Advances in Experimental Medicine and Biology 745

Michael Balls
Robert D. Combes
Nirmala Bhogal *Editors*

New Technologies for Toxicity Testing

LANDES BIOSCIENCE

Springer

New Technologies for Toxicity Testing

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Recent Volumes in this Series

Volume 737
OXYGEN TRANSPORT TO TISSUE XXXIII
Martin Wolf, Hans Ulrich Bucher, Markus Rudin, Sabine Van Huffel, Ursula Wolf, Duane F. Bruley, David K. Harrison

Volume 738
SELF AND NONSELF
Carlos López-Larrea

Volume 739
SENSING IN NATURE
Carlos López-Larrea

Volume 740
CALCIUM SIGNALING
Md. Shahidul Islam

Volume 741
STEM CELL TRANSPLANTATION
Carlos López-Larrea, Antonio López Vázquez and Beatriz Suárez Álvarez

Volume 742
ADVANCES IN MITROCHONDRIAL MEDICINE
Roberto Scatena

Volume 743
HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND BREASTFEEDING
Athena Kourtis and Marc Bulterys

Volume 744
RAMPs
William S. Spielman and Narayanan Parameswaran

Volume 745
NEW TECHNOLOGIES FOR TOXICITY TESTING
Michael Balls, Robert D. Combes and Nirmala Bhogal

New Technologies for Toxicity Testing

Edited by

Michael Balls, DPhil
FRAME, Nottingham, UK

Robert D. Combes, PhD
Independent Consultant, Norwich, UK

Nirmala Bhogal, PhD
Independent Consultant, Nottingham, UK

Springer Science+Business Media, LLC

Landes Bioscience

Springer Science+Business Media, LLC
Landes Bioscience

Printed in the USA.

Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, USA
http://www.springer.com

Please address all inquiries to the publishers:
Landes Bioscience, 1806 Rio Grande, Austin, Texas 78701, USA
Phone: 512/ 637 6050; FAX: 512/ 637 6079
http://www.landesbioscience.com

The chapters in this book are available in the Madame Curie Bioscience Database.
http://www.landesbioscience.com/curie

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes and Nirmala Bhogal. Landes Bioscience / Springer Science+Business Media, LLC dual imprint / Springer series: Advances in Experimental Medicine and Biology.

ISBN: 978-1-4614-3054-4

Library of Congress Cataloging-in-Publication Data

New technologies for toxicity testing / edited by Michael Balls, Robert D. Combes, Nirmala Bhogal.
p. ; cm. -- (Advances in experimental medicine and biology ; v. 745)
Includes bibliographical references and index.
ISBN 978-1-4614-3054-4
I. Balls, Michael, 1938- II. Combes, Robert D. III. Bhogal, Nirmala. IV. Series: Advances in experimental medicine and biology ; v. 745. 0065-2598
[DNLM: 1. Toxicity Tests--methods. W1 AD559 v.745 2012 / QV 602]

615.9'07--dc23

2011048668

PREFACE

The central theme running through this volume on *New Technologies for Toxicity Testing* is the development and application of advanced techniques for cell and tissue culture, as well as new markers and endpoints of toxicity, as alternatives to the traditional paradigm of relying on data from laboratory animal tests to undertake labelling and risk assessment.

Many such in vivo tests have been shown to be based on outdated and imprecise methodologies and experimental designs, and the large majority have never been subjected to formal validation to establish their reliability and relevance.[1] Moreover, at least as far as pharmaceutical testing is concerned, there is a growing realisation that the standard approach to pre-clinical testing is not proving sufficiently predictive of effects later seen in human volunteer studies and in patients, as shown by post-market surveillance and experience.[2]

In addition to these concerns are those caused by the demands of legislation, such as REACH (Registration, Evaluation, Assessment and Restriction of CHemicals) in the EU and the HPV (High Production Volume) programme in the USA, which require increased testing.[3,4] As a result, and due to limitations of time and money, there is an urgent need to find new ways to prioritise chemicals for further assessment that do not rely on the use of lengthy, expensive, and often unpredictive, animal experiments.

In addition, animal experiments are prone to generate data that are difficult to interpret, particularly due to the use of high dose levels, lack of knowledge of the metabolic fate and internal distribution of the test chemicals, and problems of inter-species extrapolation. Furthermore, the replication of experiments, the testing of mixtures, and one of the cornerstones of toxicology—the dose–response relationship—are more difficult to conduct when undertaking animal experiments. In vitro methods are also more suitable than in vivo systems for testing complex test materials, such as nanoparticles, which are increasingly finding applications in industry, as a result of continuing developments in nanotechnology. In addition, techniques are being developed for the treatment of cell cultures with volatile chemicals, dusts and aerosols, while allowing accurate dosimetry to be performed and the exposure of specific cell types of the respiratory tract.[5] Lastly, in vitro systems can also be used to investigate individual human differences in susceptibility and polymorphisms, to biotransformation of drugs, for example.[6]

The methods and systems described in the various chapters in this volume offer potential ways of overcoming the above problems associated with the usual panoply of rodent and other laboratory animal test methods. Many of these new methods involve the use of cultured human cells from different target organs, exposed to controlled concentrations of test chemicals. These human cells can be derived directly from tissues and organs as primary cells, by cellular immortalisation or genetic manipulation, or from embryonic and adult stem cells, so that they exhibit the in vivo functional differentiation of the tissues from which they were derived.

Tissue cultures can be grown as simple monolayers, or as more-complex three-dimensional (3D) organotypic cultures, which can contain a variety of cell types and involve cellular interactions. They can also be grown on scaffolds to resemble, both architecturally and physiologically, whole organs. Furthermore, methods are being developed to model the supply of nutrients, gases and test chemicals to cells in culture, as well as the transport of waste products, metabolites and excess test chemical away from the cells, in attempts to reproduce physiological and biochemical conditions that would be experienced by the same cells in situ. Complex though these systems are, there are also ways of miniaturising them, so that they can be designed for high-throughput screening (HTS). This also facilitates the replication of experiments under identical conditions of culturing and reduces the amounts of media and test chemicals required.

In addition, several techniques are described, which broaden the range and improve the sensitivity and relevance of toxicity endpoints, investigated both in vitro and in vivo. These include genomics and proteomics, as well as the use of quantum dot imaging biomarkers and PBPK (physiologically-based pharmacokinetic) modelling. These methods can be used to enhance the value of both in vitro and in vivo assays and can also be used with structure-activity relationship (SAR) and quantitative structure-activity relationship (QSAR) modelling to both investigate mechanisms of toxicity and predict hazard before undertaking any laboratory work. Advances in chemical and physical detection methods, such as accelerated mass spectrometry (AMS), as well as computer imaging techniques, are also permitting the earlier use of human volunteers than hitherto, for confirming safety, particularly that of new pharmaceuticals.[7,8]

QSAR studies have advanced greatly due to improved and automated methods for analysing and identifying structurally-alerting features of molecules, and, not surprisingly, they are pivotal to integrated testing schemes (ITS) for detecting chemical toxicity. Moreover, the multitude of results generated by all of these methods, particularly arising from genomic analysis and QSAR modeling, has necessitated the development of new computerised data-handling and statistical methods for comparison, spawning the newly-coined discipline of bioinformatics.

Advances in Tissue Culture

In Chapter 1 of this volume, Glyn Stacey reviews recent methods and advances in tissue culture. He stresses that systems able to reflect the range of different tissue types and diseases in vitro are the most useful for both target organ toxicity and drug efficacy testing. These systems can be obtained particularly by using both embryonic and adult stem cells, and the technique of cellular immortalisation.

Stacey also discusses techniques for scaling-up cultures to provide sufficient cells for high throughput screening, or scaling-down cultures for single cell studies, such as biosensor and 'cell-on-a-chip' applications. Other topics covered in the chapter include alternatives to using fetal calf serum as a component of tissue culture, to decrease batch to batch variation in medium content, and to avoid the need to use animals in painful procedures.[9] Stacey also discusses: the need to control the gaseous environment during tissue culturing; the advantages and disadvantages of primary vs. continuous cell lines; co-culture and organotypic cell systems; and the robustness and amenability of cell culture systems to automation.

Applications of Stem Cells

Tina Stummann and Susanne Bremer discuss the use of adult and embyonic stem (EC) cells by reference to embryotoxicity, cardiotoxicity, hepatotoxicity and neurotoxicity. These cells retain pluripotency and hence can develop into any cell type in the body. The authors note that stem cells have the advantage over primary and immortalised cells of being able to form large populations of stably-differentiated cells representative of different target species including humans.

Cardiotoxicity is investigated by studying the effects of drugs on the functions and activity of ES-derived cardiomyocytes in tissue culture. Hepatotoxicity screening can also be undertaken by using ES cells that differentiate into hepatocytes. The authors note that the susceptibility of differentiating ES cell to chemicals, relative to that of non-differentiating cells, is the basis of embryotoxicity testing in vitro. A test method (the EST–embryonic stem cell test) has been successfully validated and endorsed by ECVAM for screening potential embryotoxic chemicals. The authors discuss the various potential improvements to the EST, as well as the ethical issues concerned with using stem cells, especially ES cells of human origin.

Dynamic Bioreactors

In Chapter 3, entitled 'Trends in Cell Culture Technology,' Uwe Marx discusses dynamic bioreactor systems as a way of improving the ability of cell cultures to mimic in vivo tissue structure and physiological conditions. This requires not only an efficient oxygen and nutrient supply but also a micro-environment which models the in vivo communications between cells, as well as tissue cellular architecture and spatial organization.

Marx provides examples of the further development of this technology, which has led to commercially-available systems. Some of these are bioreactors based on a 3-dimensional network of interwoven hollow fiber membranes transporting gases, nutrients, growth factors and by-products to and away from the cells. A miniaturised membrane-based perfusion bioreactor model of the lymph node is also described. The chapter also includes a discussion of the intriguing idea of modeling an organ in culture based on systems that consist of its basic structural assembly unit, referred to as a 'sub-organoid'; examples being liver lobules and nephrons.

Lastly, Marx discusses attempts that are being made to develop 'organ-on-a-chip' (OOC) systems consisting of micro-bioreactors, which can be used in a 96-well plate format.

Tissue Engineering

Kevin Shakesheff and Felicity Rose introduce the topic of tissue engineering in Chapter 4. This includes the use of tissue scaffolds, the development of bioreactors and the stimulation and culturing of cells in complex cultures in attempts to recreate the conditions under which cells form functional tissues in vivo. Methods for tissue engineering have advanced through the use of co-cultures; extracellular matrix (ECM) signals; 3-D cell aggregates to enhance tissue functionality; provision of conditions allowing signaling between different cell types and anchorage to an ECM.

The authors provide various examples to show how tissue engineering techniques have improved over the last decade, and how they have been used in toxicology and medicine. The formation of more complex tissues in culture, including the liver, by using permeable hollow-fiber bioreactors, is discussed. Work on combining mechanical and biochemical stimuli to enhance the formation of cardiac muscle within scaffolds is also described.

Testing Nanoparticles

Nanoparticles (NPs) are becoming increasingly used in industry and in cosmetics and drugs. In Chapter 5, Amanda Schrand and colleagues discuss the principal types of NPs and their potential health effects and consider the methods available for the toxicity testing of NPs.

Changes in cellular morphology and oxidative stress are two major mechanisms involved in the toxicity of NPs, particular those that are metallised. The effects of particle size, shape, surface area, surface charge, as well as overall chemical composition and functionalisation can all contribute to toxicity.

Schrand et al. provide examples of occupational exposures to NPs. The authors also present data showing how endpoints, such as levels of reduced glutathione and apoptosis, in skin, lung and neuronal cell lines exposed to NPs can be used to quantify toxicity.

Lastly, the authors show how membrane damage (as assessed by changes in membrane potential and LDH [lactate dehydrogenase] leakage); the activation of pro-inflammatory cytokines; and fluorescent probes for fluorescent-activated cell sorting (FACS) can be used for assessing the toxicity of NPS, coupled with cell visualization by using light and scanning electron microscopy (SEM), the latter in conjunction with metal probe detection.

PBPK Modeling

Physiologically-based pharmacokinetic (PBPK) modeling uses mechanistic data on absorption, distribution, metabolism and excretion of a chemical according to mode of administration to predict the time-course of the fate and distribution of parent chemical, metabolite(s) or biomarkers within an exposed organism. In Chapter 6, John Lipscomb discusses the principles of PBPK modeling, and shows how it plays a pivotal role in both in vivo and in vitro toxicity studies.

Examples are provided of how PBPK modeling has been applied in practice, and how it has been validated and used for species extrapolation, from test organisms to humans, as well as to predict the effects of changing exposure route. The author also considers how the data generated from PBPK modeling can improve risk assessment, by allowing internal doses to be taken into account.

Such information can be used to interpret the results of in vitro toxicity experiments, with respect to concentrations tested in tissue culture, compared with the internal dose that the cells in situ would have been exposed to. The data can also be used to design in vitro experiments which involve exposing cell cultures to in vivo-like concentrations of test material.

Predicting Toxicity Using SAR and QSAR

The rationale for, and background to, in silico toxicity prediction (computational toxicology) are discussed by Robert Combes in Chapter 7. This chapter considers both quantitative structure activity relationships (QSAR) and knowledge-based and automated expert system modeling. Examples are provided to show how computational toxicology can be used to predict the toxicity of congeneric and non-congeneric series of chemicals.

The chapter also considers the advantages and disadvantages of computational toxicology, particularly the problems of using relevant molecular descriptors and reliable toxicity data, from properly-conducted experiments on relevant chemicals with clearly-defined structures using samples of high purity and unequivocal results. It is noted that the proper consideration of these issues is crucial to developing a useful training set of chemicals. Suggestions are made to address these problems, as well as how to validate models to assess their predictivity for novel chemicals.

The chapter also illustrates the application of computational models for predicting various toxicity endpoints, including their use for screening drug candidates in the pharmaceutical industry, and in the process of read-across and as part of intelligent integrated tier-testing schemes for preliminary testing of chemicals. Lastly, the chapter considers the effect on predictivity of the existence of 'activity cliffs' (when a small change in molecular properties causes a big change in toxicity in the chemical space for which a model has been developed).

Quantum Dots

In Chapter 8, Shivang Dave and colleagues discuss the use of quantum dots (QDs), which are semiconductor nanocrystals and act as very sensitive biomarkers of toxicity. QDs possess the additional advantages of high brightness, lack of autofluorescence and photo-stability, and moreover they can be used as multiple biomarkers. The authors note that multiplexed QD-based assays have been incorporated into techniques for cell imaging, such as flow cytometry; confocal laser scanning microscopy; fluorescent activated cell sorting (FACS); and fluorescence in situ hybridization (FISH).

A further advantage of QDs is the fact that they can be continuously tuned, by changing the particle size to emit single, specific, well-defined wavelengths. It is also feasible for QDs to be used for simultaneously probing and assaying multiple toxicity endpoints in cells over extended time periods. Furthermore, single QDs emitting multiple emission wavelengths conjugated onto a single microbead structure, enable the simultaneous detection of multiple toxicity events.

In Vitro Organ Models

Further progress in developing more complex and more in vivo-like tissue culture systems is discussed by Tommaso Sbrana and Arti Ahluwalia in Chapter 9, by reference

to the Quasi-Vivo® In vitro Organ Model. This tissue culture system has been designed to mimic cross talk between cells and tissues to provide a realistic physiological environment for the cells to grow in.

The Quasi-Vivo® In-Vitro organ model consists of two different cell culture chambers which simulate a blood vessel and the metabolic system, respectively, when hepatocytes are used. These can be grown as simple monolayers, or as complex 3-D cultures by seeding cells on polylactide-coglycolide scaffolds. Each chamber is environmentally-controlled in order to mimic physiological or pathological conditions in the cardiovascular system. The second chamber is a multi-compartmental device in which different cell types are separately cultured.

Using this apparatus, high-throughput experiments can be performed over several days. They also explain how allometric scaling can be used to design Quasi-Vivo® organ models to represent biotransformation and absorption and exchange processes by the liver, lungs, intestines and skin, of different species.

Validation and Regulatory Status of Non-Animal Tests

Michel Bouvier d'Yvoire and colleagues identify several problems with conventional toxicity testing paradigms in Chapter 10, namely: (a) the need for species extrapolation; (b) lack of mechanistic information; and (c) the use of very high dose levels. The chapter discusses progress in overcoming these problems in the areas of reproductive toxicity; mutagenicity and carcinogenicity; skin absorption and sensitization. With regard to the latter, Sen-si-tiv, is a project for developing a battery of assays for predicting allergenicity, within which genomics and proteomics are being employed, as well as an assessment of the importance of biotransformation in activating prohaptens. The authors refer to the Direct Peptide Reactivity Assay (DPRA), which addresses protein reactivity, the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitization Test (MUSST), which monitor the expression of cell surface markers associated with dendritic cell (DC) activation, which are also currently being evaluated in an ECVAM-coordinated study. Progress with other tests that is described covers the in vitro micronucleus test for genotoxic and non-genotoxic effects, the latter due to effects on the spindle, and the Comet assay for DNA damage in cultured mammalian cells in skin model cultures.

The authors note that four organotypic assays have already received regulatory acceptance for detecting severe ocular irritants, and the advantages of using additional endpoints such as cytokine assays and recovery are also considered. The five methods so far validated for assessing skin corrosion are compared. The EpiSkin and EpiDerm assays are also described. The former was recently validated by ECVAM as a replacement for the Draize skin test.

Other related activities include an investigation of toxico-genomics and toxico-metabonomics, coupled with mass spectrometry, to provide mechanistically-based tests for irritation, using a gene chip and the EPISKIN™ model. The use by industry of the successfully-validated 3T3 NRU in vitro phototoxicity test, as well as future improvements to the test, are also discussed. These include the application of toxicokinetics to improve the prediction of phototoxic effects on the whole organism. The authors also consider the CFU-GM assay for hematotoxicity; fetal cord blood stem cell-based assays; microelectrode arrays (MEA) for simultaneously measuring the activity of multiple neurons; and neurosensors. Lastly, the ECVAM DataBase service on ALternative Methods to animal experimentation (DB-ALM, http://ecvam-dbalm.jrc.ec.europa.eu) is described.

Chip-Based Technologies

In Chapter 11, Yuan Wen and colleagues provide a detailed introduction to microfabrication, microfluidics and chip-based technologies as means for miniaturising in vitro assays and rendering them amenable for use in high-throughput screening. Microfabrication techniques include hard micro-machining and soft lithography. Microlitre, nanolitre and even picolitre volumes of fluids can be handled with precise control provided by pumping, valving, mixing and concentration-manipulation on a micro-scale

The unique advantages of microfluidic devices include: very small consumption of reagents; rapid reaction times; a high level of replication; flexible controls for fluid transport and concentration manipulation; relevant dimensions when dealing with cells and biomolecules; wide applicability and low costs. Therefore, microfabricated devices are being widely used in the pharmaceutical and biotechnology industries.

