schemes have
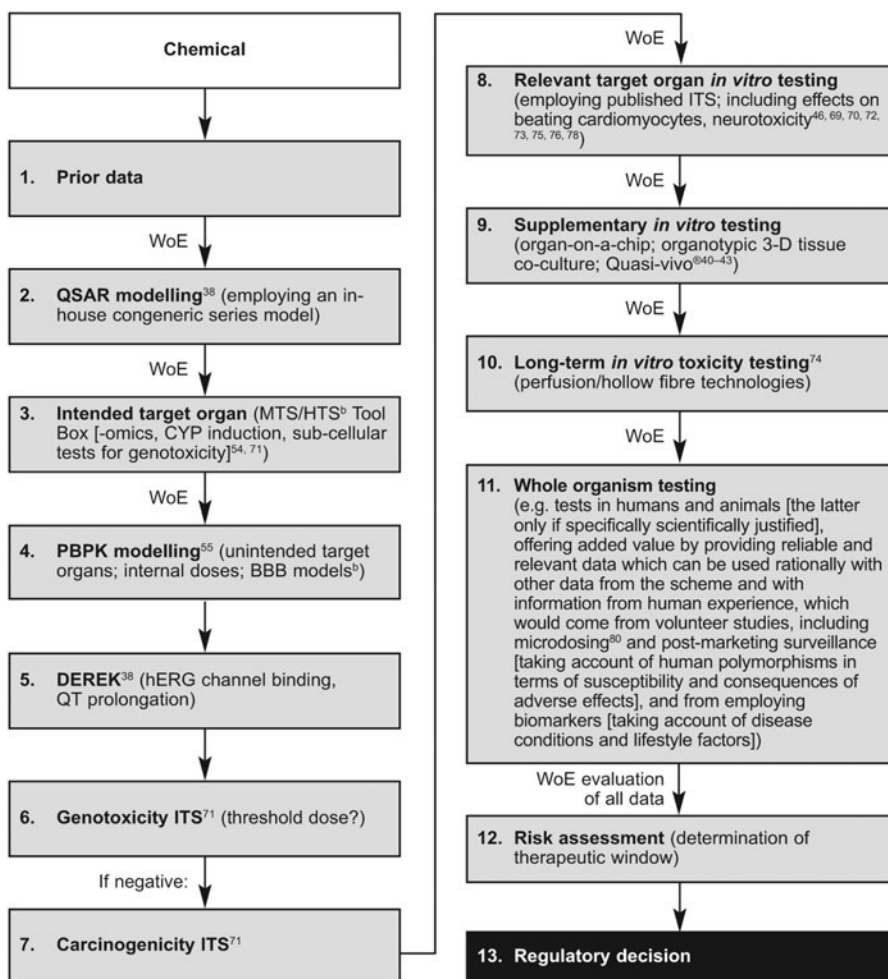
Figure 3. An ITS for candidate pharmaceuticals. This ITS scheme could be used for compliance with legislation such as the EU, US and Japanese regulations on pharmaceuticals. It is based on proposals made during the FRAME/Liverpool John Moores University/Defra REACH project, [69–77] and some of the other chapters in this book, and incorporates some of the ITS proposed as part of that project. These individual ITS schemes, which can be downloaded free from www.frame.org.uk, include animal tests, but these should only be used as a last resort (as explained in box 11). The scheme begins with the screening of large numbers of chemicals for potential utility, but the tests become more sophisticated, more important and more expensive, as the number of candidate compounds steadily decreases through the drug discovery phase and into the drug development stage, before a very small number of candidates are assessed in clinical trials. Evaluations for comparative potential toxicity are conducted in parallel with evaluations for comparative potential efficacy, along with other considerations, such as stability. Because many drugs have had to be withdrawn in the later stages of drug development and even after approval for use in patients, partly due to the inability of animal models to predict potential efficacy or serious manifestations of toxicity (e.g., cardiotoxicity, hepatotoxicity and respiratory toxicity) with sufficient accuracy, all possible steps must be taken to use procedures which are directly relevant, not only to humans, but also to variant human subpopulations. [b]MTS = medium-throughput screening; HTS = high-throughput screening; BBB = blood–brain barrier. The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68]
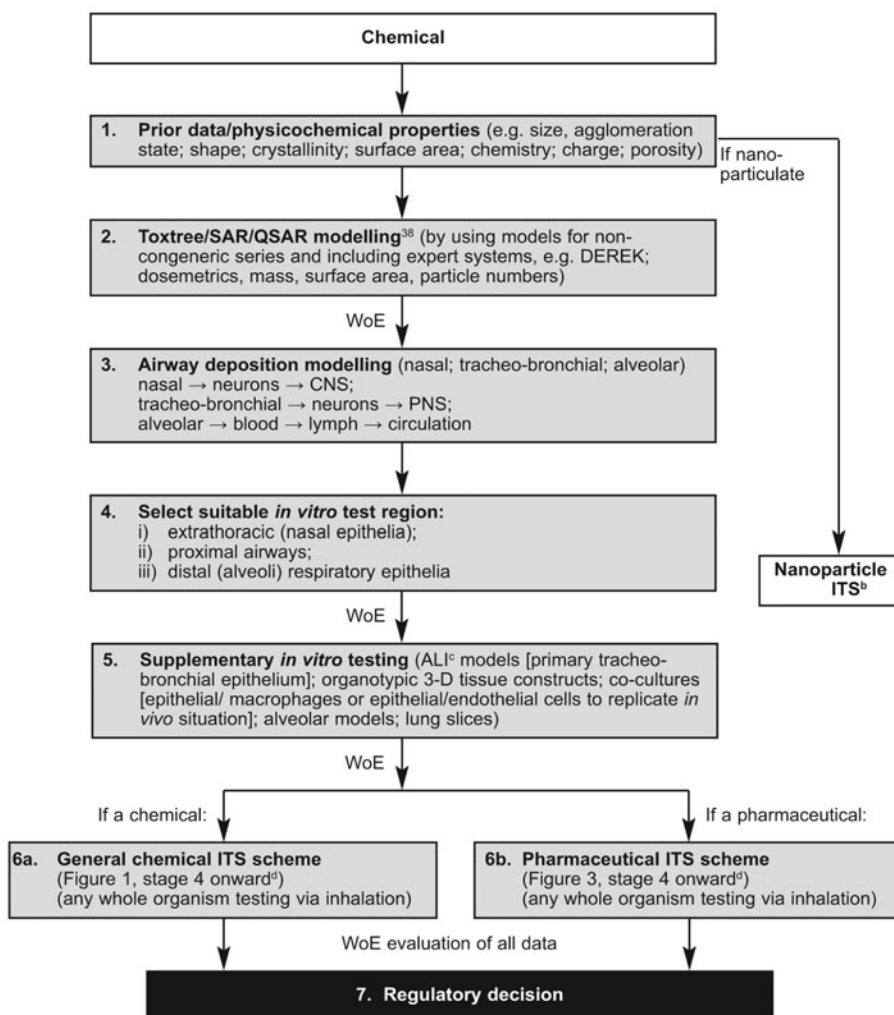
Figure 4. An ITS for inhaled chemicals. This ITS scheme is based on the report of a FRAME workshop,[82] with input from Dr Kelly BéruBé. Animal models cannot reliably reflect the responses and effects that occur in humans and in any case, such studies are very expensive and time-consuming and can only offer a very low rate of throughput. Given the serious burden of respiratory disease and the demands of regulations such as those of the REACH system and in view of the ethical limitations on human volunteer studies, it is imperative that non-animal ITS are developed, validated and accepted as rapidly as possible.[86] [b]Figure 5; [c]ALI = air–liquid interface. [d]Employing appropriate methods for application to volatiles, dusts, etc. The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68]