The chapter describes the use of microfabricated devices for creating 3D extracellular matrix (ECM) structures for cells to grow on, including tumor cells, hepatocytes, chondrocytes, neural cells and embryonic stem cells. Such systems can also be generated as tissue culture scaffolds, and they are used to assaying cytotoxicity; cardiotoxicity; neurotoxicity and metabolism.

Genomics

In Chapter 12, entitled 'The Use of Genomics in Model In Vitro Systems,' Dan Casciano reviews the current status of assays that are based on gene expression profiling, made possible by the sequencing of the human genome, which prompted the development of a new subdiscipline of toxicology termed toxicogenomics, which also includes proteomics, bioinformatics and metabonomics.

Toxicogenomics is concerned with the collection, interpretation, and storage of information about gene and protein activity within particular cells or tissues of an organism in response to toxic substances. It is also a means of identifying and characterizing mechanisms of action of known and suspected toxicants.

Genomics has been adapted for HTS through the development of DNA microarrays, which provide a large-scale medium for matching known and unknown DNA segments based on base-pairing rules. The vast amount of data generated by these collective approaches is being analyzed and interpreted by using bioinformatics, which involves computerised and statistical techniques, as well as information for comparison in the form of gene expression libraries.

Casciano describes key advances in toxicogenomics and discusses several models to study chemically-induced liver injury. He also discusses the application of toxicogenomics to the investigation of basal expression profiles of large numbers of genes in cultured primary human hepatocytes from human donors, varying in age and other factors.

Intelligent and Integrated Testing Schemes

In Chapter 13, Michael Balls, Nirmala Bhogal and Bob Combes have used the information in the preceding chapters to develop a rationale for the toxicity testing of pharmaceuticals, general chemicals, cosmetics, inhaled substances and nanoparticles, based on integrated toxicity testing schemes (ITS). These ITS involve all of the test systems, as appropriate, that are discussed in the volume, deployed in a step-wise manner.

Consideration is also given to the possibilities for using additional techniques, such as virtual tissue modeling; virtual patient populations; clinical imaging and systems biology.

A key feature of the ITS is that the testing of a chemical can be stopped at the point where a risk assessment can be performed and/or it can be classified and labeled in accordance with the requirements of the regulatory authority concerned, rather than following a checklist approach to hazard identification. In addition, the strategies are intelligent in that they are based on the fundamental premise that, in the absence of exposure, there is no hazard.

The four ITS strategies proposed incorporate 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. A general theme in the decision-tree schemes concerns making the data from in vitro tests more-relevant to predicting toxicity in vivo.

Conclusion

We hope that the reader of this volume will have gained a valuable and detailed insight into many of the latest techniques being developed and applied in *in chemico* and in silico prediction, in vitro tissue culture and endpoint detection for toxic hazard identification. We also hope that the reader will be convinced that these advanced methods provide potentially effective ways of screening large numbers of candidate chemicals and prioritising them for further assessment, while providing cheap and cost-effective approaches to testing, prior to whole animal studies, involving laboratory animals and human studies.

By being able to obtain and culture a wide range of differentiated human cells of all major target organs, the problems of species extrapolation, frequently encountered when using laboratory animals, can be overcome. Moreover, the ability to develop complex co-culture systems, and even whole organs under in situ-like physiological and biochemical conditions, is beginning to simplify in vitro to in vivo extrapolation. Added to this is the information from PBPK modeling, which not only is used for selecting relevant target organs, but also facilitates the use of in vitro data for risk assessment.[10,11]

Of course, many of the techniques and methods described in this volume are in the early stages of development, and much work will be needed to ensure their further improvement, optimisation and validation. However, we are confident that this will be achieved and that, just as with the in vitro assays that were validated and granted regulatory acceptance over the last decade, these, and many other new, advanced methods, will likewise become part of the toxicologist's improved toolbox for coping with increasingly stringent and numerous regulatory requirements and test chemicals, while placing less reliance on traditional testing paradigms.

Robert D. Combes, PhD
Michael Balls, DPhil
Nirmala Bhogal, PhD

References

1. Combes RD, Gaunt I, Balls M. A scientific and animal welfare assessment of the OECD health effects test guidelines for the safety testing of chemicals under the European Union REACH system. Altern Lab Anim—ATLA 2004; 32(3):163-208.
2. FDA. Challenge and opportunity on the critical path to new medical products. Silver Spring: US Food and Drug Administration, 2004: Available at: http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/ucm077262.htm.
3. Grindon C, Combes R. Introduction to the EU REACH legislation. Altern Lab Anim—ATLA 2006; 34(Suppl 1):11-14.
4. EPA. HPV Chemical hazard data availability study. Washington, DC: US Environmental Protection Agency, 1998: Available at: www.epa.gov/chemrtk/pubs/general/hazchem.pdf.
5. BéruBé K. Aufderheide M, Breheny D et al. In vitro models of inhalation toxicity and disease. The Report of a FRAME Workshop. Altern Lab Anim—ATLA 2009; 37(1):89-141.
6. Coecke S, Ahr H, Blaauboer BJ, et al. Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM Workshop 54. Altern Lab Anim—ATLA 2004; 34(1):49-84.
7. Combes RD, Berridge T, Connelly J, et al. Early microdose drug studies in human volunteers can minimise animal testing: Proceedings of a workshop organised by Volunteers in Research and Testing. Eur J Pharm Sci 2003; 19:1-11.
8. Langley G, Harding G, Hawkins P, et al. Volunteer studies replacing animal experiments in brain research. Report and recommendations of a Volunteers in Research and Testing Workshop. Altern Lab Anim—ATLA 2000; 28(2):315-331.
9. Jochems CEA, van der Valk JBF, Stafleu FR et al. The use of fetal bovine serum: ethical or scientific problem? Altern Lab Anim—ATLA 2002; 30(2):219-227.
10. Combes RD. Assessing risk to humans from chemical exposure by using non-animal test data. Toxicol in Vitro 2005; 19:921-4.
11. Combes R, Balls M, Illing P, et al. Possibilities for a new approach to chemicals risk assessment—The Report of a FRAME Workshop. Altern Lab Anim—ATLA 2006; 34(6):621-649.

ABOUT THE EDITORS...

MICHAEL BALLS read Zoology at Oxford University, and, after postgraduate studies in Switzerland and postdoctoral research in the USA, returned to Britain in 1966, to become a Lecturer in the School of Biological Sciences at the University of East Anglia. In 1975, he moved to the University of Nottingham Medical School, as a Senior Lecturer, and was later promoted to Reader in Medical Cell Biology and then to Professor of Medical Cell Biology. He is now an Emeritus Professor of the University.

Professor Balls became a Trustee of FRAME (Fund for the Replacement of Animals in Medical Experiments) in 1979, and has been Chairman of the Trustees since 1981. He has been Editor of ATLA (Alternatives to Laboratory Animals) since 1983. He acted as an adviser to the British Government during the drafting and passage through Parliament of the Animals (Scientific Procedures) Act 1986, and, from 1987-1995, was a founder member of the Animal Procedures Committee, which advises the Government on all matters related to animal experimentation.

In 1993, Professor Balls became the first Head of the European Centre for the Validation of Alternative Methods (ECVAM), which is part of the Institute for Health & Consumer Protection of the European Commission's Joint Research Centre, located in Ispra, Italy. ECVAM is responsible for leading and coordinating efforts at the European Union level, which are aimed at reducing, refining and replacing the use of laboratory animal procedures in research, education and testing, through the development and validation of advanced testing methods.

He retired from ECVAM at the end of June 2002, but continues to devote much of his time to FRAME.

ABOUT THE EDITORS...

ROBERT D. COMBES is an independent consultant in toxicology. He has held positions in academia and industry, where he broadened his interests to cover in vitro toxicology, biotransformation, in silico prediction, and the Three Rs (reduction, refinement and replacement) as applied to laboratory animal experimentation.

He was Scientific Director, then Director, of FRAME (Fund for the Replacement of Animals in Medical Experiments) from 1994 to 2008. He has published numerous papers, book chapters and conference proceedings, and has served on many national and international committees.

He is a Fellow of the Society of Biology in the UK, has been on the editorial board of several international journals, the secretary of the United Kingdom Environmental Mutagen Society (UKEMS) and the president of the European Society of Toxicology In Vitro (ESTIV), and is now an honorary member of ESTIV.

ABOUT THE EDITORS...

NIRMALA BHOGAL has a first degree in Biochemistry and Pharmacology, and a PhD in Biochemistry and Molecular Biology from Leeds University, where she was a research fellow from 1991 to 2004 and specialised in rational drug design and protein chemistry. She also obtained a Postgraduate Diploma in Law from Leeds Metropolitan University.

She joined FRAME (Fund for the Replacement of Animals in Medical Experiments) in 2004, and was the Charity's Scientific Director. Her work focused on the use of human volunteers and the application of biotechnology in the development of alternatives to the use of animal in biomedical research, and in particular, in the discovery, development and delivery of therapeutics.

Since leaving FRAME in 2010, Dr Bhogal has established a legal–scientific consultancy.

PARTICIPANTS

Arti Ahluwalia
Centro Interdipartimentale di Ricerca "E. Piaggio"
University of Pisa
Pisa
Italy
and
Kirkstall Ltd.
The Sheffield BioIncubator
Sheffield
UK

Michael Balls
FRAME
Nottingham
UK

Nirmala Bhogal
Independent Consulatant
Nottingham
UK

Michel Bouvier d'Yvoire
REACH Unit
DG Enterprise and Industry
European Commission
Brussels
Belgium

Susanne Bremer
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Silvia Casati
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Daniel A. Casciano
Department of Applied Science
University of Arkansas at Little Rock
Little Rock, Arkansas
USA

Mara Ceridono
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Sandra Coecke
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Robert D. Combes
Independent Consultant
Norwich
UK

Raffaella Corvi
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Liming Dai
University of Dayton
Department of Chemical and Materials Engineering
Dayton, Ohio
USA

Shivang R. Dave
Department of Bioengineering
University of Washington
Seattle, Washington
USA

Chantra Eskes
SeCAM
Agno
Switzerland

Xiaohu Gao
Department of Bioengineering
University of Washington
Seattle, Washington
USA

Laura Gribaldo
Molecular Biology and Genomics Unit
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Claudius Griesinger
European Centre for the Validation of Alternative Methods (ECVAM)
Institute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

Sami Haddad
Département de Santé Environnementale et Santé au Travail
Université de Montréal
Montréal, Québec
Canada

Saber M. Hussain
Applied Biotechnology Branch
Human Effectiveness Directorate
Air Force Research Laboratory
Wright-Patterson AFB, Ohio
USA

Terrance J. Kavanagh
Department of Environmental and Occupational Health Sciences
University of Washington
Seattle, Washington
USA

Holger Knaut
Skirball Institute
Langone Medical Center
New York University
New York, New York
USA

Kannan Krishnan
Département de Santé Environnementale et Santé au Travail
Université de Montréal
Montréal, Québec
Canada

Jens P. Linge
Global Security and Crisis Management Unit
Institute for the Protection and Security of the Citizen (IPSC)
DG Joint Research Centre (JRC)
European Commission
Ispra
Italy

John C. Lipscomb
US Environmental Protection Agency
Office of Research and Development
National Center for Environmental Assessment
Cincinnati, Ohio
USA

Uwe Marx
ProBioGen AG
Berlin
Germany

Torka Poet
Center for Biological Monitoring and Modeling
Battelle Pacific Northwest Division
Richland, Washington
USA

Annett Roi
REACH Unit
DG Enterprise and Industry
European Commission
Brussels
Belgium

Felicity R.A.J. Rose
Wolfson Centre for Stem Cells
Tissue Engineering and Modelling (STEM)
Centre Biomolecular for Studies
School of Pharmacy
University of Nottingham
Nottingham
UK

Tommaso Sbrana
Centro Interdipartimentale di Ricerca "E. Piaggio"
University of Pisa
Pisa
Italy

John J. Schlager
Applied Biotechnology Branch
Human Effectiveness Directorate
Air Force Research Laboratory
Wright-Patterson AFB, Ohio
USA

Amanda M. Schrand
Applied Biotechnology Branch
Human Effectiveness Directorate
Air Force Research Laboratory
Wright-Patterson AFB, Ohio
and
University of Dayton
Department of Chemical and Materials Engineering
Dayton, Ohio
USA

Kevin M. Shakesheff
Wolfson Centre for Stem Cells
Tissue Engineering and Modelling (STEM)
Centre Biomolecular for Studies
School of Pharmacy
University of Nottingham
Nottingham
UK

Glyn Stacey
Division of Cell Biology and Imaging
National Institute for Biological Standards and Control
South Mimms
UK

Tina C. Stummann
European Centre for the Validation of Alternative Methods (ECVAM)
Insitute for Health and Consumer Protection (IHCP)
DG Joint Research Centre (JRC)
Eruopean Commission
Ispra
Italy

Yuan Wen
William G. Lowrie Department
of Chemical and Biomolecular
Engineering
The Ohio State University
Columbus, Ohio
USA

Collin C. White
Department of Environmental
and Occupational Health Sciences
University of Washington
Seattle, Washington
USA

Shang-Tian Yang
William G. Lowrie Department
of Chemical and Biomolecular
Engineering
The Ohio State University
Columbus, Ohio
USA

Xudong Zhang
William G. Lowrie Department
of Chemical and Biomolecular
Engineering
The Ohio State University
Columbus, Ohio
USA

Valérie Zuang
REACH Unit
DG Enterprise and Industry
European Commission
Brussels
Belgium

CONTENTS

CHAPTER 1

CURRENT DEVELOPMENTS IN CELL CULTURE TECHNOLOGY

Glyn Stacey
Division of Cell Biology and Imaging, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, UK
Email: gstacey@nibsc.ac.uk

Abstract: The ideal features of a cell culture system for in vitro investigation depend on what questions the system is to address. However, in general, highly valuable systems will replicate the characteristics and more specifically, the responses, of normal human tissues. Systems that can faithfully replicate different tissue types provide tremendous potential value for in vitro research and have been the subject of much research effort in this area over many years. Furthermore, a range of such systems that could mimic key genetic variations or diseases would have special value for toxicology and drug discovery. In the pursuit of such model systems, there are a number of significant practical issues to consider for their application, which includes ability to deliver with ease, the required quantities of cells at the time needed. In addition any cell culture assay will need to be robust and reliable and provide readily interpreted and quantified endpoints. Other general criteria for cell culture systems include scalability to provide the very large cell numbers that may be required for high throughput systems, with a high degree of reliability and reproducibility. The amenability of the cell culture for down-scaling may also be important, to permit the use of very small test samples (e.g., in 96-well arrays), even down to the level of single cell analysis. This chapter explores the range of new cell culture systems for scaling up cell cultures that will be needed for high throughput toxicology and drug discovery assays. It also reviews the increasing range of novel systems that enable high content analysis from small cell numbers or even single cells. The hopes and challenges for the use of human stem cell lines are also investigated in comparison with the range of eukaryotic cells types currently in use in toxicology.

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

INTRODUCTION

Fundamental Features of Cell Culture Systems for In Vitro Testing

The ideal features of a cell culture system for in vitro investigation depend on what questions the system is required to address. However, in general, the most valuable systems will be those that replicate the characteristics and more specifically, the responses, of normal human tissues. Systems that can faithfully replicate different tissue types provide tremendous potential value for in vitro research and have been the subject of much research effort in this area over many years. Furthermore, a range of such systems that could mimic key genetic variations or disease models would have special value for toxicology and drug discovery.

In the pursuit of such model systems, there are a number of significant practical issues to consider for their final application, which include:

- Availability of appropriate source materials at the time and in the quantities needed;
- Ease and speed of preparation and use;
- Readily interpreted and measured outputs and endpoints;
- Robust and reproducible assay systems; and
- Assays that relate to key cellular functions.

Other general criteria for cell culture systems include scalability of starting cultures to provide the very large cell numbers that may be required for high throughput systems, with a high degree of reliability and reproducibility. The amenability of the cell culture for down-scaling may also be important to permit the use of very small test samples (e.g., in 96-well arrays), even down to the level of single cell analysis.

Shortfalls in Current Systems

When using primary cells or tissues, gametes and embryos, the inherent variability between donors (e.g., age, sex and genotype) can significantly influence the reliability and broad application of the data from the resultant studies. Whilst the use of cell lines can help to overcome these issues, commonly used cell lines are typically of tumor origin, infected with viral agents or otherwise altered from the native state and often have a "transformed" or "dedifferentiated" phenotype. In addition, cell lines passed between a number of laboratories and subjected to large numbers of passages, may show changes in their characteristics due to variations in culture conditions, or become cross-contaminated or switched with another culture.[1,2] They are also known to acquire contaminating micro-organisms, most commonly mycoplasma from other cell cultures, that may adversely affect cell responses.[3] To overcome these problems, it is an important principle of assay development to screen a number of cell lines to ensure the most useful of them are taken up and to obtain these lines only from quality controlled sources such as culture collections (e.g., American Type Culture Collection [ATCC; USA], German Collection of Microorganisms and Cell Cultures [DSMZ; Germany], European Collection of Cell Cultures [ECACC; UK], Interlab Cell Line Collection [ICLC; Italy], Japanese Collection of Research Bioresources [JCRB; Japan], UK Stem Cell Bank [UKSCB]). For general references on sourcing human cells, tissues and cell lines, see Stacey (2007)[4] Stacey and Hartung (2006).[5]

NEW TECHNOLOGIES FOR CELL DELIVERY

Culture Vessels

An ever increasing diversity of cell culture vessel systems have been developed; some are aimed to provide systems for the analysis of live cells, some are for real time studies and others are designed for the more-efficient or more-reliable scaling up of cell cultures. Various systems have been developed for high content analysis by using different combinations of standard culture vessels containing biosensors or ultra-scaled down systems, including 'cell on a chip' techniques, which can give measurements from single cells (for a recent review, see Robitski and Rothermerl).[6] Numerous analytical systems are available commercially and some examples are listed in Table 1. New small volume automated culture chamber systems offer on-line monitoring of culture conditions and in some cases exquisite control of culture conditions and sampling mechanisms for experimental purposes. Others are focused on the automation of the cell culture process (Table 2) and different systems are focused on different solutions including:

- The ability to grow more than one cell culture independently (e.g., 'CompacT', 'SelecT' [TAP]);
- The ability to screen, pick and expand clones of recombinant cells (e.g., 'Cello' [TAP)]; and
- The ability to passage more-challenging cultures, such as human embryonic stem cells, that grow as colonies (e.g., 'Cellhost' [Hamilton].