a number of WoE stages, at which such a decision can be taken, as and when sufficient data have been accumulated to inform the process. The second step in the ITS schemes is usually an assessment of bioavailability to determine the extent to which the chemical can enter biological systems; for example, via percutaneous absorption, gastrointestinal uptake, or passage across the blood–brain barrier (BBB). It is often possible to predict
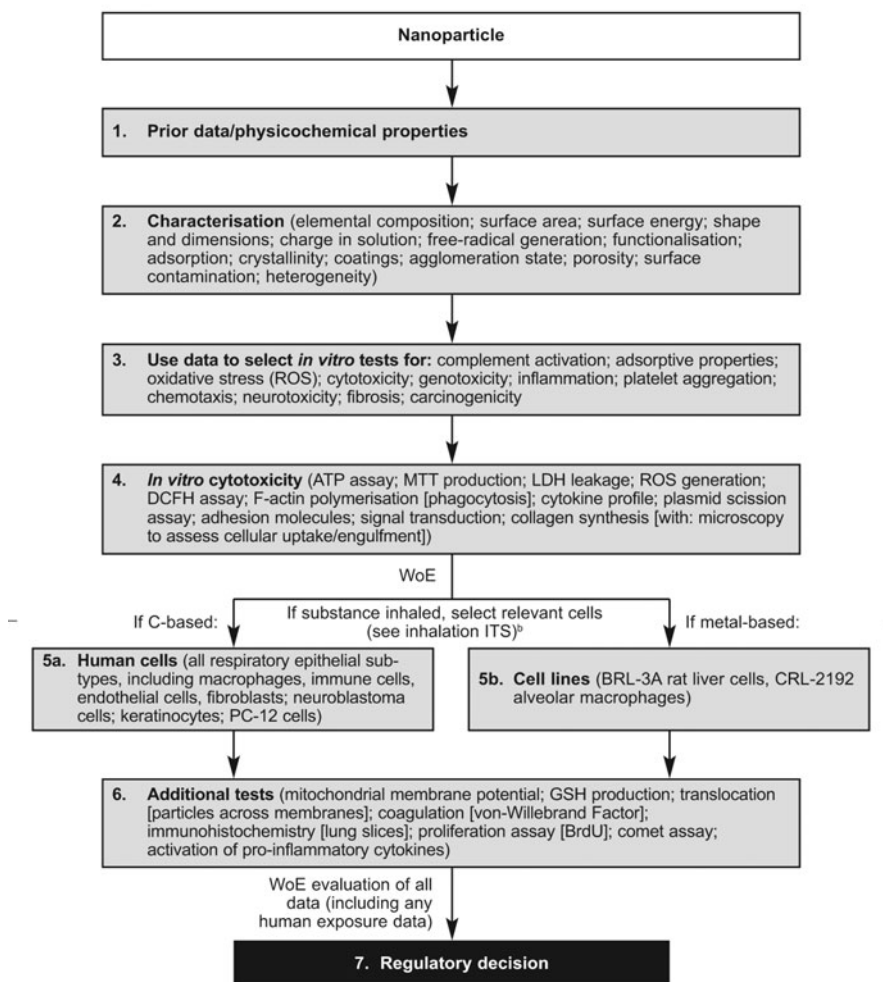
Figure 5. An ITS for nanoparticles. This ITS scheme is based on those in the chapter by Schrand et al[32] and in BéruBé et al.[82] The toxicity testing of nanoparticles presents a significant new challenge, which is unlikely to be met via conventional laboratory animal tests, which were designed for the testing of high doses of macroparticles. Abbreviations: GSH = reduced glutathione; ROS = reactive oxygen species; MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; LDH = lactate dehydrogenase; DCFH = 2',7'-dichlorofluorescein; BrdU = bromodeoxyuridine. [b]Figure 4. The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68] Suggestions made by Dr Kelly BéruBé are gratefully acknowledged.