Some of these systems can employ multiple cell types, with the aim to reproduce some of the complex cell-cell interactions and tissue-like structures observed in vitro. In particular, culture-well inserts permit the coculture of different cells types, both in direct cell-cell contact or communicating solely via secreted factors which can pass across a separating membrane. The principles for establishing such systems have been in place since before the 1970s,[7] and have been applied for the in vitro simulation of functional epithelial polarity[8] and tissue invasion of immune and malignant cells.[9,10] A variety of different insert filter types are available (some are transparent, to facilitate visualization by microscopy) and the type of filter used may influence the response and integrity of the cell monolayer. Measurement of the trans-epithelial resistance (TEER) of such cultures is important, to establish when the functional integrity of the cell layer has been achieved and to monitor the effects of experimental conditions. For a review of these issues, see Lazarovici et al, 2006.[11] Flow cell systems can mimic the intermittent pulses of hydrodynamic pressure and the sheer stress induced by natural blood flow and can reproduce patterns of gene expression and phenotypic features more typical of in vivo tissues.[12] An alternative approach is adopted in low sheer-stress culture systems, where a silicone membrane replaces the usual direct air-medium gas-liquid interface and the medium/cells are rotated to prevent gravitational settling. Cells of different buoyant densities can be grown as structures in suspension with enhanced mass transport characteristics.[13] Yet another system enables metabolic activity to be measured without turbulence in the growth medium, in which cells be grown in "orbital" or "free-fall" modes[14] or a combination of these approaches as the culture develops.[15] Examples of such systems are the Rotating Wall Vessel, Cellon and 'Nova Pod', Novathera (see Table 2).

The scaling up of cell cultures to provide sufficient cells for high throughput test systems is traditionally achieved, for adherent cell cultures, by passaging into larger

Table 1. Automated monitoring and analysis systems for cell culture*

20/20 Technology
Bionomic system: An environmental control stage for live cell, real-time imaging.

Amnis
ImageStream®: Combines the visual capability of microscopy and the statistical rigor of flow cytometry in a single platform, permitting the quantitation of cell structure features not accessible to traditional flow cytometry.

Beckman Coulters
Cellomics KineticScan HCS Reader: The temporal and high-resolution spatial analysis of the physiological processes, dynamic distribution and activity of cellular constituents and morphological features in individual live cells.

Capsant
Hi Spot™ 3Dl aggregates of primary cells, reformed on semi-permeable membranes in multiwell formats to provide 'organ-like' microdots on a biochip for multi-readout, high throughput drug screening.

CompuCyte
iCyte® Automated Imaging Cytometer: Automated high-content cellular analyzers, also designed for higher throughput. Capability to precisely measure cellular DNA content and simultaneously combine these measurements with other molecular markers and cell morphology.

Essen
IncuCyte™: An automated imaging platform that fits within a laboratory incubator and is designed to provide non-invasive live cell imaging; useful for visualizing changes in cells and multicellular structures over time.

Luminex Corporation
Luminex 100 IS System: Designed to permit the simultaneous assay of up to 100 analytes in the single well of a microtiter plate. The system can deliver many assay formats, including nucleic acid assays, receptor-ligand assays, immunoassays and enzymatic assays.

Nikon
BioStation IM: A compact cell incubation and monitoring system that permits live cell imaging. Long-term time-lapse experiments (including studies of cell growth, morphology and protein expression) are possible, through its capacity to provide the consistent environmental control of temperature, humidity and gas concentration and observation by phase-contrast and fluorescence imaging.

Nova Biomedical
BioProfile 400A: A metabolite analyzer for use with mammalian cell cultures.

*The author would like to thank Ms M Gillett (University College London and NIBSC) for assistance in collating this information.

classical cell culture flasks. These flasks, based on the original use of glass tubes and 'medical-flat' bottles, are now obtainable in a variety of forms, made of plastic treated in different ways to promote attachment and growth. Commonly used scale-up systems include roller bottles that are continuously rotated, ridged and layered-surfaces for culture vessels that increase the culture surface area by more than 2-fold (available from numerous suppliers) and stacked static-flask systems (e.g., Cell Cube™ [NUNC]). In addition,

there are well established scale up systems for the culture of cell lines which grow as cell suspensions. These systems are largely focused on the means to maintain growing cultures of homogenous cell suspensions and can employ the use of an internal impellor device within the suspension of cells (e.g., spinner flasks), growth of cells in porous membrane compartments (examples include 'miniPerm' vessel,[16] Dialysis tubing systems[17] and hollow fiber systems such as the Biovest AcuSyst culture system) and the culture of cells in agitated flexible culture bags (e.g., Wave bioreactors [GE Healthcare]). Adherent cells can also be grown on "microcarriers", which enables their growth in bioreactor formats normally used for suspension cell cultures or in 'perfused' bioreactor systems with recirculated culture medium. All of these systems have been developed for culturing of cells to secrete useful products, but could also be used to expand cell cultures for high throughput toxicology experiments, or possibly, for more- complex 3D cell culture assays. For more details, see the recent reviews on such systems by Davis[18] and Portner and Geise.[19]

Table 2. Examples of new systems for cell culture scale-up*

Automation Partnership
CompacT SelecT™: Expansion and maintenance of multiple cell lines, sub-culturing, expanding cell numbers through the seeding of a number of flasks and performing transient transfections.
SelecT: Automated cell culture lines are maintained and expanded in T-175 flasks. The system is designed to prevent cross contamination, even when many cell lines (up to 182) are cultured in parallel. The system can be set to harvest, count and seed without operator intervention—at any time, day or night—to help optimize screening productivity.
Cello™: Cello is designed to culture multiple cell lines in parallel, from seeding through expansion and sub-cloning. The system is aimed to provide the throughput and capacity needed to support several projects running in parallel, with minimal operator involvement. Cello involves the culture of both adherent and non-adherent cell lines with the capability to utilize 384-, 96-, 24- and 6-well plates.
Piccolo™: A fully automated cell culture system for the rapid optimization of recombinant protein production in microbial or insect cell cultures.
Cellmate: Full automation of processes needed to culture cells in roller bottles and T-flasks, by using a robotic arm. It simulates normal cell culture manipulation, thus avoiding the need for process change.

Cellon
RollerCell™/Roller Cell Max™: A roller bottle culture system designed to automate cell seeding, medium changes and trypsinization in units of up to 200 roller bottles that can be automatically coordinated as ten units with a computer. Cellon also supplies the 'Synthecon' 3D bioreactors (see below).

Corning
CellSTACK™: Multilayered (the operator handles up to 10 at a time) stacked culture surfaces.
Cell Cube™: Stacking multilayer tissue culture vessels, providing large surface areas (up to 340000 cm^2) for the culture of adherent cells, operated as a perfusion culture system.

Hamilton
Cellhost™: A fully automated system for colony dissection, culture and expansion of hESCs.

NovaThera Ltd/MedCell
NovaPod™: The 3D culture of cells in air-liquid interface-free medium. Also used with microcarriers.

table continued on following page

Table 2. Continued

NUNC
'Cell Factory': Multilayered (the operator handles up to 10 at a time) stacked culture surfaces with an 'active gassed' system to ensure adequate and uniform buffering.

Synthecon
Rotary Cell Culture Systems™: 'Rotating wall vessel' systems that achieve the low sheer stress culture of cells in air-liquid interface-free growth medium that permits the generation of high viability 3D cell structures. Can be used with cells on microcarriers in small batch cultures (from 10 ml) and perfusion culture systems that permit the 'online' monitoring of pH, oxygen and glucose levels.

Tecan
Cellerity™: Fully automated cell-line maintenance, expansion, harvesting and plating. A modular system that incorporates an integrated CO_2 incubator, cell-counter for determining cell number and viability and refrigerated media storage with in-line warming immediately prior to dispensing.

Wave Biotech LLC
Wave Bioreactor™: Flexible, nongas permeable cell culture bags, which contain growth medium and gas headspace on a rocking platform. Capacity from a 100 ml to 500 l.

*The author would like to thank Ms M Gillett (University College London and NIBSC) for assistance in collating this information.

Culture Environment

The basal media formulations developed in the 1960s, combined with the use of bovine serum, have continued to be used for the culture of most cells, with only minor modifications. However, there have been significant developments in the use of alternative defined media, which remove the need for serum. Such developments were driven initially by the desire to avoid the use of bovine materials for the manufacture of medicinal products (e.g., vaccines, recombinant proteins, gene therapy vectors). This is because serum is an undefined and relatively variable component of cell culture media. Its replacement with defined alternatives is very important for standardizing any work with cell cultures. Serum-free formulations are now available for many different cell culture types.[20,21]

For some time, it has been known that it is possible to enhance the differentiated phenotypes of cell cultures for in vitro assays by adding certain bioactive molecules to the culture medium, such as all-trans retinoic acid or dimethyl sulfoxide (for a summary, see Chapter 14 in Freshney).[22] The further development of such systems for inducing differentiation has been slow, although systems yielding improved neural and hematopoietic cultures have been published.[23,24]

Culture surfaces can clearly have a dramatic effect on the ways in which cells grow. The culture surface treatment may therefore need to be tailored to the particular cell type involved. New techniques for the physical modification of cell culture surfaces are also being investigated for tissue engineering purposes, including polymer preparation[12] and plasma treatment.[24] These may find applications to promote differentiation in cell culture toxicology assays.

Adjusting the gaseous environment can have a significant influence on the biology and performance of a cell culture system and can permit in vitro culture responses that more accurately mimic in vivo tissue responses. Low oxygen tension has long been known to benefit the growth of cell lines,[25] and to promote proliferation and stable characteristics in special cell types such as undifferentiated human stem cell cultures.[27,28] Such environments can be achieved in 'multigas' incubators, which depress environmental oxygen levels by introducing nitrogen and carbon dioxide gases at a controlled rate. Multigas incubators are increasingly used in cell culture, but present a number of technical challenges including:

- The need to 'pregas' culture media to maintain the low oxygen environment;
- The exposure of cultures to intermittent high oxygen levels during culture passage and manipulation; and
- The complexity of the calibration and maintenance of multigas incubators.

An alternative is to use sealable, gas-tight boxes, primed with a mixture of air, carbon dioxide and nitrogen, which can then be placed in any warm-air incubator. A more expensive option is to use 'isolators', which are sealed work environments that can maintain a stable atmosphere and temperature. These have been developed for aseptic processes and for the containment of pathogens. They have also been used for the culture of anaerobic organisms that are highly sensitive to oxygen toxicity and require a stable anaerobic environment. Such isolator systems are also now produced commercially for cell culture and in vitro fertilization procedures (see, for example, the Biospherix website, http://www.biospherix.com).

Changes to other culture conditions, such as incubation temperature, media composition and culture surface, can also be used to modify cell performance. Incubation temperatures below 37 °C can be used to optimize or arrest cell proliferation,[29–31] and may, in some instances, increase the capacity of cells to manufacture secreted proteins.[32]

NEW CELL CULTURES FOR TOXICOLOGY

Primary Cells and Tissues

It is clear that the use of animals as a source of primary tissues and cells is no longer acceptable in many countries,[33,34] or requires careful justification.[5] However, fish and amphibian embryos still appear to present an acceptable source of primary tissues, some of which have value in toxicology.[35] The generation of embryos from fertilized zebra fish (*Danio danio*) eggs enables the production of thousands of embryos in short periods of time, thus making statistically qualified experimentation relatively straightforward, although potentially very time consuming, depending on the difficulty of the assay and the endpoints used. However, it is important to recognize that these are still primary cells from animals and the use of reliable cryopreservation methods would also promote the Three Rs principles by avoiding the wastage of large numbers of cells/embryos that can occur.

Stem Cell Lines

Experimental models for embryotoxicity have been used for many years and attract attention due to the public health significance of toxic events in the early human embryo. Clearly, the use of animal embryos for toxicology has been controversial and the advent

of embryonic stem cell lines has provided an important opportunity to investigate models of embryotoxicity without the ongoing use of embryos. Mouse embryonic stem (mES) cell lines have been used for some years in toxicology and have received attention as the basis of validated test methods which employ undifferentiated mES cells in place of established in vivo embryotoxicity studies.[36,37] The availability of high throughput approaches and the serum-free culture of these cell lines provide significant advantages in method standardization and the potential for testing germline competency (i.e., the ability to regenerate mouse functional embryos by using mES cells) may have additional attractions for the use of these cells in toxicology. However, potential differences from human responses remain a challenge for in vitro toxicology systems based on their use.

Whilst mES cultures were established in the early 1990s, human embryonic stem cells (hESCs) were only isolated relatively recently.[38] They have received a great deal of attention, primarily with regard to their clinical potential. They could also provide a scientifically more valuable alternative to mES cells for toxicology, but methods involving hESCs have yet to be developed and validated for use in embryotoxicity testing. The use of embryoid bodies derived from hESCs may prove valuable since they represent all three germ layers. HESCs certainly have significant theoretical potential, in that they are believed to be pluripotent stem cells and should therefore be able to regenerate cells from almost any tissue. The necessary conditions to regulate cell differentiation to efficiently deliver tissue specific cells in a reproducible way have yet to be resolved. Nevertheless, a number of differentiation protocols are now being established for the production of specific cells types from hESC lines[39-45] and Table 3 illustrates areas where some progress has been made.

hESCs may provide further revolutionary developments in relation to the understanding of disease processes, since they can be derived from embryos affected by genetic disorders. These could potentially provide in vitro models of affected tissues, leading to unprecedented systems with which to study the effects of new drugs in the tissues of individuals suffering from diseases such as cystic fibrosis (CF), fragile X syndrome (Fra-X) and Huntington's disease (HD). The isolation of hESC lines from such sources has already been achieved[46] **(**the example quoted is available from the UK Stem Cell Bank, www.ukstemcellbank.org.uk/**)**.

Table 3. Protocols for derivation of different cell types from hESC lines

Cells Types Derived from hESCs	Examples of Published Detailed Protocols and Discussions of Differentiation Methods
Hepatocyte–like cells	Sullivan et al (2007)[39]
Cardiac cells	Freshney et al (2007)[40]; Loring et al (2007)[41]; Masters et al (2007)[42]; Sullivan et al (2007)[39]
Neural cells	Chojnacki and Weiss. (2008)[43]; Freshney et al (2007)[40]; Loring et al (2007)[41]; Masters et al (2007)[42]; Sullivan et al (2007)[39]; Turksen (2006)[44]
Osteogenic cells	Masters et al (2007)[42]; Sullivan et al (2007)[39]
Haematopoietic stem cells	Loring et al (2007)[41]; Masters et al (2007)[42]; Sullivan et al (2007)[39]; Turksen, (2006)[44]
Pancreatic islet cells	Masters et al (2007)[42]; Sullivan et al (2007)[39] (for experimental design see also Roche et al (2007)[45])
Endothelial cell lineages	Sullivan et al (2007)[39]

Cultures of hESCs also potentially offer an unprecedented opportunity to study human embryonic tissues during the early stages of human development. In the past, the study of embryonic development by using human embryos was considered unethical and even today, many countries, whilst permitting research on early-stage cells, do not allow research on blastocysts cultured for more than 14 days. The establishment of hESC lines permits studies of cells from this critical window of human development, which could yield significant benefits for the health of unborn children. Nevertheless, it will be critical to establish means of determining whether the hESC cultures actually reflect early human development with sufficient precision.

Cloning techniques to provide hESC lines that have specified genotypes, by the nuclear transfer technique, could provide panels of hESC lines for use in a broad range of cell-based systems for drug discovery and toxicology. However, the first breakthroughs in this technology (in Korea) were largely discredited and the associated publications were withdrawn. So far, the efforts of other workers have not revealed an effective method and it is clear this approach will take some time to yield a technique that is efficient. An alternative and potentially more fruitful approach to the development of reliable human nuclear transfer technology is to transfer human nuclei into enucleated animal eggs.[47] This approach may provide useful cell lines for research and testing purposes and may also provide the methodology required to generate pure hESC lines of defined genotypes that may also be appropriate for therapeutic applications. The availability of these cells for research also offers novel potential tools for drug discovery and toxicology, but will require careful validation, to evaluate the influence of the nonhuman elements of the cells, such as mitochondria, which could influence the relevance of such cells to human toxicology. The development of nuclear transfer technology to generate individual specific hESC lines would be a significant step, since, not only would this permit patient-specific cell therapy, it would also offer the potential to deliver hESC lines with the characteristics of individuals from specific genotypic groups or with specific genetic disorders. This would be a key step in the development of new cell and tissue models that would permit the study of toxicological systems in different population groups, as well as being useful in in vitro drug discovery research on specific disorders. The development of human nuclear transfer techniques has been slow, but promise has been demonstrated in the establishment of more-efficient techniques involving primate cells.

Whilst hESCs have a particularly wide potential in toxicology, it should be borne in mind that many tissues derived from them may well retain features of cells in the early stages of development that may not closely relate to responses or resistance to toxicological effects in adult somatic tissues. Accordingly, it will be important to explore the potential of non-embryonic stem cell lines.

Another, more-recent development has been the publication of new technology to manipulate somatic cell types, in order to derive stem cell lines which have the characteristics of hESCs.[48,49] This involves the genetic modification of the donor cell nuclei to over-express a combination of two or more reprogramming factors or oncogenes, to induce a pluripotent state (induced pluripotency stem cells or iPS cells). This approach is still under development,[50] but may offer an extremely efficient way of establishing genotype-specified cell lines with the potential of hESCs. It will be important to coordinate the data being generated on pluripotent cell lines, to enable researchers to compare information on different cell lines and this is currently being captured in a number of databases. One database designed to list internationally available human pluripotent cell

lines is called hESCreg (www.hesreg.eu). This active database has data on more than 600 cell lines, with documented data on derivation, culture and characterization.

Immortalized Primary Cells and Recombinant Cell Lines

A number of continuous cell lines have been proposed for use in toxicology, including Madin Darby Canine Kidney (MDCK) cells, the hepatocyte line, HepG2 and Chinese Hamster Ovary (CHO) cells. However, the available cell lines are primarily of tumor origin (e.g., MDCK, HepG2), may not show phenotypes typical of mature tissue and may be deficient in certain key functions. Over the last two decades, considerable efforts have been made to generate immortalized primary cultures by using various recombinant vectors, but these have suffered, due to the transforming effects of the mechanism of unlimited replication in these cells (e.g., SV40 Large T antigen) and, as in other cell lines, there are key failings in the biochemical transformations and phenotypes of many of these cells. Attempts to provide better cell substrates by this approach have been made by using recombinant DNA vectors with conditional expression, where the transformed replication phenotype is suppressed under certain conditions (temperature sensitive SV40 antigen, heavy metals and ecdysone in baculovirus vectors).[51] Thus, each recombinant cell line grows normally under permissive conditions, but when the conditions are altered, the cells stop growing and, hopefully, express more-differentiated characteristics. However, the cell lines established by using these systems have not seen a very active uptake in toxicology; in some cases, this may have been due to the leaky control of expression, which allows the growth of a proportion of the cells, even when restrictive conditions are applied. However, one system that seems to offer hope for the generation of bespoke cell lines with "normal" characteristics, is the use of telomerase-expressing vectors that subvert the normal mechanisms of telomere shortening that are closely associated with cell ageing and senescence—a natural restriction in the use of primary cells. Several examples of cell lines generated from different tissue cell types have been established by using this system,[52,53] although cotransfection with oncogene-expressing vectors may be required.[54]

THE IMPORTANCE OF GOOD CELL CULTURE PRACTICE

Drawing on the experience of experts in animal cell culture, Coecke et al[21] described the principles of Good Cell Culture Practice, i.e., a minimum standard of practice that should be adopted by anyone working with in vitro cell cultures. These widely applicable principles recognized that more-detailed relevant guidance might need to be developed for complex stem cell cultures. Also in 2007, a group called the International Stem Cell Banking Initiative was set up with funding from the International Stem Cell Forum (ISCF, see www.stemcellforum.org). This group coordinated input from stem cell researchers and stem cell banking centers in over 17 countries and produced a consensus on best practice for the banking, characterization, safety testing, quality control and distribution of hESC lines. This guidance has now been published.[55]

The International Stem Cell Initiative, coordinated by Professor Peter Andrews at the University of Sheffield, UK, has published standardized protocols for the characterization of a large number of hESC lines (International Stem Cell Initiative, www.stemcellforum.org/).[55] This project has now entered a second phase, in which a large number of laboratories are evaluating defined growth media for hESC lines and

studying their genetic stability under the different culture conditions. Such international collaborations are important for providing standardization in a field where the cell cultures used are highly challenging and require new and more stringent approaches to the delivery of reliable and reproducible data from in vitro culture experiments.

REGULATION

The routine use of animal tissues for toxicology has been a central element in testing for many years, but is now questioned widely from an ethical and scientific perspectives.[33,34] There are formal governmental and international efforts to eliminate the use of animals for testing purposes, as exemplified by the European Directive 86/609/EEC. For a review of the international frameworks for developing alternatives to the use of animals, see Speilmann.[57] The progressive move toward the use of human tissues and cells also raises a range of issues which are vital to their efficient procurement and use. An expert group coordinated by the European Centre for Validation Alternative Methods (ECVAM; www.ecvam.jrc.eu) delivered 23 recommendations relating to the legal and ethical issues, biohazards, logistics and end user requirements.[58] These recommendations address the need for the more-effective and safe use of human tissues. In addition, toxicologists using cells and tissues must now address requirements for traceability and accountability when using human tissues, to ensure compliance with legal requirements for obtaining fully informed consent from donors and, in some cases, for obtaining licenses for the storage of the tissue (e.g., www.hta.org.uk). Specific approaches and protocols are also needed to ensure the appropriate establishment, standardization and validation of cell-based assays, to address the key challenges of tissue procurement, microbiological contamination, cell line stability and cell line cross-contamination.[4,5,55] In addition, due to controversy over the use of human embryos in research, some countries require researchers to obtain formal permission for the work they wish to perform on embryos or hESC lines and researchers may be expected to comply with formal codes of practice for hESC line work (e.g., Code of Practice for the Use of Human Stem Cell Lines, 2010, www.ukstemcellbank.org.uk/). It is likely that, as new developments such as iPSC technology show potential for personal medical benefits, public awareness will grow and potentially demand greater scrutiny over the use of human tissues for research and development.

CONCLUSION

The practice of cell and tissue culture, once thought to be dependent on "green-fingers", may be more amenable to automation than was envisaged and the potential consequence of this will be to provide much more-reproducible toxicology data from in vitro cell-based assays. In addition, the development of human stem cell lines with the potential to generate human models of specific cell types and possibly to mimic tissues, raises an unprecedented potential for developing in vitro assays that will hopefully produce data of direct relevance to the toxicology of human cells in the body. Further developments in the derivation of stem cells lines with bespoke genotypes will further enhance the possibilities for studying inherited disease and drug discovery. However, careful evaluation of the cell systems generated from stem cell types will be needed to assure they truly represent mature cells and have sensitivities to toxicants that reflect real-world susceptibility.

REFERENCES

1. MacLeod RAF, Dirks WG, MatsuoY et al. Widespread intra-species cross-contamination of human tumour cell lines arising at source. Int J Cancer 1999; 83:555-563.
2. Masters JR, Thomson,JA, Daly-Burns B et al. Short tandem repeat profiling provides an international reference standard for human cell lines. Proc Natl Acad Sci USA 2001; 98:8012-8017.
3. Rottem S, Naot Y. Subversion and exploitation of host cells by mycoplasma. Trends in Microbiol 1998; 6:436-440.
4. Stacey GN. Sourcing human embryonic stem cell lines. In: Sullivan S, Cowan C. and Eggan K, eds. Human Embryonic Stem Cells: A Practical Handbook. Chichester: John Wiley and Sons, 2007:11-24.
5. Stacey GN, Hartung T. Availability, standardisation and safety of human cells and tissues for drug screening and testing. In: Marx U, Sandig V, eds. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH Verlag, 2006:231-246.
6. Robitski AA, Rothermerl A. An overview on bioelectronic and biosensoric microstructures supporting high-content screening in cell cultures. In: Marx U, Sandig V, eds. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH Verlag, 2006:79-97.
7. Dickson JA, Suzangar M. A predictive in vitro assay for the sensitivity of human solid tumours to hyperthermia (42 degrees C) and its value in patient management. Clin Oncol 1976; 2:141-55
8. Chambard M, Verrier B, Gabrion J et al. Polarization of thyroid cells in culture: evidence for the basolateral localization of the iodide "pump" and of the thyroid-stimulating hormone receptor-adenyl cyclase complex. J Cell Biol 1983; 96:1172-1177.
9. Elvin P, Wong V, Evans CW. A study of the adhesive, locomotory and invasive behaviour of Walker 256 carcinosarcoma cells. Exp Cell Biol 1985; 53:9-18.
10. McCall E, Povey J, Dumonde DC. The culture of vascular endothelial cells to confluence on microporous membranes. Thromb Res 1981; 24:417-31.
11. Lazarovici P, Li M, Perets A et al. Intelligent biomatrices and engineered tissue constructs: in vitro models for drug discovery and toxicity Testing. In: Marx U, Sandig V, eds. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH Verlag, 2006:1-51.
12. McKinney VZ, Rinker KD, Truskey GA. Normal and shear stresses influence the spatial distribution of intracellular adhesion molecule-1 expression in human umbilical vein endothelial cells exposed to sudden expansion flow. J Biomech 2006; 39:806-817.
13. Martin Y, Vermette P. Bioreactors for tissue mass culture: design, characterization and recent advances. Biomaterials 2005; 26:7481-7503.
14. Kofidis T, Lenz A, Buoblik J et al. Pulsatile perfusion and cardiomyocyte viability in a solid three-dimensional matrix. Biomaterials 2003; 24:5009-5014.
15. Klaus DM. Clinostats and bioreactors. Gravit Space Biol Bull 2001; 14:55-64.
16. Bruce MP, Boyd V, Duch C et al. Dialysis-based bioreactor systems for the production of monoclonal antibodies—alternatives to ascites production in mice. J Immunol Methods 2001; 264:59-68.
17. Pannell R, Milstein C. An oscillating bubble chamber for laboratory scale production of monoclonal antibodies as an alternative to ascitic tumours. J Immunol Methods 1992; 146:43-48.
18. Davis J. Systems for cell culture scale-up. In: Stacey GN, Davis J, eds. Medicines from Animal Cells. Chichester: John Wiley and Sons, 2007:1435-172.
19. Portner R, Geise C. An overview of bioreactor design, prototyping and process control for reproducible three-dimensional tissue culture. In: Marx U, Sandig V, eds. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH Verlag, 2006:53-70.
20. Jayme D. Development and optimization of serum-free and protein-free media. In: Stacey GN, Davis J, eds. Medicines from Animal Cells. Chichester: John Wiley and Sons, 2007:29-44.
21. Coecke S, Balls M, Bowe G et al. Guidance on good cell culture practice. A report of the second ECVAM task force on good cell culture practice. Altern Lab Anim—ATLA 2005; 33(1):1-27.
22. Freshney IR. Culture of Animal Cells: A Manual of Basic Technique, 4th ed. Chichester: John Wiley and Sons, 2004.
23. Greene JJ, Brophy CI. Induction of protein disulfide isomerase during proliferation arrest and differentiation of SH5Y neuroblastoma cells. Cell Mol Biol 1995; 41:473-80.
24. Fleck RA, Athwal H, Bygraves J et al. Optimisation of NB-4 and HL-60 differentiation for use in opsonophagocytosis assays. In Vitro Cell Dev Biol Anim 2003; 39:235-242.
25. Howard D, Buttery LD, Shakesheff KM et al. Tissue engineering: strategies, stem cells and scaffolds. J Anat 2008; 213:61-72.
26. Carswell KS, Weiss JW, Papoutsakis ET. Low oxygen tension enhances the stimulation and proliferation of human T-lymphocytes in the presence of IL-2. Cytotherapy 2000; 2:25-37.
27. D'Ippolito G, Diabira S, Howard GA et al. Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone 2006; 39:513-22.

28. Ezashi T, Das P, Roberts RM. Low O_2 tensions and the prevention of differentiation of hESCs. Proc Natl Acad Sci USA 2005; 102:4783-4788.
29. Enninga IC, Groenendijk RT, van Zeeland AA et al. Use of low temperature for growth arrest and synchronization of human diploid fibroblasts. Mutat Res 1984; 130:343-352.
30. Fischer SM, Viaje A, Harris KL et al. Improved conditions for murine epidermal cell culture. In Vitro 1980; 16:180-188.
31. Yang QR, Berghe DV. Effect of temperature on in vitro proliferative activity of human umbilical vein endothelial cells. Experientia 1995; 51:126-132.
32. Takahashi K, Tereda S, Ueda H et al. Growth rate suppression of cultured mammalian cells enhances protein productivity. Cytotechnology 1994; 15:57-64.
33. Milstein, J, Grachev V, Padilla A et al. WHO activities towards the three Rs in the development and control of biological medicines. Dev Biol Stand 1996; 86:31-39.
34. Hartung T. Three Rs potential in the development and quality control of pharmaceuticals. ALTEX 2001; 18:3-11.
35. Rubenstein AL. Zebrafish assays for drug toxicity screening. Expert Opin Drug Met Toxicol 2006; 2:231-240.
36. Scholtz G, Pohl I, Genschow E et al. Embryotoxicity screening using embryonic stem cells in vitro: correlations with in vivo teratogenicity. Cells Tissues Organs 1999; 165:203-211.
37. Rolletscheck A, Blyszczuk P, Wobus AM. Embryonic stem cell derived cardiac neuronal and pancreatic cells as model systems to study toxicological effects. Physiol Rev 2005; 85:635-67.
38. Thomson J, Itskovitz-Eldor J, Shapiro S et al, Embryonic stem cell lines derived from human blastocysts. Science 1998; 282:1145-1147.
39. Sullivan S, Cowan CA, Eggan K, eds. Human Embryonic Stem Cells: The Practical Handbook. Chichester: John Wiley and Sons, 2007.
40. Freshney IR, Stacey GN, Auerbach J, eds. Culture of Human Stem Cells. Hoboken: John Wiley and Sons, 2007.
41. Loring JF, Wesselschmidt RL, Schwartz PH, eds. Human Stem Cell Manual, A Laboratory Guide. New York: Academic Press, 2007.
42. Masters JR, Palsson BO, Thomson JA, eds. Human Cell Culture V6, Embryonic Stem Cells. Dordrecht: Springer, 2007.
43. Chojnacki A, Weiss S. Production of neurons, astrocytes and oligodendrocytes from mammalian CNS stem cells. Nat Protoc 2008; 3:935-40.
44. Turksen K. Human Embryonic Stem Cell Protocols. Methods Mol Biol 2006; 331:1-12.
45. Roche E, Ensenat-Waser R, Vicente-Salar N et al. Insulin-producing cells from embryonic stem cells experimental considerations. Methods Mol Biol 2007; 407:295-309.
46. Pickering SJ, Minger S, Patel M et al. Generation of human embryonic stem cell line encoding the cystic fibrosis mutation deltaF508, using preimplantation genetic diagnosis. Reprod Biomed Online 2003; 10:390-397.
47. Chen Y, He ZX, Liu A et al. Embryonic stem cells generated by nuclear transfer of human somatic nuclei into rabbit oocytes. Cell Res 2003; 13:251-63.
48. Takahashi K, Tanabe K, Ohnuki M et al;. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131:861-872.
49. Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318:1917-1920.
50. Nakagawa M, Koyanagi M, Tanabe K et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 2008; 26:101-106.
51. Jeffs SA. Expression of recombinant biomedical products from continuous mammalian cell lines. In: Stacey GN, Davis J, eds. Medicines from Animal Cells. Chichester: John Wiley and Sons, 2007: 61-78.
52. Chapman EJ, Hurst CD, Pitt E et al. Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. Oncogene 2006; 25:5037-5045.
53. Morales CP, Gandia KG, Ramirez RD et al. Characterisation of telomerase immortalised normal human oesophageal squamous cells. Gut 2003; 52:327-333.
54. Maeda T, Tashiro H, Katabuchi H et al. Establishment of an immortalised human ovarian surface epithelial cell line without chromosomal instability. Br J Cancer 2005; 93:116-123.
55. International Stem Cell Banking Initiative . Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes. Stem Cell Rev Rep 2009; 5:301-314.
56. International Stem Cell Initiative, Adewumi O, Aflatoonian B, Ahrlund-Richter L et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 2007; 25:803-816.
57. Spielmann H. Ethical environment and scientific rationale towards in vitro alternatives to animal testing: where are we going. In: Marx U, Sandig V, eds. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH Verlag, 2006:251-267.
58. Anderson R, O'Hare M, Balls M et al. The availability of human tissue for biomedical research—the report and recommendations of ECVAM workshop. Altern Lab Anim—ATLA 1998; 26(6):763-777.

CHAPTER 2

EMBRYONIC STEM CELLS IN SAFETY PHARMACOLOGY AND TOXICOLOGY

Tina C. Stummann and Susanne Bremer*
European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy
**Corresponding Author: Susanne Bremer—Email: susanne.bremer@jrc.it*

Abstract: Embryonic stem (ES) cells undergo self-renewal and are pluripotent, i.e., they can give rise to all the types of specialised cells in the body. Scientific knowledge on ES cells is increasing rapidly, leading to opportunities for establishment of ES cell-based in vitro tests for drug discovery, preclinical safety pharmacology and toxicology. The main properties of ES cells making them useful in in vitro assays are that they have a normal diploid karyotype and can provide a large number of cells for high-throughput assays. Human ES cells additionally have the potential to provide solutions to problems related to interspecies differences and methods for screening for human polymorphisms, thus supporting robust human hazard identification and optimised drug discovery strategies. Importantly, ES cell based assays could be potential tools to reduce and perhaps replace, animal experiments. This chapter will describe ongoing research in the use of ES cells in toxicology and safety pharmacology, focusing on the major areas of progress, namely, embryotoxicology, cardiotoxicology and hepatoxicology.

INTRODUCTION

Stem cells are defined as cells capable of both self-renewal and multi-lineage differentiation, i.e., the process whereby they give rise to the various differentiated cell types of the body tissues. They can be broadly classified into embryonic or adult stem cells, depending on their developmental status, as reviewed in detail by the US National Institutes of Health.[1] Adult stem cells are multipotent, i.e., they can yield the specialised cell types of the tissues from which they originate. Embryonic stem (ES) cells, isolated from the morula or the inner cell mass of blastocyst-stage embryos, are the only stem cells

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

considered to be pluripotent, as they can give rise to the differentiated cell lineages of all three germ layers (endoderm, mesoderm and ectoderm), as well as to germ cells (Fig. 1).

In 1981, the first ES cell lines were isolated from murine embryos,[2,3] while the isolation of human ES cells occurred two decades later.[4] Based on experience with the mouse cells, knowledge of human stem cell derivation, propagation and in vitro differentiation into specific cell lineages has increased rapidly. During the last decade, a variety of studies have demonstrated spontaneous and directed differentiation of human ES cells in vitro, into cells with morphological and functional characteristics resembling those of many different cell types, including various neuronal subtypes and glial cells,[5,6] insulin-producing β-cells,[7] endothelial cells and haematopoietic cells,[8] cardiomyocytes[9,10] and hepatocytes.[11]

The prior establishment of reproducible and relevant maintenance and differentiation protocols is of great importance to the practical use of ES cells as tools in drug discovery and safety assessments. ES cells, as compared to primary cell cultures or immortalised cells, offer considerable advantages for use in in vitro model systems. They are genetically normal (diploid) and do not exhibit the donor-dependent variability which is characteristic of primary cells. ES cells also have a strong proliferation capacity as compared to primary cells and they can be maintained in culture for a long time and grown on a large scale, avoiding problems of irregular availability. Furthermore, a unique strength of mouse ES cells is their

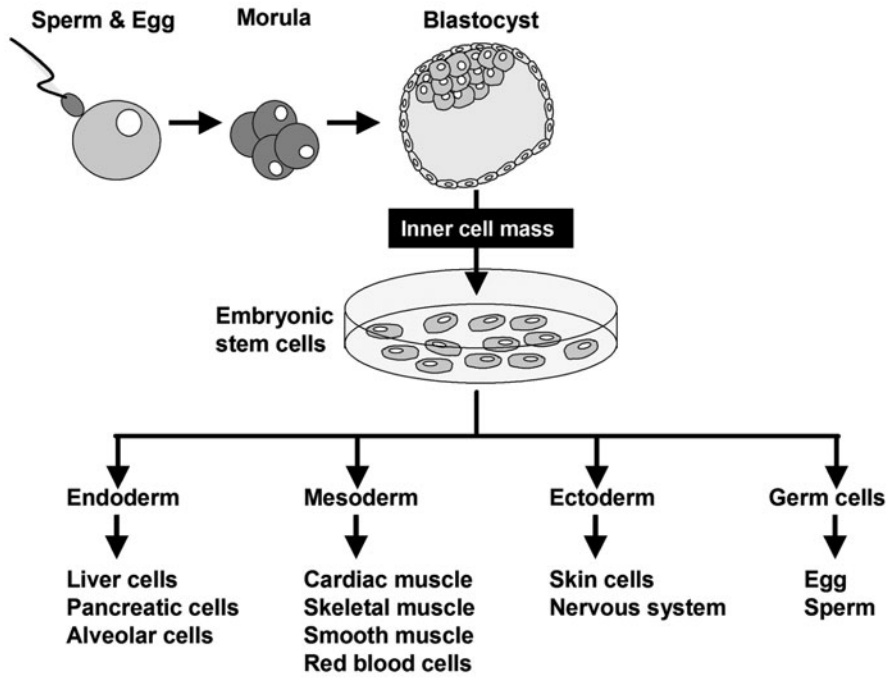

Figure 1. Embryonic stem cells can be isolated from the inner cell mass of blastocyst stage embryos. They are pluripotent, as they can differentiate into the cell lineages of all three germ layers (endoderm, mesoderm and ectoderm), as well as into germ cells. Modified with permission from Stummann and Bremer. Current Stem Cell Research and Therapy 2008; 3:118-131.[19]

ability to undergo mitotic recombination (in which homologous chromosomes exchange DNA) at a relatively high frequency, permitting the selection of homozygous genetically modified clones, which can be used to derive precisely genetically modified mice.[12]

The isolation of the first human ES cell line opened the door to an increasingly important branch of ES cell research,[4] since human ES cells hold great promise as a source of cells for transplantation and for gene therapy. In addition, human ES cells provide a model for studying early human embryonic development, which was previously largely confined to the use of animal models. Importantly, human ES cells could contribute to avoiding the difficulties in identifying therapeutic effects and toxicological hazard, which result from interspecies differences. Furthermore, they may provide improved understanding of human inter-individual differences due to genetic polymorphisms.