bioavailability by using relevant algorithms or, as in the case of the BBB, via the use of a coculture system. Once inside the body, the main target organs, as well as likely internal concentrations at these sites, in relation to applied doses, can be predicted by PBPK modelling.[55]

The next stage of the ITS strategies is to undertake SAR/QSAR and expert system modelling, by using the information from the previous steps to focus on predicting

target organ toxicity, as well as structural alerts for hERG K^+ channel affinity, related to QT prolongation interval, by using the rulebase in DEREK.[38] Apart from the testing of candidate pharmaceuticals, it is likely that this stage will involve the use of a series of models for noncongeneric series of chemicals that have overlapping applicability domains and are based on different mechanisms of toxicity. It is suggested that, for general chemicals and cosmetic ingredients, SAR/QSAR modelling should start with the use of the freely-available decision-tree system called Toxtree.[39] In addition, it might be possible, based on the information obtained, to predict hazard for the chemical concerned by performing read-across.[39]

It is suggested that the SAR/QSAR modelling stage of the ITS strategies is followed by an estimation of mutagenicity using a published ITS for this endpoint.[71] This is because positive genotoxicity is of great importance for risk assessment, as it is generally assumed that such chemicals can exert genotoxicity in the absence of a threshold dose. In addition, genotoxicity implies the potential for carcinogenicity. However, it is suggested that, in the absence of genotoxicity, the carcinogenicity of the chemical is investigated by following a published ITS for carcinogenicity.[71] A positive result would suggest that the chemical could be a nongenotoxic carcinogen, with a threshold dose, and this should inform subsequent decisions as to continuation of testing, chemical rejection or classification and labelling.