MAJOR PROGRESS IN THE USE OF EMBRYONIC STEM CELLS IN SAFETY PHARMACOLOGY AND TOXICOLOGY

ES cells and especially human ES cells, offer great advantages in pharmacological studies and safety assessments, as described in the Introduction. The opportunities for using pluripotent ES cells, specific cell types differentiated from ES cells, or cell lines established from ES cells for the establishment of in vitro tests in the various areas of drug discovery and safety assessments have, however, only been investigated to a limited extent, as is evident from previous reviews.[13-19] This chapter will describe ongoing European Commission (EC) supported research on establishment of safety tests focusing on embryotoxicity, cardiotoxicity and hepatotoxicity.

Screening for Cardiotoxicity

Screening for cardiotoxicity is not specifically requested by the new European Union (EU) Chemicals Policy, but is addressed for industrial chemicals produced or marketed at more than 10 tonnes/year, as part of the repeated dose in vivo studies.[20] In contrast, assessment of cardiotoxicity is a key activity in drug development programmes in the pharmaceutical industry.[21,22] Drug-induced delayed ventricular repolarisation is manifested as QT interval prolongation in the electrocardiogram. It is considered to be an important risk factor for the potential life threatening form of ventricular tachycardia, torsades de pointes. Due to the potential lethal effect of this condition, the European Medicines Agency (EMA) recommends that new drugs are tested according to the guideline for nonclinical testing for drug-induced ventricular repolarisation disturbances.[22] The guideline includes electrophysiological studies in vivo, as well as in vitro. The in vitro assays can be at the molecular level (affinity to heterologously expressed hERG channels), at the cellular level (hERG channel blockage in primary cardiomyocytes), at the tissue level (repolarisation assays on papillary muscle or Purkinje fibres), or at the organ level (Langendorff heart). Currently, mainly animal tissue and cell preparations are used, as human preparations are only occasionally available.

The unintended cardiac toxicity of new drugs is among the leading causes of market withdrawals.[23,24] In fact, several noncardiac blockbuster drugs have been taken of the market as they were found to cause torsades de pointes in humans, although no indications for this was found in animals. A multi-national pharmaceutical survey has demonstrated that the

negative concordance of human cardiotoxicity with animal cardiotoxicity is approximately 20%.[25] These facts strongly support the use of cardiotoxicity tests based on human tissue.

Human ES cells can be differentiated into cells that have structural properties and functional activities of cardiac cells, including long action potential duration, cardiac ion channels and responsiveness to β-adrenergic and muscarinic pharmacological modulation.[9,10] These findings provided the scientific rationale for establishment of the Specific Targeted Research Project, "InVitroHeart" (www.invitroheart.org), which is funded by the EC. The project aims to develop in vitro assays for cardiotoxicity based on human ES cells derived cardiomyocytes, which reflect the properties of human primary cardiac tissue. Standardised human cardiomyocyte cultures will be combined with electrophysiology and cytotoxicity micro-sensor platforms, in order to allow for rapid drug screening. The advantages of such assays would be their high-throughput format, the assessment of functional endpoints and the avoidance of interspecies differences (Fig. 2). Furthermore, the screening of human ES cell-derived cardiomyocytes for several polymorphisms may help to identify predisposition to drug-induced torsades de pointes due to genetic polymorphism.[26] Such assays could provide valuable information, not only on cardiac safety assessments, but also for development of cardiac pharmaceuticals.

Screening for Hepatotoxicity

Drug-induced hepatotoxicity is among the most frequent reasons cited for the withdrawal of approved drugs and accounts for more than 50% of cases of acute liver failure, making screening for hepatotoxicity of high priority in safety pharmacology.[23,27,28] Specific assessment of hepatotoxicity is not required by the new EU Chemicals Policy, but it is addressed for industrial chemicals produced or marketed at more than 10 tonnes/ year, as part of the repeated dose in vivo animal studies.[20]

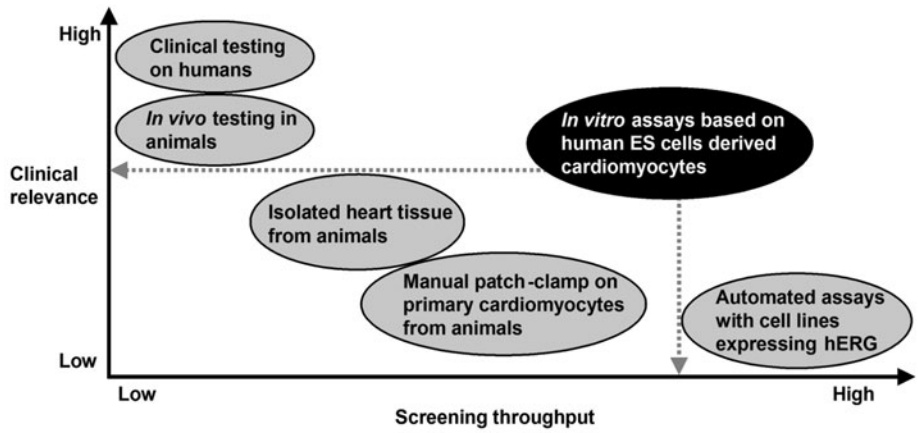

Figure 2. In vitro cardiotoxicity assays based on cardiomyocytes derived from human ES cells have the advantage of combining high screening capacity with high clinical relevance, as compared to current safety pharmacological methods, which mainly involve tissue and cell preparations of animal origin. Modified with permission from Stummann and Bremer. Current Stem Cell Research and Therapy 2008; 3:118-131.[19]

A multi-national pharmaceutical company survey on the toxicity of drugs showed little (~55%) positive concordance between human and animal hepatotoxicity.[25] The use of human cell-based systems as surrogate models for liver toxicity in vivo is, therefore, of great importance. The potential availability of an unlimited source of human cells was suggested by the demonstration of human ES cell differentiation into cells with hepatocyte structural properties and functional activities, such as cytochrome P-450 (CYP P-450) metabolism, albumin production, glycogen storage and indocyanine green uptake and release.[11,29] Furthermore, the exposure of hepatocyte-like cells derived from human ES cells to the hepatotoxicant, CCl_4, resulted in an elevation of liver enzymes, supporting the potential usefulness of this kind of in vitro model.[30]

"Vitrocellomics" is a Specific Targeted Research Project funded by the EC. The aim is to establish in vitro hepatotoxicity assays based on hepatic cells derived from human ES cells, which reliably reflect the properties of human hepatic tissue. Screening assays will be developed by combining the hepatic model system with micro-sensor platforms for measuring absorption, cytotoxicity and metabolism. Such an approach would allow a panel of endpoints to be evaluated in parallel, which would be of great importance due to the multiple mechanisms involved in liver damage.[27] Other advantages would be the availability of high-throughput formats, the assessment of functional endpoints and the avoidance of interspecies differences. Furthermore, even though most patients may tolerate a drug well, others may suffer severe liver damage due to their specific genetic variations in drug metabolising enzymes.[31] Screening for polymorphisms in the hepatocytes derived from human ES cells could offer a solution to this problem.

THE USE OF EMBRYONIC STEM CELLS IN EMBRYOTOXICITY TESTS

The occurrence of birth defects and malformations in humans is an important social and healthcare issue. Major birth defects are observed in 2 to 8% of infants and fetuses.[32,33] They can be inherited or can result from exposure of the mother and embryo to infections, drugs and occupational and environmental chemicals, or to other ingested substances during critical and sensitive periods of development. Thalidomide, a sedative given to pregnant women in the late 1950s and early 1960s to counter early morning sickness, is a teratogenic agent that caused more than 7,000 children to be born with malformations.[34] This disaster led to the implementation of rigid regulatory requirements in order to increase the protection of users, consumers and patients.

Detailed reproductive/developmental toxicity testing is part of drug development programmes.[35] The 3-study design is considered adequate for most medical products and the guideline suggests the use of 16-20 litters of each species per dose tested and the inclusion of one control and two dose levels.[35] Hence, 96-120 animals per assay per 3-study design are required as minimum. Also, industrial chemicals must be tested for reproductive/developmental toxicity according to the regulations set by the new EU Chemicals Policy. The relevant guidelines are the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 414 (Prenatal Developmental Toxicity Study), TG 416 (Two-Generation Reproduction Toxicity Study) and TG 421 or 422 (Reproduction/Developmental Toxicity Screening Test).[36-39] TG 414, TG 416 and TG 421 require up to 150, 3,200 and 560 animals/test, respectively.[40] Hence, the new EU Chemicals Policy legislation will probably result in the use of large numbers of animals, as it is likely to require detailed reproductive/developmental toxicity testing for chemicals

produced or marketed at >100 tonnes/year.[20] In addition, more than 5,000 chemicals, which are already on the market, must be assessed for reproductive/developmental toxicity, as these data are missing.[20,41] It should be mentioned that only in vivo experiments are requested by the OECD and EMA guidelines for reproductive/developmental toxicity testing of industrial chemicals and human pharmaceuticals.

The thalidomide tragedy was not only a bitter lesson on the vulnerability of the human embryo, it also demonstrated the relevance of addressing interspecies differences when making developmental toxicity assessments. Although positive animal teratogenic responses can be indicative of human teratogenicity, the between-species concordance is far from ideal.[42-44] Hurtt et al analysed veterinary drugs from which residues may be present in human food, and found overall positive concordances between rat, rabbit and mice of 74% for teratogenicity and 56% for fetotoxicity.[44] In another study, substances identified as human teratogens showed levels of positive concordances between human and mouse (85%), rat (80%), rabbit (60%), but only 45% for hamster and 30% for monkey.[42] For compounds which specifically had been reported to have no evidence of teratogenicity in humans, the positive concordance was even lower, namely, 35 % for mouse, 50% for rat, 70% for rabbit and 35% for hamster, but 80% for monkey.[42]

In order to overcome the problem of species differences, regulatory bodies request developmental toxicity testing in a second species, but the consequence is an enormous increase in the number of false positives, resulting in unwanted restrictions.[45] The use of large numbers of laboratory animals and the difficulties of hazard identification due to interspecies differences strongly encourage the use of in vitro tests which address the modes of action of human developmental toxicity and which are based on human cells.

The Embryonic Stem Cell Test

The fact that ES cells differentiate in vitro into many cell lineages and thus, to some extent, mimic the in vivo development of the embryo, provides the scientific rationale for using these cells to establish in vitro tests for teratogenicity to the early embryo. The first studies on an embryotoxicity test based on ES cells, compared the effects of 28 known teratogens on the survival of mouse ES cells and primary mouse fibroblasts from day-14 mouse embryos, in order to detect differences in sensitivity between undifferentiated and differentiated cells.[46] The ES cells had a higher sensitivity to the teratogens. This was seen as an indication of teratogenicity, although the system classified some compounds as false negatives. The approach was further developed by replacing the primary fibroblasts with a mouse fibroblast cell line, including inhibition of the cardiac differentiation of mouse ES cells as a toxicological endpoint and developing a prediction model, i.e., an algorithm describing the conversion of the in vitro results into a prediction of no, weak or strong in vivo embryotoxicity (Fig. 3).[47] This protocol, namely the Embryonic Stem cell Test (EST), showed promising results, leading to the initiation of a validation study according to the requirements of ECVAM.[48]

The formal validation trial was carried out between 1998 and 2000. 20 chemicals with different embryotoxic potentials, i.e., non, weakly or strongly embryotoxic, were tested under blind conditions in four different laboratories from governmental institutions and industry.[49] Although, the EST showed good reproducibility and achieved a good overall accuracy of 78%, the expert panel in the validation study follow-up workshop recommended further development of the EST before its acceptance for use for regulatory purposes.[50] Among the requested optimisations was a revision of the prediction model

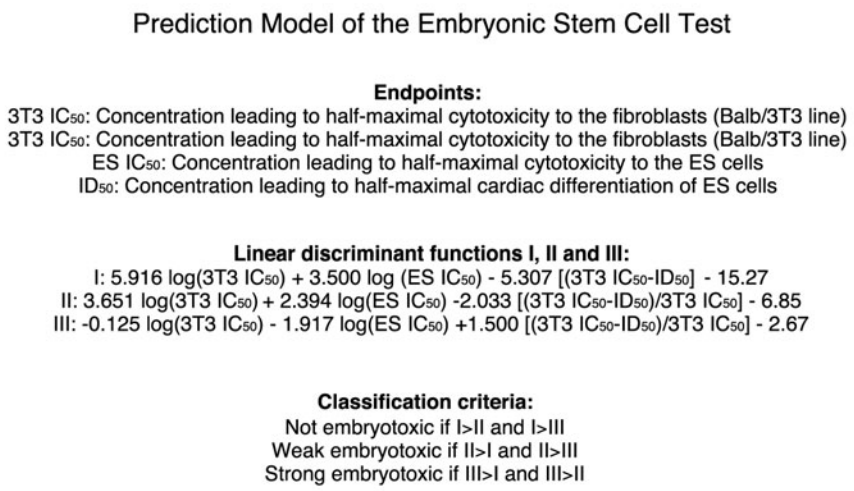

Figure 3. The prediction model of the EST is based on the three endpoints of the test. i.e., cytotoxicity to mouse embryonic stem (ES) cells and 3T3 fibroblasts and inhibition of ES cell cardiac differentiation. The endpoint values are determined from the compound concentration-inhibition curves and used in the three linear discrimination functions. The function giving the highest values determines the classification of the compound.

to improve the separation of non and weak embryotoxicants. Also requested were the inclusion of additional differentiation lineages, the integration of a metabolic system and the testing of more chemicals, in order to explore the applicability domain of the test.[50] Despite these limitations, this EST is currently the only validated in vitro test based on an ES cell line and it is likely to play a role in a weight-of-evidence approach in the assessment of developmental toxicity hazard.

Ongoing Research Activities in Improvement of Embryotoxicity Tests Based on Embryonic Stem Cells

The major limitations of the EST, identified in the validation study follow-up workshop, have been taken into account in the workpackage, Early Prenatal Development, under the umbrella of ReProTect, an integrated project of the EC.[50,51] The workpackage will enlarge the chemical database for the validated EST, in order to explore its applicability domain. Previous studies indicate that embryonic stem cell-based assays can detect neuronal and skeletal embryotoxicity.[52-55] This will be further developed and standardised in the workpackage. In addition, the possibility of adapting the murine system to human embryonic stem cells will be investigated, in order to obtain a better prediction of developmental toxic hazard in humans and increase the effectiveness of protecting human embryos from hazardous chemicals. A previous study has shown that the EST can predict the embryotoxic hazard of metabolically activated cyclophosphamide.[56] The Early Prenatal Development workpackage will further explore the possibilities for the integration of a biotransformation system into the EST. Moreover, the workpackage will contribute to a better understanding of toxicological modes of action at the molecular

level, since ultrasensitive differential protein expression analysis will be applied in addition to conventional immunohistochemical and molecular biological markers, in order to identify relevant toxicological endpoints.

Additional research initiatives have been exploring the applicability of ES cells for in vitro embryotoxicity testing. A number of basic research studies have been published on different approaches for the use of mouse ES cell differentiation assays for assessment of hazard to neuronal, osteogenic, chondrogenic and cardiac development in embryogenesis.[52-55,57] In particular, we would like to stress the research on the use of mouse ES cells with a fluorescent reporter gene driven by the cardiac specific promoter for the α-myosin heavy chain. This feature permits easier detection of cardiac differentiation than the morphological assessment (counting of beating colonies) used by the EST. Moreover, the approach seems reasonable, since the development of cardiac beating and myosin heavy chain mRNA expression show high correlation.[57,58] Similar approaches could most likely be applicable to other tissue endpoints, as it has been shown that mRNA expression analysis can give indications for toxicity to neuronal, osteogenic and chondrogenic development in embryogenesis.[52-55] Embryotoxicity tested based on ES cells expressing tissue specific fluorescent reporter genes have consensual appeal as they may provide the basis for high-throughput screening assays.

A Battery Approach Must Be Used for Embryotoxicity Testing in Vitro

The life of every mammal begins as a single cell, the progeny of which form complex tissues and organs that are themselves integrated into larger systems. This is accomplished by continuous changes and developments in cell subtypes and cell-cell interactions during embryogenesis. Due to the complexity of the development of the child during pregnancy, only a strategy of combined test systems will provide sufficient information to permit the identification of chemicals likely to be embryotoxic. Such a strategy would have to include assays for detecting toxic effects on lineage differentiation, as well as on developmental mechanisms. The emerging "omics" technologies (toxicogenomics, proteomics and metabolomics) may prove useful in such a battery approach, as they can address a large range of toxicological endpoints.[59]

FURTHER CONSIDERATIONS

Quality Criteria for Embryonic Stem Cells Used in Toxicology and Safety Pharmacology

One great advantage in the toxicological application of the highly proliferative ES cells is that they can provide large numbers of cells with a diploid karyotype. However, all cell lines, even those considered to proliferate indefinitely, have a limited life-span and are likely to die out or change their characteristics with repeated passage. In the case of cultured human ES cells, which are of relatively new origin, a detailed analysis is needed concerning the stability of their characteristics in vitro, in order to ensure reproducibility of assays based on these cells. One major limitation for standardised high-throughput assays based on ES cells is the prominent heterogeneity of cell lineages that arise during differentiation. Controlled differentiation and a clearer characterisation

of the derivatives must be achieved, if ES cells are to be used as the basis for toxicological and pharmacological assays.

The OECD and EMA require that safety studies, carried out for regulatory purposes, are planned, conducted, recorded, reported and archived in accordance with Principles of Good Laboratory Practice (GLP).[60] An OECD advisory document provides specific guidance for the application of these principles to in vitro safety studies.[61] Complementary information is given in an ECVAM Task force report on Good Cell Culture Practice (GCCP), which was developed to support best practice in the use of cell and tissue in vitro studies conducted for regulatory, basic research and other purposes.[62] These GLP and GCCP principles are fully applicable to ES cells, but need to be expanded in order to cover the unique properties of these cells. During an ECVAM workshop held in January 2007, inputs from those in academia and industry involved in the establishment of ES cell lines and related products and from regulatory bodies were brought together, in order to define quality criteria for the maintenance and differentiation of human ES cell to be used in toxicity testing. The detailed report will help to promote internationally harmonised quality and safety criteria.[63]

Ethical Issues

Ethical issues on the establishment of in vitro tests based on human ES cells should be discussed separately from issues related to their medical applications. In contrast to their use for medical applications, where the donor and patient have to be tissue matched and xenofree cell lines must be established, the establishment of new ES cell lines is not essential for their use in safety evaluations. Thus, additional destruction of embryos and foetuses and creation of new embryos seems not to be necessary.