It is important to note that, as with all of the other published ITS strategies, the emphasis with the use of both of the genotoxicity and carcinogenicity schemes should be on the avoidance of laboratory rodent tests, as far as possible, and that any whole organism testing should be undertaken on the basis of all prior information, in the context of the overall ITS now being proposed.

In the case of the scheme for general chemicals, in vitro carcinogenicity testing is followed by relevant in vitro target organ toxicity tests, according to published ITS strategies, with the above corollary. These tests are to be selected and used in relation to previous data on target organs and are as follows: acute systemic toxicity;[70] skin sensitisation;[76] skin corrosion and irritation;[75] eye irritation;[73] and developmental and reproductive toxicity.[72] In addition, the chemical needs to be tested for environmental toxicity.[77]

In each of these individual ITS strategies, testing should begin at the stage immediately following the use of in silico modelling and is usually based on the use of monocultures of mammalian cells in culture.

These initial tests are succeeded by supplementary in vitro methods, involving the use of more-complex and in vivo-like systems, such as 3D-organotypic coculture systems, particularly as models of likely or known target organs. In addition, exposure of the cells is achieved by using methods designed to simulate the in vivo situation as far as possible, such as air–liquid interface (ALI) models. These systems include complex whole organ models in which different cell types exist, retaining their specific in vivo functions. Some of these systems could be based on whole organ cultures, such as the liver, developed by tissue engineering techniques, including the use of tissue scaffolds, anchorage to extracellular matrix (ECM) and specific growth factors to stimulate cells, in an attempt to recreate the conditions under which functional tissues are formed in vivo.[43]

Further testing then involves assessing repeat-dose toxicity by using long-term culture systems, such as hollow fibre and perfusion cultures, in which cells can be grown for extended periods of time with chronic dosing of the test chemical and in which recovery from initial toxicity can be measured.

It will be noted that, as far as possible, hazard data are obtained from the use of human cells and cell tissue systems, increasing the relevance of the information for protecting

human populations.[40] These human cells can be used as primary cultures, be derived from immortalisation or be obtained through the specific development of stem cells.[45,46] The exception to this is the ITS strategies for environmental toxicity, where target organisms are used as much as possible, for obvious reasons.[77]

The ITS for candidate pharmaceuticals assumes that a high number of structures are being screened both for efficacy and for toxicity via HTS and MTS (medium throughput screening systems) and, as such, relies heavily on genomic and proteomic analyses, as well as bioinformatics,[54] as a result of which a high rate of attrition is expected.[43] This part of the ITS could also be supplemented with several other emerging methods described in this volume, particularly if a series of candidates are being developed with respect to a specific target organ, to provide an HTS platform by which to test both efficacy and toxicity. Examples of such methods include the dynamic bioreactor tissue culture system—'organ-on-a-chip'—described by Marx.[40] This provides an in vivo-like micro-environment for cells and has been designed for use in a 96-well format. Likewise, the Quasi-Vivo® multi-compartment reactor, which is designed to mimic cross-talk between cells and tissues to represent a more realistic physiological environment and is described by Sbrana and Ahluwalia,[41] is also available for use in a 96-well format and could be used to study hepatoxicity and liver biotransformation in liver cell cultures. In addition to these systems, other HTS chip-based methods involving micro-scale tissue culture systems of target organs of interest could be developed by using micro-fabrication and micro-fluidic technologies, as discussed by Wang et al.[42]

Figure 6 presents a scheme for neurotoxicity testing, which is an expanded part of the ITS for candidate pharmaceuticals that can also be incorporated into any of the other ITS strategies, where effects on the neural system are to be investigated. It will be seen that the neurotoxicity strategy, which is based on previously-suggested schemes,[78,79] follows the same principles as those for the other ITS schemes, with measurements of cytotoxicity and specialised functions, including neurite growth and production of glial fibrillary acidic protein (GFAP). However, the scheme also includes a range of other studies, such as metabonomics, to measure effects on neural-specific metabolites, as well as the use of invertebrate and vertebrate models to study effects on intact nervous systems. There is also the possibility of undertaking HTS for neurotoxicity by using, for example, rat hippocampal brain slice cultures in multi-electrode arrays in conjunction with growth on silicon chips.[42] Also, developmental neurotoxicity (DNT) can be studied by using these models as well as by investigating effects on the activities of neural stem cells.

Once all of the non-animal data have been obtained from performing an ITS scheme, they are evaluated with a view to deciding on whether to continue testing and, if so, which, if any, whole organism studies would be appropriate and scientifically-justified. The emphasis here is on maximising the use of any pre-existing human data from occupational exposures and volunteer studies, but only to confirm the negative results obtained from earlier experiments. In the case of testing of pharmaceutical candidates, which are nongenotoxic, it is suggested that first studies in humans could involve microdosing,[80] in which very low dose levels of a chemical are administered and metabolic fate is followed by using extremely sensitive analytical techniques. In addition, extreme care should be taken when selecting human subjects for clinical testing, to avoid the problems that occurred with TGN1412.[29] The use of laboratory animal tests should only be required where the other available information is insufficient to justify human studies or where it is unable to provide a basis for making a decision as to whether to reject the chemical or make a regulatory decision. Even then, it should be specifically and scientifically established
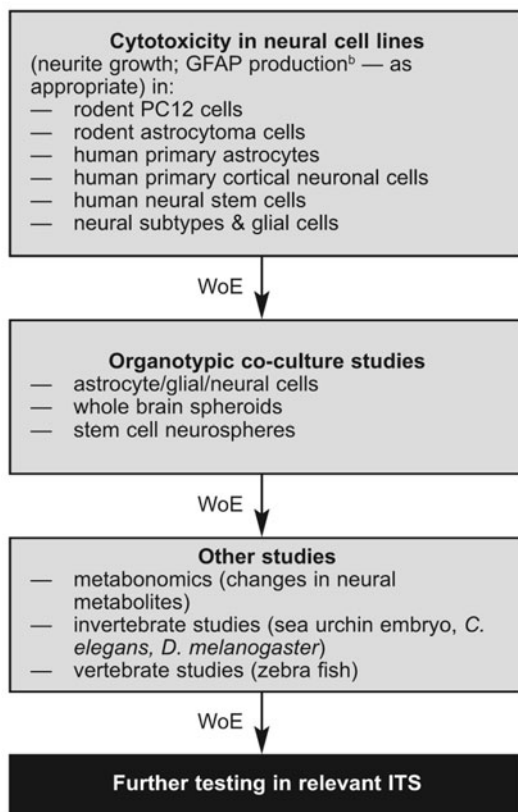
Figure 6. An ITS for neurotoxicity. This ITS scheme is based on those of Coecke et al.[79] Neurotoxicity is one of the most serious effects of exposure to high doses of chemicals and is a major cause of acute toxicity. Developmental neurotoxicity to humans is very difficult to assess by using animal models; in vitro methods employing human stem cells are extremely useful. [b]GFAP = glial fibrillary acidic protein. The scheme was adapted, with permission, from Altern Lab Anim—ATLA 2011; 39(3):213-225.[68]

that the proposed animal testing is likely to provide the information that is required. In addition, any whole organism testing should make full usage of the most-modern analytical and diagnostic techniques, such as biomonitoring for biomarkers of exposure and effect and the use of molecular labelling methods, such as quantum dots.[81]

Figure 4 presents an ITS scheme for inhaled chemicals. This is based on the discussions held at a FRAME workshop in 2007, in which methods for assessing the toxic and health effects of chemicals entering via the inhalation route were discussed.[81] A key stage of the scheme involves airway deposition monitoring, information which is used to select suitable cellular models for further studies, depending on the area of the respiratory system targeted. For purposes of the strategy, three scenarios determine the overall course of testing of an inhaled substance, depending on whether it is a general chemical, a drug (intended to be delivered by the nasal route) or a nanoparticle. In the first two cases, the scheme follows the respective ITS, but involves the use of respiratory cells and cellular systems as far as possible. In the latter case, the ITS follows a strategy

devised specifically for nanoparticles (NPs; Fig. 5) and is based on tests and results described by Schrand et al.[32] The scheme involves the characterisation of the NP in question, by using a range of criteria, some of which were discussed elsewhere.[82] The resulting information is then used to choose the most appropriate in vitro and biochemical tests and toxicity endpoints to measure. Choice of target cells depends on whether the NP is carbon-based or metal-based.

It is important to note that the testing of a chemical can be stopped at the point where it can be classified and labelled in accordance with the requirements of the regulatory authority concerned. If, at the target organ/system testing stage, an effect on one organ or system (e.g., the liver or the nervous system) meant that the chemical must be given a certain label, there may be no need to test its effects on other organs or systems (e.g., the kidney or the reproductive system).