Importantly, as exemplified in this chapter, ES cell-based in vitro assays could reduce and potentially replace the extensive use of animal experiments in the areas of drug discovery, safety pharmacology and toxicology. Furthermore, assays based on human ES cells may contribute to resolving the problems resulting from interspecies differences, leading to better identification of human hazard and hence to improvement of the drug development process.[19]

CONCLUSION

The rapidly increasing knowledge on ES cells offers the possibility to establish in vitro tests in a range of toxicological and pharmacological areas. Currently, prominent progress is taking place in the fields of embryotoxicology, cardiotoxicology and hepatoxicology, as international collaboration research projects are funded by the EC in these areas. The outcome will hopefully be robust ES cell-based assays which are ready to undergo validation, which in itself would provide proof of principle for the use of human ES cells in in vitro tests.

Major advantages of in vitro assays based on ES cells would be the use of cells with a normal diploid karyotype and a high proliferative capacity, making large numbers of cells available for high-throughput assays. Human ES cells further offer the possibilities of avoiding interspecies differences and screening for human inter-individual differences, which would be of great benefit for identification of human hazards and improvement of the drug development process. In addition, human ES cell-based assays could be a

valuable tool to reduce and possibly replace, the current ethical-problematic animal experimentation in drug discovery, safety pharmacology and toxicology.

REFERENCES

1. NIH. Stem cells: Scientific progress and future research directions. Bethesda: National Institutes of Health, 2001. Available at: http://stemcells.nih.gov/info/scireport/2001report.htm.
2. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292:154-156.
3. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. 1981; 78:7634-7638.
4. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282:1145-1147.
5. Zhang SC, Wernig M, Duncan ID et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001; 19:1129-1133.
6. Pomp O, Brokhman I, Ben Dor I et al. Generation of peripheral sensory and sympathetic neurons and neural crest cells from human embryonic stem cells. Stem Cells 2005; 23:923-930.
7. Assady S, Maor G, Amit M et al. Insulin production by human embryonic stem cells. Diabetes 2001; 50:1691-1697.
8. Wang L. Endothelial and hematopoietic cell fate of human embryonic stem cells. Trends Cardiovasc Med 2006; 16:89-94.
9. Reppel M, Boettinger C, Hescheler J. Beta-adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes. Cell Physiol Biochem 2004; 14:187-196.
10. Capi O, Gepstein L. Myocardial regeneration strategies using human embryonic stem cell-derived cardiomyocytes. J Control Release 2006; 116:211-218.
11. Hay DC, Zhao D, Ross A et al. Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities. Cloning Stem Cells 2007; 9:51-62.
12. Liu P, Jenkins NA, Copeland NG. Efficient Cre-loxP-induced mitotic recombination in mouse embryonic stem cells. Nat Genet 2002; 30:66-72.
13. Cezar GG. Can human embryonic stem cells contribute to the discovery of safer and more effective drugs? Curr Opin Chem Biol 2007; 11:405-409.
14. Pouton CW, Haynes JM. Embryonic stem cells as a source of models for drug discovery. Nat Rev Drug Discov 2007; 6:605-616.
15. Davila JC, Cezar GG, Thiede M et al. Use and application of stem cells in toxicology. Toxicol Sci 2004; 79:214-223.
16. McNeish J. Embryonic stem cells in drug discovery. Nat Rev Drug Discov 2004; 3:70-80.
17. Piersma AH. Alternative methods for developmental toxicity testing. Basic Clin Pharmacol Toxicol 2006; 98:427-431.
18. Rohwedel J, Guan K, Hegert C et al. Embryonic stem cells as an in vitro model for mutagenicity, cytotoxicity and embryotoxicity studies: present state and future prospects. Toxicol In Vitro 2001; 15:741-753.
19. Stummann TC, Bremer S. The possible impact of human embryonic stem cells on safety pharmacological and toxicological assessments in drug discovery and drug development. Curr Stem Cell Res Ther 2008; 3:118-131.
20. EC. Regulation (EC) No 1907/2006 of the European Parliament and of the Council. Brussels: European Commission (EC), 2006: Available at: http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_396/l_39620061230en00010849.pdf.
21. EMA. ICH Topic M3(R2) Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. London: European Medicines Agency, 2009: Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf.
22. EMA. ICH Topic S7B The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. London: European Medicines Agency, 2005: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002841.pdf.
23. Lasser KE, Allen PD, Woolhandler SJ et al. Timing of new black box warnings and withdrawals for prescription medications. JAMA 2002; 287:2215-2220.
24. Lexchin J. Drug withdrawals from the Canadian market for safety reasons, 1963-2004. CMAJ 2005; 172:765-767.
25. Olson H, Betton G, Robinson D et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 2000; 32:56-67.

26. Fitzgerald PT, Ackerman MJ. Drug-induced torsades de pointes: the evolving role of pharmacogenetics. Heart Rhythm 2005; 2:S30-S37.
27. Lee WM. Drug-induced hepatotoxicity. N Engl J Med 2003; 349:474-485.
28. EMEA. Draft guideline on detection of early signals of drug-induced hepatotoxicty in nonclinical studies. London: European Agency for the Evaluation of Medicinal Products, 2006: Available at: http://www.emea.europa.eu/pdfs/human/swp/15011506en.pdf.
29. Soderdahl T, Kuppers-Munther B, Heins N et al. Glutathione transferases in hepatocyte-like cells derived from human embryonic stem cells. Toxicol In Vitro 2007; 21:929-937.
30. Kulkarni JS, Khanna A. Functional hepatocyte-like cells derived from mouse embryonic stem cells: a novel in vitro hepatotoxicity model for drug screening. Toxicol In Vitro 2006; 20:1014-1022.
31. Lee WM, Senior JR. Recognizing drug-induced liver injury: current problems, possible solutions. Toxicol Pathol 2005; 33:155-164.
32. Queisser-Luft A, Stolz G, Wiesel A et al. Malformations in newborn: results based on 30,940 infants and fetuses from the Mainz congenital birth defect monitoring system (1990-1998). Arch Gynecol Obstet 2002; 266:163-167.
33. EUROCAT. Eurocat report 8: Surveillance of congenital anomalies in Europe 1980-1999. Newtownabbey: EUROCAT Central Registry, 2002: Available at: http://www.eurocat-network.eu/content/EUROCAT-Report-8-Part-1.pdf.
34. Gilbert SF. Developmental Biology. Sunderland, Massachussets: Sinauer Associates, 2006.
35. EMEA. ICH Topic S5(R2) detection of toxicity to reproduction for medicinal products and toxicity to male fertility. London: European Agency for the Evaluation of Medicinal Products, 1994: Available at: http://www.emea.europa.eu/pdfs/human/ich/038695en.pdf.
36. OECD. OECD Guidelines for the testing of chemicals. Test no. 414: Prenatal development toxicity study. Paris: Organisation for Economic Co-operation and Development, 2001: Available at: http://oberon.sourceoecd.org/vl=1320423/cl=11/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s15/p1.
37. OECD. OECD Guidelines for the testing of chemicals. Test no. 416: Two-generation reproduction toxicity. Paris: Organisation for Economic Co-operation and Development, 2001: Available at: http://oberon.sourceoecd.org/vl=1320423/cl=11/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s17/p1.
38. OECD. OECD Guidelines for the testing of chemicals. Test no. 421: Reproduction/developmental toxicity screening test. Paris: Organisation for Economic Co-operation and Development, 1995: Available at: http://oberon.sourceoecd.org/vl=1320423/cl=11/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s22/p1.
39. OECD. OECD Guidelines for the testing of chemicals. Test no. 422: Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test. Paris: Organisation for Economic Co-operation and Development, 1996: Available at: http://oberon.sourceoecd.org/vl=1320423/cl=11/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s23/p1.
40. Hofer T, Gerner I, Gundert-Remy U et al. Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. Arch Toxicol 2004; 78:549-564.
41. Pedersen F, de Bruijn J, Munn S et al. Assessment of additional testing needs under REACH. Brussels: European Commission, 2003: Available at: http://ecb.jrc.it/DOCUMENTS/REACH/PUBLICATIONS/REACH_testing_needs_final.pdf.
42. Frankos VH. FDA perspectives on the use of teratology data for human risk assessment. Fundam Appl Toxicol 1985; 5:615-625.
43. Schardein JL. Chemically induced birth defects. New York: Marcel Dekker, 2000.
44. Hurtt ME, Cappon GD, Browning A. Proposal for a tiered approach to developmental toxicity testing for veterinary pharmaceutical products for food-producing animals. Food Chem Toxicol 2003; 41:611-619.
45. Bremer S, Hartung T. The use of embryonic stem cells for regulatory developmental toxicity testing in vitro—the current status of test development. Curr Pharm Des 2004; 10:2733-2747.
46. Laschinski G, Vogel R, Spielmann H. Cytotoxicity test using blastocyst-derived euploid embryonal stem cells: a new approach to in vitro teratogenesis screening. Reprod Toxicol 1991; 5:57-64.
47. Scholz G, Pohl I, Genschow E et al. Embryotoxicity screening using embryonic stem cells in vitro: correlation to in vivo teratogenicity. Cells Tissues Organs 1999; 165:203-211.
48. Balls M, Blaauboer BJ, Fentem JH et al. Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. Altern Lab Anim—ATLA 1995; 23(1):129-147.
49. Genschow E, Spielmann H, Scholz G et al. Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim—ATLA 2004; 32(3):209-244.
50. Spielmann H, Seiler A, Bremer S et al. The practical application of three validated in vitro embryotoxicity tests. The report and recommendations of an ECVAM/ZEBET workshop (ECVAM workshop 57). Altern Lab Anim—ATLA 2006; 34(5):527-538.
51. Hareng L, Pellizzer C, Bremer S et al. The integrated project ReProTect: a novel approach in reproductive toxicity hazard assessment. Reprod Toxicol 2005; 20:441-452.

52. Schmidt MM, Guan K, Wobus AM. Lithium influences differentiation and tissue-specific gene expression of mouse embryonic stem (ES) cells in vitro. Int J Dev Biol 2001; 45:421-429.
53. Pellizzer C, Bello E, Adler S et al. Detection of tissue-specific effects by methotrexate on differentiating mouse embryonic stem cells. Birth Defects Res B Dev Reprod Toxicol 2004; 71:331-341.
54. zur Nieden NI, Kempka G, Ahr HJ. Molecular multiple endpoint embryonic stem cell test—a possible approach to test for the teratogenic potential of compounds. Toxicol Appl Pharmacol 2004; 194:257-269.
55. Stummann TC, Hareng L, Bremer S. Embryotoxicity hazard assessment of methylmercury and chromium using embryonic stem cells. Toxicology 2007; 242:130-134.
56. Bremer S, Pellizzer C, Coecke S et al. Detection of the embryotoxic potential of cyclophosphamide by using a combined system of metabolic competent cells and embryonic stem cells. Altern Lab Anim—ATLA 2002; 30(1):77-85.
57. Bremer S, Worth AP, Paparella M et al. Establishment of an in vitro reporter gene assay for developmental cardiac toxicity. Toxicol In Vitro 2001; 15:215-223.
58. Schwengberg S, Bohlen H, Kleinsasser N et al. In vitro embryotoxicity assessment with dental restorative materials. J Dent 2005; 33:49-55.
59. Leighton JK. Application of emerging technologies in toxicology and safety assessment: regulatory perspectives. Int J Toxicol 2005; 24:153-155.
60. OECD. OECD principles on good laboratory practice. Paris: Organisation for Economic Co-operation and Development, 1998: Available at: http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem(98)17.
61. OECD. The application of the principles of GLP to in vitro studies. Paris: Organisation for Economic Co-operation and Development, 2004: Available at: http://appli1.oecd.org/olis/2004doc.nsf/43bb6130e5e86e5fc12569fa005d004c/58d5c8b13297a995c1256f5c006008a7/$FILE/JT00174939.pdf.
62. Coecke S, Balls M, Bowe G et al. Guidance on good cell culture practice a report of the second ECVAM task force on good cell culture practice. Altern Lab Anim—ATLA 2005; 33:261-287.
63. Adler S, Allsopp T, Bremer S et al. hESC Technology for Toxicology and Drug Development: Summary of Current Status and Recommendations for Best Practice and Standardization. The Report and Recommendations of an ECVAM Workshop. Unpublished report. Available at: http://ecvam.jrc.it/publication/hESC_%20010711.pdf.

CHAPTER 3

TRENDS IN CELL CULTURE TECHNOLOGY

Uwe Marx
ProBioGen AG, Berlin, Germany
Email: uwe.marx@probiogen.de

Abstract: Dynamic macroscale bioreactor systems are the most recent breakthrough in cell culture technology. This major achievement, at the beginning of the 21st century, fortunately coincided with an embarrassing gap in the measures to predict the safety and modes of action of chemicals, cosmetics, air particles and pharmaceuticals. The major hurdles to the translation of these breakthrough achievements of cell culture technology into meaningful solutions for predictive high throughput substance testing remain miniaturization from the milliliter to the microliter scale and the supply of relevant amounts of standardized human tissue. This chapter provides insights into the latest developments in this area, illustrates an original multi-micro-organ bioreactor concept and identifies highways for closing the gap.

INTRODUCTION: THE 21ST CENTURY TEST DILEMMA

There has quite clearly been an embarrassing gap in the provision of adequate measures to predict the interactions of consumer-relevant synthetic or natural substances with the human body in its typical environment and with its individual genotypic specificity prior to human exposure. The results of this have included:

- false predictions of the toxicity of chemicals and chemical products;
- devastating misjudgments concerning the risks of using recently-recalled drugs;
- dramatic increases in allergies within the worldwide population; and
- the recent indication of the tumor risk induction potential of nano-sized particles in diesel engine exhausts.

At the beginning of the 21st century, consumer health is suffering substantially from this major obstacle, which affects the chemical, cosmetics and pharmaceutical industries

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

equally. In the pharmaceutical industry, a prime example is the super-agonist antibody, TGN1412, which was developed to direct the immune system to fight cancer cells or reduce arthritis pain. This triggered multiple organ failure in 6 healthy volunteers that participated in Phase I clinical testing. By binding to the CD28-receptor, the antibody overrides the basic control mechanisms of the whole immune system.[1] Tested according to the standard clinical research guidelines, the drug showed no adverse effects in animal studies. At other times, significant drawbacks of pharmaceuticals, such as severe side-effects and lack of efficacy, are often only evident after drugs have entered the market. There is increasing evidence that specific genetic predisposition is one of the key reasons for the now common and highly publicized drug withdrawals. This human genetic diversity is rarely addressed in preclinical and clinical safety studies at the present time. A sound hypothesis on the correlation of the morbidity of patients treated with roferoxib (Vioxx) with polymorphic genotypes for 5-LOX and 5-LOX activating proteins is one of many examples. In general, the last 10 years have provided increasing evidence that the adsorption, distribution, metabolism, excretion and toxicity (ADMET), immunogenicity and efficacy of a variety of substances to which consumers are exposed, are often human-specific and even individual-specific more so than has been anticipated in the past. In view of this dramatic situation, both the US and European regulatory bodies have reacted by instigating a number of actions and programs. In Europe, legislative pressures, such as the 7th Amendment to the Cosmetics Directive and the retrospective REACH (Registration, Evaluation and Authorisation of Chemicals) program for application to approximately 30,000 chemicals, have dramatically increased the industrial demand for predictive test procedures which are more reliable. The Critical Path Initiative, introduced by the US Food and Drug Administration (FDA) in 2004, and the later risk-based approach of the European Medicines Agency (EMA), are radically changing how the safety and efficacy of medicinal products are evaluated during drug development. Any sound proposal for closing the striking gap between prediction and reality in substance testing and experience in use is to be welcomed.

DO CELL CULTURE TECHNOLOGIES PROVIDE MEANINGFUL SOLUTIONS?

Historical Sketch

A retrospective overview of the short history of in vitro cell culture might be helpful to assess the potential of modern cell culture technologies to provide a meaningful solution for the testing dilemma. Over the last hundred years, scientists have been trying to culture human tissue in vitro, in order to gain mechanistic knowledge and to assist with the development of new medicines. Interestingly, in 1912, Alexis Carrel (Rockefeller Institute for Medical Research, New York) said "On the permanent life of tissues outside of the organism",[2] that some in vitro "cultures could be maintained in active life for fifty, fifty-five and even for sixty days". These results showed that the early death of tissues cultivated in vitro was preventable and "therefore that their permanent life was not impossible". At that time, synthetic cell culture media, antibiotics, disposable tissue culture flasks, aseptic techniques and bioreactors were not available. About two decades later, an avian bone more than seven millimeters long and with clear signs of calcification could be produced in vitro from embryonic cells (for a review, see ref. 3). Subsequently,

scientists concentrated on research on tumor cell lines in suspension or monolayer cultures. Histotypic cultures of primary cells of human and rodent origin in the late 1960s were made possible when the crucial role of an efficient oxygen supply was recognized and systems that improved oxygen distribution in cell culture were developed.[4] Interestingly, some of the early human histotypic cultures, such as Dexter and Lajtha's culture of human haematopoietic stem cells on feeder layers, demonstrated the importance of the interaction of different cell types with each other for growth and functionality.[5]

In the late-1990s, tissue engineering was used to develop a functional substitute for damaged human tissue, which raised hopes of therapeutic solutions that were not realized, even though crucial initial knowledge of how to engineer tissue emulating its human counterpart was gained. Recently, the hope of finding ultimate solutions for organ and tissue repair has been heavily associated with stem cell technologies.

It has become clear that, in addition to efficient oxygen and nutrient supply, a local microenvironment with appropriate mechanicochemical coupling (achieved by regulating interstitial flow or applying external stresses) is a crucial prerequisite for mimicking in vivo biology.[6] Thus, rather than homogeneous culture systems, there is more focus on producing heterogeneous culture models with an emphasis on controlled, continuously adjustable, long term culture processes. Dynamic bioreactors stand at the centre of the latest successful developments of in vitro models.

Dynamic Bioreactor Systems

A major breakthrough in in vitro liver organotypic culture technology was achieved in the last decade by liver tissue engineers exposed to a very specific research and development environment. Their motivation was to provide patients with acute liver failure with an extracorporeal bio-artificial liver, to bridge the time for the patients' liver self-regeneration or the availability of a matching donor liver. However, the health care costs associated with liver failure and liver transplantation are extremely high, so liver transplant centers were seeking and investing in alternatives. As the resulting liver culture systems connected to a patient's bloodstream would have to take over crucial life functions over several weeks, patient survival was the most significant factor in the evaluation of bio-artificial livers. Hepatectomy studies suggest that, to be effective in the treatment of acute liver failure, tissue engineered liver constructs should perform metabolic functions quantitatively equivalent to at least 30% of the natural liver mass, so the scale of the liver bioreactor was set at one hosting an average of at least 0.5 kg cells. As the liver is the prime organ in which to study the metabolism and detoxification of substances, achievements with such large scale in vitro systems has inadvertently impacted on the metabolism and toxicity testing of substances in vitro.