The testing requirements for pharmaceuticals are very different and lend themselves less readily to generalisation. Both efficacy and toxicity have to be evaluated in a cost–benefit approach. While toxicity to the liver might be a reason for halting the development of a drug, concluding that the hepatotoxicity was acceptable and manageable in the light of its benefits would not be sufficient, since its adverse effects on, say, the cardiovascular system, the respiratory system or the nervous system might be more serious than those on the liver.

The challenge to effectively harness the new technologies in drug development is particularly acute. The question is whether sufficient knowledge of sufficiently high quality, of sufficient breadth and depth and of sufficient direct relevance to humans, about both desired and adverse effects, can be gathered, scientifically, efficiently and acceptably in terms of time and cost, so that a convincing case can be presented for embarking on clinical trials. The pressing need is to devise better ways of determining, during drug development, the probability that these predicted benefits and adverse effects would actually occur.

THE INTELLIGENT USE OF ITS

There has been much recent interest in promoting so-called intelligent integrated testing strategies, which involve the sequential use of non-animal and in vivo tests, especially as a means of addressing the testing requirements of the EU REACH legislation.[39] While intelligent testing strategies are based on integrated schemes, they are designed to encourage a bottom-up approach to risk assessment, starting with exposure information, in order to avoid the use of strategies based on collecting all possible hazard data (nonscientific check-list testing). Intelligent testing schemes are based on the assumptions that: (a) there is no risk without exposure; and (b) that testing should be dictated primarily by the bioavailability of the test substance. Therefore, only the hazard data needed to make a regulatory decision are required, with the cessation of testing when such a decision can be taken.

The ITS schemes we have developed are in a decision-tree style, whereby, at certain stages in each scheme, a decision on whether to classify and label and/or to undertake a risk assessment with respect to the test substance is made via a WoE process. Essentially, WoE evaluation, as used here, refers to the process of achieving a consensus decision as to the hazard and/or risk associated with a certain type of exposure to a chemical, after a detailed scientific assessment of all of the available evidence, based on any considerations of the strengths and weaknesses of the information. It is assumed that the WoE process will: (a) be transparent; (b) take into account the scientific validity, quality and relevance

of the data; (c) be undertaken by a group of individuals with relevant expertise by using their professional judgement; and (d) be iterative, so that new data can be taken into account, as and when they appear. It is further recommended that those undertaking WoE evaluations should, wherever possible, discuss them with the relevant authorities at the prenotification stage. However, even with such stipulations, it is clear that WoE evaluation is open to considerable variability, requires harmonisation and should include the application of consistent criteria for the acceptance and rejection of pre-existing data.[83] Ideally, decisions as to whether to stop or continue testing in any ITS scheme, should be taken at every step. However, most of the schemes include several steps, the order of which is arbitrary and between which it is difficult for a decision to be made.

Tests for chemicals and certain chemical products that have been validated and approved for regulatory use, are indicated by inclusion of the respective OECD Test Guideline (TG) number in the original publications of the individual ITS strategies. It will be noted that we have included many tests that have not been formally validated according to internationally accepted criteria, even though most of these have standardised and optimised protocols. While we are firmly committed to the validation process, particularly for regulatory toxicology, we justify the inclusion of such nonvalidated test methods on the basis that they are able to produce data that can be used in WoE evaluations, particularly for classification and labelling purposes. Also, the methods are particularly useful for prioritising chemicals for further safety assessment. This is especially the case for the chemical methods (in silico prediction and read-across approaches) which are an integral part of all of the ITS schemes. We do, however, caution that nonvalidated methods should be used judiciously, especially the chemical methods, in view of their important limitations at the present time.[84] The ITS also serve to show what tests are potentially available for inclusion in testing strategies. We urge that those tests that are nonvalidated are subjected to formal validation as rapidly as possible, so that this can lead to the eventual validation of the ITS strategies themselves.[85]

A general theme in the decision-tree schemes concerns making the data from in vitro tests more-relevant to predicting toxicity in vivo. The main possible approaches to this problem are to use: (a) cells of target organisms (e.g., human cells or fish cells); (b) cells in culture from target tissues; (c) metabolising systems from target organisms and tissues; (d) organotypic 3D coculture systems, sometimes involving the use of whole organ cultures; and (e) test substance concentrations adjusted for levels predicted to arise at target tissue sites in vivo from biokinetic modelling and metabolism prediction.