In the liver parenchyma, the hepatocytes perform most of the liver-specific metabolic functions. They are arranged in repeating units, as plates called "lobules", in which they spread outward from a central vein. At the lobule vertices, a bile duct branches of the hepatic artery and the portal vein are located close to one another in an arrangement called the portal triads. Blood flows from the two vessel branches toward the central vein through small vascular channels (sinusoids) lined with a fenestrated layer of endothelial cells. Plasma filters through the endothelium into the space of Disse that separates it from the hepatocytes and exchanges nutrients and metabolites with the hepatocytes through their apical surfaces. Bile is secreted into canaliculi formed between the basal surfaces of adjacent hepatocytes and flows through the bile ducts into the common bile

duct that delivers its contents into the duodenum. Kuppfer cells and extra cellular matrix (ECM)-producing stellate cells, biliary epithelial cells, hepatocyte precursor cells and fibroblasts are also present and perform important metabolic functions. Thus, the liver cells are spatially organized to optimize communication and transport. The cells communicate through cellular and gap junction pathways using chemical signals. The metabolic (e.g. carbohydrate metabolism) and detoxification (e.g. via CYP450 enzymes) activities of the hepatocytes change spatially along the length of the sinusoid, where they are regulated by gradients of oxygen, hormones and ECM composition, which result in liver zonation. For these reasons, the design of extracorporeal bio-artificial livers was based on the liver micro-architecture. However, reproducing the whole liver architecture in extracorporeal livers is unnecessary for the cells to perform a subset of hepatic functions relevant to progressive, acute liver failure. The liver cells must be cultured at the high density seen in the natural liver, which itself is much higher than in many other tissues. They also have demanding nutrient requirements and are highly sensitive to the accumulation of metabolic by-products. In vivo, the liver is richly vascularised and is provided with soluble nutrients by a high blood flow that reaches the innermost cells in the organ with the diffusion distance between liver cells and the blood supply being a few hundred microns.

Creating an analogous system that supplies basic substrates (e.g. oxygen, glucose and amino acids) and clears waste metabolites (e.g. CO_2, ammonia, urea and lactate) from liver cells in large 3D constructs is a formidable challenge. This has been met by several systems which are available commercially, such as Vitagen ELAD®, Vital Therapy ELAD®, Arbios Systems HepatAssist® and MELS CellModule. The latter of these is a four-compartment bioreactor based on a network of interwoven hollow fiber membranes, which was developed by Gerlach and coworkers in the 1990s.[7,8] The bioreactor consists of a 3D network of hollow fiber membranes with different separation and transport properties, woven in orderly planar mats enclosed in polyurethane housing with the aim of reproducing the liver vascular network. Oxygen is supplied to the cells via the medium and through the hydrophobic microporous membranes, thus creating physiologically-relevant oxygen gradients across the cell mass. Pressure-driven, direct cell perfusion enhances the transport of large solutes and species rapidly produced by the cells and is intended to lead to the prompt return of large liver-specific factors to the plasma, the reduced accumulation of waste metabolites near the cells, enhanced cell survival and functions and the efficient use of the available cellular activity. Liver cells cultured in this 3D membrane network were shown to spontaneously re-organize into liver-like aggregates, forming sinusoid-like microchannels with a neo-space of Disse underlying the self-organizing capacity of human tissues in adequate microenvironments. The cells produced biomatrices and expressed liver-specific functions consistently for several weeks. Bioreactors seeded with porcine liver cells were used in bio-artificial livers as a bridge to orthotopic liver transplantation to treat a number of acute liver failure patients (coma Stage III-IV), all of whom survived for three years posttransplantation.[9] A pilot study, in which the same bioreactor was used to culture human liver cells harvested from donor organs discarded for steatosis, cirrhosis or mechanical injury, is currently under way and is giving promising results.[10]

The ideal bioreactor for promoting liver cell re-organization into liver-like structures and the expression of the same enzymatic activities as in the natural liver must feature fluid dynamics. Such a bioreactor should also minimize resistance to metabolite transport to the cells and permit reaction rate measurements to be made, since multiple reaction steps may be involved in the biotransformation and elimination of a xenobiotic: a scaled-down

version of the dynamic 3D membrane bioreactor described above. This arrangement reduces tissue culture space to approximately 5 ml and is seeded with porcine liver cells and operated by filtering the medium across the cell compartment in dead-end mode. It was also able to promote cell re-arrangement into liver-like structures, providing a down-scaled tool for testing the metabolism and toxicity of substances.[11] More recently, a number of small-scale dynamic liver bioreactors have been developed that have culture spaces of more than a cubic centimeter. An exhaustive overview of liver tissue engineering is provided by Capatano and Gerlach.[12]

Another dynamic bioreactor system, fulfilling all the criteria for the self-assembly of functional organotypic tissue in vitro, was developed by Giese et al.[13] The human lymph node can be described as an interface between a stationary network of antigen-presenting cells, such as dendritic cells and a population of suspended and highly migratory lymphocytes. The interface is embedded in a suitable environment of stroma cells and ECM. Pathogens, antigen-loaded macrophages and dendritic cells enter the lymph node via lymphatic flow. Resting lymphocytes circulate in the bloodstream, entering the lymph node via a specialized endothelium and migrate to the network of antigen-presenting cells, guided by cytokine and chemokine gradients in the T-cell areas. Activated T-cells have a high clonal proliferation capacity and act as activators and modulators for B-lymphocyte reaction. They swarm to the B-cell areas of the lymph node, where they facilitate an effective and persistent B-cell response, leading to antibody forming and expression. Giese and coworkers designed a disposable, miniaturized membrane-based perfusion bioreactor system consisting of a matrix-assisted central culture space of about 1 ml and an outer culture space for suspended cells of about 4 ml. The central culture space is supported by a planar set of microporous hollow fibers for media and gas supply and exchange. Two dendritic cell-loaded sheets of matrices are mounted in the central cell culture space and are stabilized by a macro-porous membrane. Lymphocytes can be fed in via the outer cell culture space and recirculated via a separate fluidic system. They can pass through the porous membranes and interact with the immobilized dendritic cells within the matrix. This design ensures a sufficient residence time of lymphocytes within the matrix-supported dendritic network and a short residence time within the supporting fluidics. If the antigen is recognized, naïve or resting T-cells are activated. This results in a massive clonal proliferation and enhanced migration. In a next step, activated T-cells bind to those B-lymphocytes, which carry antigen specific antibodies on their surface. In response to activation, the spontaneous re-organization of micro-organoid follicle-like structures, which are composed of B-cell and T-cell clusters, takes place in a way that resembles the in vivo situation. Polysulfone housing, microporous polyethersulfone fibers and polyurethane potting and bonding were evaluated and found to be appropriate materials for use in this artificial lymph node device. Exposure to an adequately dynamic substance for immunogenicity evaluation is ensured by substance distribution to the circulating fluids. This mimics the original lymphatic pathogen distribution pathway. Monocytes and lymphocytes are derived from a donor's blood, usually by leukophoresis. Specific donor panels can be selected by gender, age, genotype or other relevant parameters. Monitoring variations in human immunological responses to various substances fits well with the trend towards personalized medicines. Figure 1A shows how dendritic cells mounted onto matrix sheets are loaded into the bioreactor for exposure to the test substance on day 2, followed by monitoring for a further 14 days. In addition to histological endpoint measurements (Fig. 1B-E), the system supports a variety of in-process measurements for monitoring time-related dynamic changes within the lymph nodes in response to toxic or pathogenic compounds. Among them, cytokine profiles are

of particular interest, since they allow, for example, the induction of Th2 cell and Th1 cell differentiation pathways to be studied. The artificial lymph node system of Giese and coworkers operates with 4-6 parallel bioreactor devices within a central bioreactor control unit. A single donor leukophoresis preparation can feed between 10 and 12 bioreactor runs with autologous leukocytes.[14] In addition to the evaluation of immunogenicity and immunotoxicity of substances, the system is well-suited to provide basic knowledge on immune mechanisms in man.

The dynamic bioreactor systems described so far have been mainly perfused with blood, plasma, synthetic media, nutrients or oxygen through various artificial hollow fiber membranes. The endothelium-covered vasculature of organs in humans is not only a biological solution to provide blood to the organs, but plays a crucial role in other key biological processes, such as cell migration. With this in mind, researchers are attempting to replace technical membranes in high performance dynamic organ bioreactors with natural vasculature.

A cutting-edge concept for providing natural vascularisation for organotypic tissues of small size was proposed by Mertsching and coworkers (Fig. 2).[15] They standardized the repopulation of a decellularized porcine jejunal segment, including preserved vascular structures, with porcine and, more recently, human microvascular endothelial cells. Endothelial cells almost fully repopulated the fine natural vessel network. In this set-up, integrated inlet and outlet ports on the two larger collecting vessels allow for the easy circulation of matter through the capillary network established, thus emulating human blood circulation (Fig. 2B). The inner part of the acellularized jejunal segment can be loaded with cells of different origins to permit the evaluation of re-organization into respective organotypic clusters. Mertsching and coworkers (for review, see ref. 16) have succeeded in generating liver tissue by re-organizing cells in liver-like functional organotypic units (Fig. 2D). They recently demonstrated the re-organization of an entire mucosal structure, achieved by seeding a human mucosa single cell suspension into the inner lumen of the jejunal segment (Fig. 2C). Computerized control allows more than six vascularized culture devices the size of a culture dish to be maintained (Fig. 2A). It is likely that similar coculture systems of liver and gut mucosa, connected through a common human capillary network, will soon be available. This would have an immediate impact on the availability of organ-based ADMET assessments of xenobiotics in vitro and would lead the way for developing small scale vascularized multi-organ culture systems/ bioreactors that connect several ‘organs’ of interest through a natural capillary network, for use in determining the whole-body ADMET profiles of substances.

Miniaturization and “Humanization”—Hurdles for High Throughput in In Vitro Testing

Logistically, predictive in vitro test systems should be cost effective, validated and have high throughput performance. However, the currently-available dynamic bioreactors, which support the re-organization of organ tissues with adequate functionality, cannot cope with these high throughput and cost requirements. This is because each individual organ culture space requires, at least, a full set of pumping means, tools to stabilize temperature, an oxygen sensor, a pH sensor and an adequate control unit. As the simultaneous operation of a minimum of six organ cultures for statistically valid testing is essential, monitoring hardware and control hardware need to be multiplied when designing bioreactor equipment suited for commercial scale substance testing. To our knowledge, such parallelized

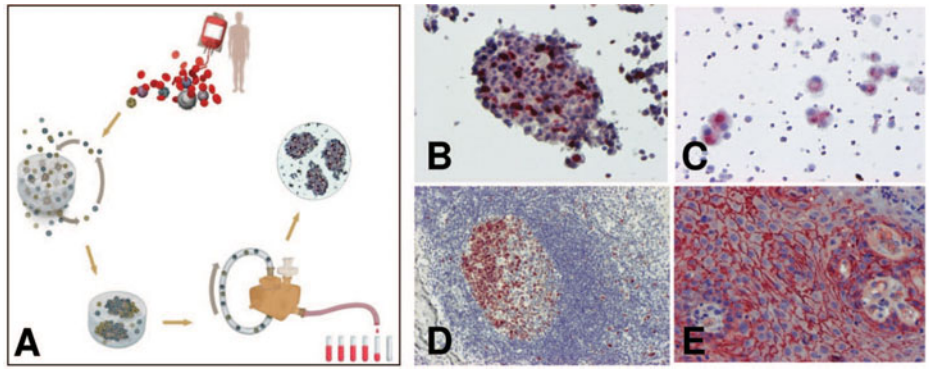

Figure 1. See legend on following page.

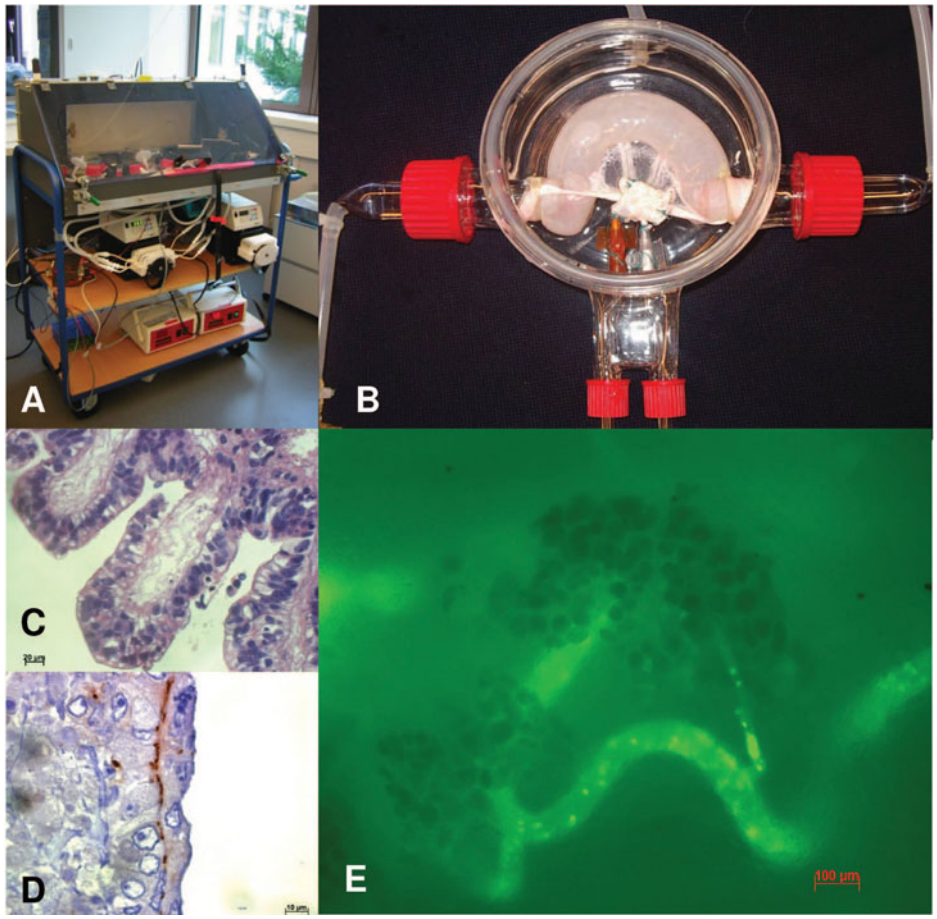

Figure 2. See legend on following page.

Figure 1, viewed on previous page. A) Schematic overview of the use of a human artificial lymph node for substance testing. Monocytes are separated from donor leukocytes and differentiated into dendritic cells for use in the human artificial lymph node device. They are then integrated into a semisolid matrix support and, finally, mounted into the bioreactor device. Continuous media perfusion, oxygen supply and lymphocyte perfusion are provided over a 14- to 16-day period. Xenobiotic exposure is usually carried out on day two, although the timing of exposure can be changed to be more reflective of natural exposure situations. Daily samples are taken for in-process analyses. Micro-organoid formation and analysis is carried out by histological inspection of the lymph node slices. B) A micro-organoid derived from artificial lymph node culture stained positively for Ki67 proliferating lymphocytes embedded in a nonproliferating organoid environment. C) Ki67 staining of a human tonsil slice exhibiting a natural follicle of comparable composition. D) Single differentiated CD138-positive plasma cells derived from the same 14-day artificial lymph node culture. E) A human tonsil slice with large plasma cells positively stained for CD138 and embedded in the reticular network of the tonsil. (Illustration courtesy of Dr. C. Giese, ProBioGen AG, Berlin.)

Figure 2, viewed on previous page. A) A computer-controlled bioreactor system, capable of operating. B) A single "organ-in-a-dish" device with an almost completely accelularized jejunal segment, including vascular structures, mounted onto a dish and connected with inlet and outlet ports for medium or blood perfusion on the lower part and a connector at each end of the jejuna segment for access into the inner lumen of the segment. Endothelial cell repopulation is carried out through the medium inlet and outlet, whereas organ-specific cells are seeded in the inner lumen of the gut segment. C) Haematoxylin-eosin histostaining of a human mucosa segment, derived through re-organization of human mucosa cell suspension within a single "organ-in-a-dish" bioreactor over a culture period of 14 days. D) Immuno histostaining (CK18) of a human liver segment, derived from a single "organ-in-a-dish" bioreactor after 21 days. Liver-specific structures, including bile canaliculi, can be identified. E) Carboxyfluorescein succinimidyl ester fluorescence staining of a segment of a vascularised "organ-in-a-dish" liver cell culture, showing the capillaries and including green fluorescent cells and hepatocyte cell clusters (black shadows) surrounding the capillaries. (Illustration courtesy of Dr. H. Mertsching, Fraunhofer IGB, Stuttgart.)

organ culture bioreactor systems are not yet commercially available, due to significant development and operation costs. A rough estimate of the capital costs of such systems can be drawn by analogy to the biopharmaceutical manufacturing industry, where a parallelized small scale stirred-tank bioreactor system, Cellferm-pro® (DASGIP AG, Jülich, Germany), that provides the necessary periphery for the individual operation, control and monitoring of eight small scale stirred mammalian cell tanks costs in excess of €150,000 as well as requiring operation by highly skilled personnel. In addition, the standardization requirements for substance testing may not allow for the re-use of organ culture devices. The need for disposable culture ware further increases the operational costs of test procedures involving the use of such equipment.

In addition to prohibitive capital costs, a second factor which frustrates high throughput substance testing is associated with the currently available dynamic organ culture bioreactors. The conventional fabrication technologies do not allow for the reduction of individual organ culture spaces significantly below the cubic centimeter tissue culture range. As this translates to a need for a gram-range of human tissue in order to produce a single data point within a substance testing program, many tons of standardized human tissue would be required to meet global annual testing demands. Neither the volume nor the standardization of living human tissue could be provided on this extreme scale in the foreseeable future.

Consequently, the two main objectives for tissue engineers and scientists at the beginning of this century are:

- to miniaturize organ culture space further from the milliliter to the microliter scale, and
- to provide substantial sources of relevant standardized human tissue.

On the technical side, the further miniaturization of organ culture space requires new fabrication technologies since it is clear that most of the actuators and sensors used are ineffectual at the necessary organ culture micro scale. Biologically, it remains unclear to what degree a human organ of, for example, 1.5 kg weight (e.g. the liver), can be miniaturized in vitro, without a loss of relevance to the in vivo situation. Hence, coming back to the initial question of "do modern cell culture technologies provide meaningful solutions to close the substance testing gap today?" the answer has to be both "yes" and "no". Cell culture technologies have provided medicine with breakthrough solutions for the robust in vitro generation of transplantable tissues, such as liver tissue, in a natural 3D environment with all the characteristics of self-organisation and self-remodelling. In addition, the latest cell culture achievements have provided proof that multi-organ systems with linked vascularisation and functionalities can be developed to assist with the study of whole-body ADMET. However, the cell culture technologies are at their limits of miniaturization of culture space for dynamic bioreactors, as are actuators, sensors and other pieces of supporting equipment. Therefore, new technologies are needed as a means of developing meaningful solutions to these outstanding problems.

OVERCOMING THE GAP IN IN VITRO TESTING

Stem Cell Niches and Sub-Organoid Self-Assembly

To develop a theoretical understanding concerning the degree to which human organs can be miniaturized in vitro whilst retaining essential functionality, a short excursion into organ development and architecture is necessary. Architecture and functionality are related in all organisms and biological complexity has progressively increased during evolution. In humans, the organization of molecules, cells, tissues, and organs is thought to represent the most advanced levels of evolution (Fig. 3).