Lastly, our proposals focus on whole organism testing, with the emphasis on obtaining as much information on human exposure and effects from both occupational exposure and volunteer studies, by using modern and sensitive analytical techniques, without compromising human safety and rather than relying on traditional laboratory animal testing. We consider that it is time to abandon the existing paradigm for risk assessment and regulatory toxicology, which, we firmly believe, is too rigid and outdated, particularly as most of the in vivo test guidelines are outdated and in urgent need of revision.[6]

A SPECIFIC EXAMPLE: DRUG-INDUCED LIVER INJURY

Drug-induced liver injury (DILI) is an excellent example of a problem which desperately requires the intelligent application of the new biotechnologies. It is one of the leading causes of termination of clinical trials of new therapeutic compounds and of

refusal of market approval.[87] DILI is also a major cause of the withdrawal of drugs from the market, well after they have been approved for population-scale use with patients. The failure to detect DILI at a sufficiently early stage results in both a huge financial cost for the pharmaceutical industry and a real human cost for the patient—75% of the individuals who suffer idiosyncratic liver injury either die or require a transplant. Clearly, the traditional preclinical animal testing used in drug discovery and development fails to identify the potential for DILI in humans—indeed, the concordance between animal toxicity and adverse effects in humans is so poor that animal studies do not contribute effectively and accurately to the decision-making process.[88] This is partly because of irreconcilable differences between the enzyme complements involved in drug metabolism in animals and in humans.[89] However, variation among humans is another important contributory factor, which affects drug efficacy as well as susceptibility to adverse effects and their consequences and which therefore affects the usefulness of clinical trials as a background to population-scale use.[90]

Advances in computer modelling and in vitro systems, as well as improved ADME techniques, have made significant contributions to toxicology over the last decade. However, the ability to predict DILI remains a frustratingly elusive target, although some progress is being made in identifying structural alerts for hepatotoxicity as a basis for predictive expert systems.[91] DILI is therefore a key area of focus, not only for the FDA and the IMI, but also for a number of academic and research institutions and international collaborations. For example, the EU Vitrocellomics project[92] involves the development of systems for preclinical predictive drug testing for metabolism and hepatotoxicity, based on in vitro models derived from hESCs and human hepatocyte cell lines, and DILI is a main focus of research at the Hamner–UNC Institute for Drug Safety Sciences, Research Triangle Park, NC, USA,[93] and at the MRC Centre for Drug Safety Sciences, University of Liverpool, UK.[94] Another important collaboration is the International Drug-induced Liver Injury Consortium (iDILIC), which is studying genetic susceptibility to idiosyncratic drug-induced liver injury, with a UK arm of the study, DILIGEN, funded by the Department of Health. One initiative involves collecting DNA from DILI cases and suitable controls for a Genome Wide Association Study (GWAS), with the aim of identifying polymorphisms predictive of the development of drug-related liver injury, which will open up the possibility of prevention by identifying patients at high risk of developing DILI, by means of a simple test performed before treatment with a particular drug begins.[95,96] One encouraging aspect of this initiative is that the data obtained are being made publicly available through the Genevar (GENe Expression VARiation) database (www.sanger.ac.uk/resources/software/genevar/), so that they can be used by other academic and industrial institutions.[97]

The new technologies offer the prospect of solutions to these problems, via directly human-based approaches.[98] For example, human polymorphisms could be revealed and studied in omics systems, leading to the identification of biomarkers of susceptibility and effect.[99] It would be particularly useful, if hepatocytes could be routinely produced from iPSCs, since, not only could this provide a readily-available source of hepatocytes on a large scale, for use in pharmaceutical research and testing in general, but also, some of the iPSCs could be derived from human sub-populations with a greater susceptibility or greater resistance to DILI, or from patients who had already suffered adverse effects in the liver.

The importance of two-way interactions between what happens in the laboratory and in the clinic could not be exaggerated. However, hitherto, there has been no commonly-adopted system for classifying drugs according to their DILI potentials.

However, Chen et al[100] have now proposed a systematic and objective classification scheme, based on 287 drugs representing a wide range of therapeutic categories and daily dosages, that have been marketed for 10 years or more. These authors have provided a method for consistently and constantly annotating the ever-increasing number of drugs in the future, which promises to be of great value in drug discovery and biomarker development.

CONCLUSION

Those with the responsibility for considering the potential benefits and costs of human exposure to chemicals and chemical products are now faced with an increasing complexity and variety of methods based on mechanisms of action and modes of action at the molecular, cellular and tissue/organ/system/organism levels.

For the pharmacotoxicologist, the challenge is to tackle the problems confronting the pharmaceutical industry and especially that of providing new and relatively safe ways of dealing with a number of serious and complex diseases which threaten the quality of life in ageing human populations. The reliability of predictions made for a drug accepted for clinical use is progressively revealed by experience in the clinic and postmarketing surveillance. Indeed, it is the stark revelation of the truth about the inadequacy of preclinical animal testing which has led to great concern and to some dramatic new initiatives.[22-24] While generalised ITS may be appropriate for chemicals and certain kinds of products, it is hard to see how they can be appropriate for pharmaceuticals, where unique ITS specifically designed for particular circumstances are undoubtedly more appropriate. This is partly because of the need to balance likely benefit and potential harm, but also because the human population to be treated can be precisely known, so that factors such as predisposition, disease states and stages, other drugs, age and lifestyle factors can more easily be taken into account. Also, rather than a linear progression from in silico to in vitro to in vivo, with all kinds of uncertainties and unsatisfactory extrapolations, highly-relevant and detailed information, ethically and safely obtained from human subjects themselves, can be applied directly to the tools in order to facilitate their most-meaningful application.

The situation with regard to industrial chemicals and chemicals products such as pesticides, is much less straightforward, since exposure levels and exposed populations are not so predictable and the truth emerges over time, as a result, for example, of epidemiological and occupational evidence, if it ever emerges at all. Here, given the enormous number of chemicals to be evaluated, a more standardised approach is not only unavoidable, but essential.

Cosmetic products represent an in-between situation, as they are intended to be applied to, but not taken up by, the human body, and their ingredients should be relatively biologically inert. However, that situation is becoming more complicated, since cosmetics companies are now developing 'cosmeceuticals', i.e., products which have biologically active ingredients and medical or drug-like benefits. In the USA, they are regarded as pharmaceuticals, whereas in Europe, they are still seen as cosmetics. A crucial question is the extent to which cosmetic ingredients cross the skin or other barriers and whether they accumulate in certain tissues at levels which can approach TTCs. One important route of entry, e.g., for aerosols, is via the respiratory system, for which there are no adequate animal models of the human situation. Happily, there are encouraging opportunities for applying the new technologies with human tissues.[82,86]

The new technologies offer a variety of tools, which tend to reflect the reductionist approach on which progress in science is usually based: understanding a problem usually involves breaking it down into its component parts, then using the information gained to reconsider the whole issue or reconstruct the object of focus or concern. The available biotechnology tools certainly reflect this. Most of them are concerned with measurements on biological material at a lower level of organisation than the intact human body and they can offer answers to only a limited number of specific questions. Thus, as when any craftsman is faced with a full toolbox of complementary tools, it is essential to use them intelligently, according to a scheme which reflects what they individually can or cannot offer, to progress toward the completion of the job to be done, which itself has been clearly defined.

The new technology tools are sophisticated and scientifically advanced, offering the prospect of a mechanistic understanding of the questions being asked. It is vital that the high-quality information they can provide is not seen as a mere prelude to what are really important—the traditional tests in rodents, dogs and nonhuman primates. Animal tests should never be used in attempts to "confirm" the predictions from the non-animal tests and evaluations. In particular, they should never be done because they always have been, or because regulators and company lawyers want to see them done.

The keys to success will be the use of the systems biology approach, backed by bioinformatics, to support the integrated use of in silico and in vitro systems, the omics approaches, and evaluations of epigenetic factors, to permit the use of biomarkers of susceptibility, response and effect to optimise the management of chemicals and chemical products and to promote the well-being and protection of individual patients, workers and citizens.

In the case of medicines, where much of the new effort is focused, e.g., by the FDA and the IMI, the translation of the new technologies from the laboratory to the clinic and back to the laboratory will be vital to their successful application. In the future, population-based treatments, such as the universal use of a small number of antibiotics with a very large number of patients, will progressively be replaced by individualised treatments, e.g., for cancer or cardiovascular disease patients. The drugs developed will be based on precise knowledge, rather than on intuition, and the markets for them will be smaller and themselves more targeted. The days of the animal-models-tell-us-all and one-drug-suits-all philosophies are over.

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