Early in human embryonic development, embryonic stem cells give rise to ectoderm, mesoderm and endoderm. Rapid pluripotent stem cell proliferation and cell differentiation into various tissues, which is induced by local microenvironments, continues from fertilization to beyond adolescence, during which organs mature at different rates before functional homeostasis is reached. Multiple and lifelong exposure to xenobiotics is schematically depicted in Figure 4. Should a xenobiotic cause organ or tissue damage, regenerative processes attempt to restore this homeostasis by the renewal of damaged tissues. Thus, a detailed understanding of biological substrates for both organ functionality and organ regeneration may provide the cue for novel solutions in substance testing.

It has been proven that almost all organs are built of multiple, identical, functionally self-reliant, structural units (namely sub-organoids and adult quiescence-promoting stem cells; Fig. 5). Sub-organoids can be composed of several cell layers up to 1mm thick, which corresponds to volumes of less than one microliter. Liver lobules, kidney nephrons, skin dermis and epidermis, gut mucosa, pancreatic islets of Langerhans and the grey and white matter of brain cortex and cerebellum, are some examples of human sub-organoid structures which display functionality and highly variable conglomerate geometry. Due to their organ-specific functionality, their independence from each other, the independence of identical suborganoids within a single organ and a high degree of self-reliance and the multiplicity of such sub-organoids, their reactivity patterns to any substances seem to be representative of the whole organ. This is not surprising, since

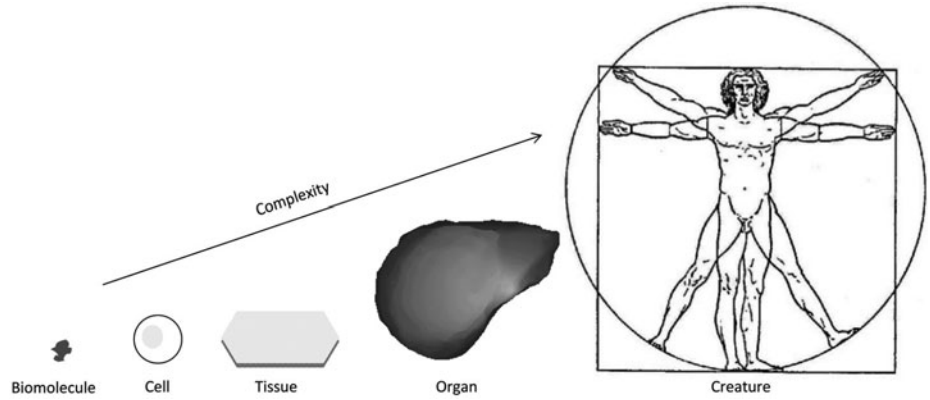

Figure 3. Biological levels of human complexity.

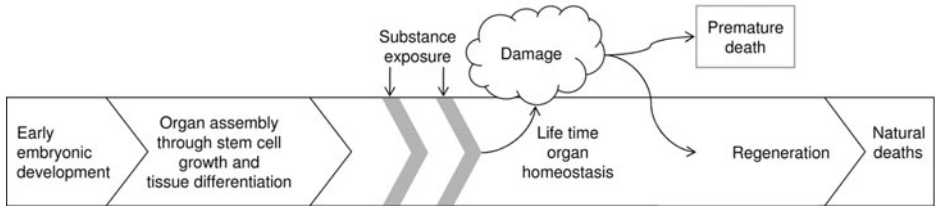

Figure 4. Natural human organ fate. Partial organ damage caused, for example, by toxic substances, is rapidly regenerated by organ-specific mechanisms, resulting in the liver, for example, in full re-organization of the hepatic tissue.

sub-organoids are found naturally, as these structures within a given organ represent nature's risk management tool to prevent the total loss of functionality during partial organ damage, as well as a way for the body to adjust organ size and shape to meet the needs of a given species or, indeed, individual, whilst the same master plan is used to build the single functional sub-organoids.

It can be hypothesised that adult quiescent stem cell niches are distributed within each human organ. Being of exceptionally small size, in the nanoliter range, they represent sorts of germinal crystallization centers for the almost unlimited reproduction of sub-organoids. Almost all the known types of human adult stem cell niches consist of essentially two components which provide quiescence-promoting stem cell niche homeostasis. These are the stem cells themselves and the specific stem cell niche support provided, for example, by the basal membrane and/or feeder cells. An overview of the components which make up the adult physiological stem cell niches of different organs has been provided by Jones and Wagers.[17] Examples of human stem cell niches include: the follicular bulge stem cell niche in the skin, a crypt base, columnar stem cell niche in the small intestine, a broncho-alveolar stem cell niche in the lung, a hematopoietic stem cell niche for blood reconstitution, a sub-ventricular zone stem cell niche for the regeneration of nerve tissue and a stem cell niche for the maintenance of hormone glands.[18-24] The mechanical properties of the stem cell niches influence stem cell

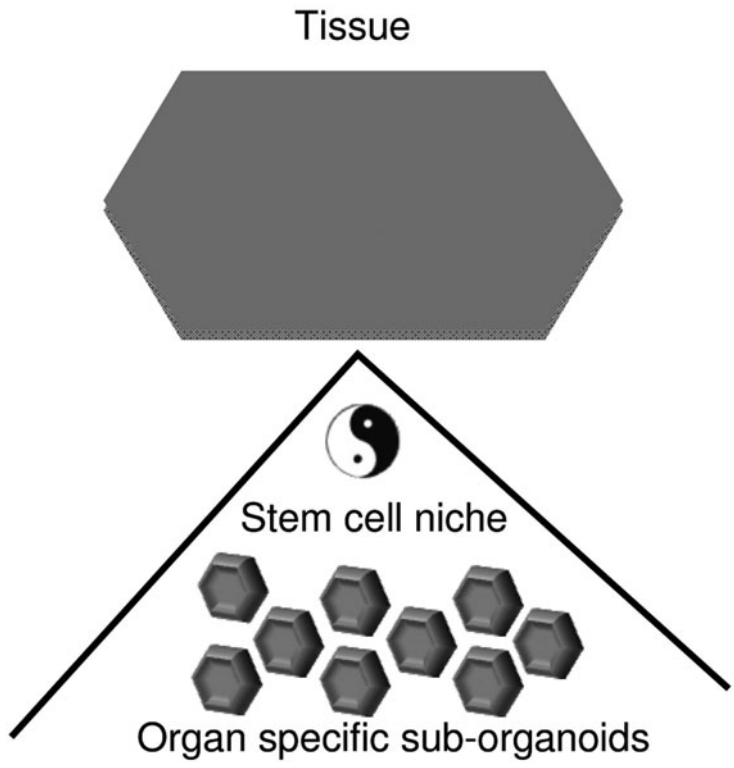

Figure 5. Structural micro-compounds of an organ tissue. Organ-specific sub-organoids and stem cell niches are the smallest building blocks of each human organ. Each microliter sized, self-reliant sub-organoid provides the essential functionality of the respective organ, whereas the nanoliter sized, stem cell niche ensures the rapid renewal of damaged sub-organoids. A Yin-Yang-like quiescence promoting stem cell niche homeostasis is provided by the two essential components of a niche—the stem cells themselves and a support, for example, a basal lamina and/or feeder cells or molecules. Examples are as follows: osteoblasts are suitable as supports for haematopoietic stem cells; vascular cells and astrocytes for sub-ventricular zone stem cells and sub-granular zone stem cells; crypt fibroblasts and Paneth cells for crypt base columnar cells; dermal fibroblasts for follicular bulge stem cells; and Sertoli cells and interstitial cells for spermatogonial stem cells. Under physiological conditions, the number of tissue stem cells remains relatively constant. Divisional asymmetry is caused by intrinsic cellular factors within the cell division process, whereas the exposure of two identical daughter stem cells to different extrinsic signals may lead to environmentally driven differentiation.

function. The relative microstructure and elasticity or stiffness of a stem cell niche, in particular, can directly modify stem cell differentiation decisions. Substantial knowledge has been acquired about how stem cells self-renew and produce differentiated progeny under homeostatic conditions, both during ontogeny and in adults. Therefore, there is a unique opportunity to exploit this knowledge for predicting how xenobiotics affect the micro-structure of human organs. This calls for hybrid micro scale culture systems that allow sub-organoids to be cocultured with their respective stem cell niches, in order to create the “smallest” biological in vitro organ equivalents which might prove useful in overcoming the prohibitive bottlenecks of miniaturization and humanization, whilst at the same time permitting predictive ADMET testing.

Impact of Micro- and Nano-Technologies on the Success of In Vitro Testing

Micro-electro-mechanical systems (MEMS) technology is a multiple-disciplinary approach that can provide high performance micro- and nanosystems for use in various applications, by combining micro- and nanosystem research with microfluidic technology. MEMS are in their early stages of development yet are already proving useful. "Lab-on-a-chip" devices, with their superior analytical performance, are efficient tools for monitoring or performing complex tasks that relate to genetic sequencing, proteomics and drug delivery. However, at present, there are many areas in which the use of these technologies is significantly impeded. Fabricating a microsystem for the dynamic long term culture of tissues of very small size requires more than just assembling together high performance individual functions. The challenge of integrating all the necessary steps in bioanalysis is increased by the fact that signal-to-noise ratios and sensitivity tend to get smaller at reduced scales such that samples have to be relatively large to provide statistically sound data. Today, many cell-based microsystems include the use of "lab-on-a-chip" or "micro-total-analysis-system" (μTAS) that incorporate all assay procedures in a single system. Within this framework, microfluidic technologies that allow the manipulation of nanoliter to femtoliter amounts of fluids by using micrometer scale channels have developed quickly over the past few years and is an essential prerequisite for the fabrication of a number of microscale cell and tissue culture chambers. Other examples of such systems include a microcavity array-based biosensor chip for functional drug screening[25] and microfluidic channels fabricated by lithographic technologies for the in vitro formation of capillary networks.[26] The matrix-dependent adhesion of vascular and valvular endothelial cells was shown in microfluidic channels by Young et al[27] and a digital microfluidic technology for cell-based assays was recently described by Barbulovic-Nad et al.[28] As these technologies were primarily developed for single cell analyses, their adaptation to the handling of larger microliter scale volumes for sub-organoid and stem cell niche culture has still to be achieved. For a review, see reference 29.

With regard to the fabrication materials used in MEMS, silicon-based devices are likely to be complemented with devices made of polymer, textile, glass, ceramic and finally, biological entities. This transition has already started, since biomaterials have been used extensively as parts of electrical and optical biosensors. In recent years, new types of actuators have been developed that are based on polymer materials able to change dimension and/or shape in response to a specific external stimulus (thermal, chemical, electrical, magnetic, electro-chemical, electro-magnetic, or optical). The so-called ElectroActive polymers represent a relevant class of such materials. These polymers exhibit interesting properties, such as sizable active strains and/or stresses in response to an electrical stimulus, low specific gravity, high grade of processability and down-scalability and, in most cases, low costs. An example of how fluid-dynamic gradients of signaling proteins can be integrated in dynamic tissue culture devices is the gradient supporting dynamic tissue chamber of Sonntag and coworkers (Fig. 6). This flexible tissue chamber permits the study of cell-cell interactions in the fluidic interface of signalling biomolecule gradients in a dynamic setting, thus emulating the in vivo situation of rapid hormone release from endocrine glands into the body tissue.

Finally, a prime example of MEMS application for substance testing is a micro-bioreactor that supports the formation of a 3D liver tissue model, which was recently used in drug safety and efficacy assessments.[30] The bioreactor core is a 3D scaffold produced by the deep reactive ion etching of silicon wafers, featuring through-micro-channels with

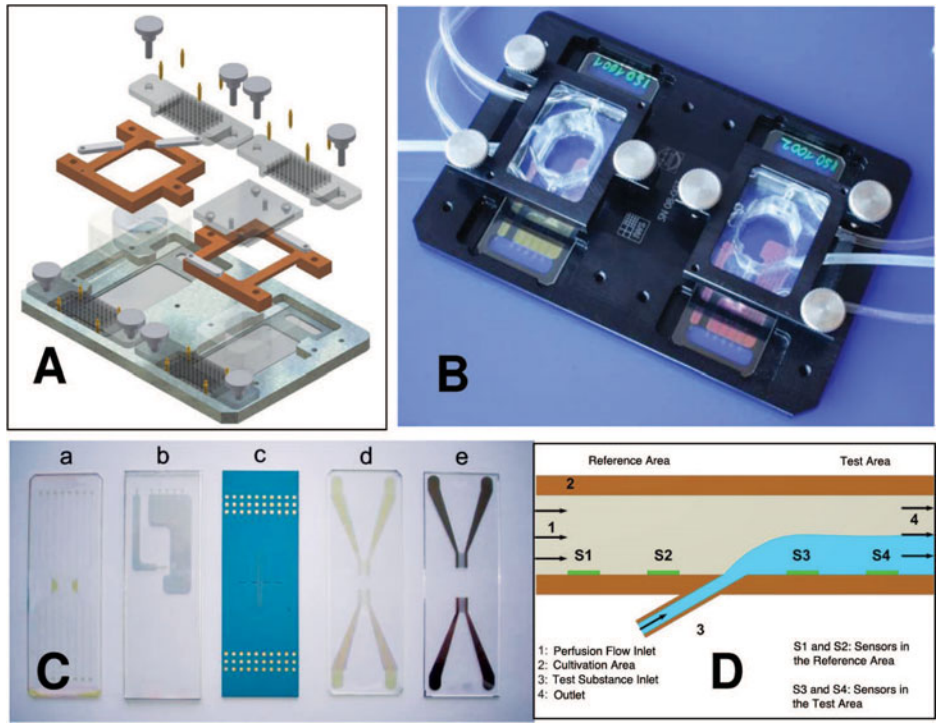

Figure 6. Gradient supporting dynamic tissue culture chamber. Two identical miniaturized microscopic tissue culture flow chambers (A), fabricated in a holder with a 96-well plate format (B) and equipped with different electrical means for cell measuring (C) allow for in-process dynamic microgradients within the tissue culture space, through the defined continuous lateral infusion (D) of fluid-dynamically controlled solutions containing soluble signaling factors. (Illustration courtesy of Frank Sonntag, Fraunhofer IWS, Dresden.)

dimensions of approximately 300 μm × 300 μm. When the scaffold pores are perfused at flow rates where oxygen transport rate matched the estimated oxygen consumption rate, rat hepatocytes adhere to the collagen-coated cell adhesive walls and re-arrange into liver-like structures. However, the cells are still exposed to physiological shear stresses. Seeding cell spheroids was reported to prolong the maintenance of tissue-like architecture and viability. The authors reported that, in this microbioreactor, the hepatocytes expressed quasi-in vivo levels of metabolic competence, unlike the situation with many other bioreactor systems. Comparable solutions are described elsewhere.[31,32]

The above technologies meet the size, shape and microfluidic requirements of microsystems and closely resemble in vivo surfaces and ECM architecture at nano-scale. Technologies for nanostructuring surfaces and generating protein-coated nanoparticles for local signaling have appeared in the last few years.[33] "Tagged" nanoparticles, for instance, can be applied as contrast agents for highlighting specific cells. Examples of their use include fluorescent markers, which can be applied for the observation of biological processes down to the molecular level, by using optical molecular imaging and provide for the extremely sensitive detection of analytes in in vitro microsystems.

In terms of micro-actuation and online monitoring, MEMS technologies are useful. Actuating systems can monitor changes in the physical state of the cells by exerting pressure on the cell mass, as required. Examples include a system that pumps fluids back and forth to simulate capillary flow for bone and cartilage formation, or a tissue interface as found in the gut simulated by providing heat or electrical stimulation. In addition, the electrical sensors discussed above, small size optical sensors for pH and pO_2, have already been introduced into use by the industry. MEMS technology-based microsensors composed of cytokine-specific antibodies coupled on multiple microsurfaces and positioned in the outlet channels of dynamic microbioreactors, are ideal tools for non-invasive cytokine measurement.

In conclusion, the availability of cost-effective technologies is essential for the widespread introduction of micro-systems for sub-organoid and stem cell niche culture. Robust, simple and "stand-alone" approaches are needed for such complex applications. Miniaturized, integrated, "organ-on-a-chip" tools based on microfluidic solutions and enabled by advances in microtechnology and nanotechnology may satisfy this need.

Fully Integrated Human Multi-Micro-Organ Systems

Dynamic macro-scale liver bioreactors have illustrated how fully functional single human organ equivalents can be established and maintained long-term in vitro. For the first time, the holistic approach to modelling human biology in vitro has taken over the differential approach of using immortalized cell lines or individual primary cell cultures. The major hurdle to the translation of these breakthrough achievements of cell culture technology into meaningful solutions for high throughput testing remain miniaturization from the milliliter to the microliter scale and the supply of relevant amounts of standardized human tissue.

Human organ growth, maintenance and regeneration in vivo rely on a balanced interaction between nanoliter-sized adult stem cell niches and surrounding self-reliant sub-organoids of microliter size. The vascular network of microcapillaries of less than 10 μm in diameter reaches each and every sub-organoid. Armed with this knowledge, it is, in theory, possible to link together different human micro-organs at the sub-organoid scale through human vasculature into dynamic multi-micro-organ systems. Cell culture technology, together with MEMS technologies, could be the key for success in this respect.

"Organ-on-a-Chip"

Fundamental paradigms of the in vivo behavior of human organs, as described above, can be translated into rational design principles for dynamic multi-micro-organ bioreactors for in vitro substance testing within three categories; device, architecture and process.

On the basis of these three design principles, we have developed an "organ-on-a-chip" (OOC) platform concept and have prototyped the first dynamic microbioreactor systems aimed at closing the gap in predictive in vitro substance testing.

The OOC is a self-contained, sensor-controlled multi-micro-organ device, the shape of a standard microscope slide, with a total height of less than 3 mm. It fits into an autonomous supply unit (Fig. 7). A portable, battery-based supply unit ensures that the operation of the OOC is independent of any cell culture incubator or power socket. It fits a standard 96-well plate format in width and depth and, being approximately 3 cm in height, matches the appropriate objective distance of high performance microscopes for live tissue imaging into 1 mm tissue depths. It maintains and monitors the temperature

Figure 7. Photograph of a self-contained organ-on-a-chip prototype, composed of a supply unit in black and an "organ-on-a-chip" (OOC) of the standard size of a microscope slide. A holder allows for the easy and exact positioning of the OOC within the supply unit. Electrical connectors ensure the stable temperature of the OOC through sensor controlled heating at its base. Prototypes of supply units, as well as OOC devices, have been fabricated by GESIM mbH, Grosserkmannsdorf, Germany, in collaboration with F. Sonntag and coworkers at the Fraunhofer IWS, Dresden, Germany.

of the six microbioreactors operated on an OOC over at least 14 days. The OOC (Fig. 8) is designed to operate six identical microbioreactor systems simultaneously. All the microbioreactors are provided with nutrients from a common central medium reservoir with a volume capacity of about 1 ml. Each single microbioreactor consists of an organ growth section (Fig. 8A[3]), composed of a central stem cell niche (Fig. 8A[9]), three different organ cavities (Fig. 8A[4,4a,4b]) and three sensor segments, each dedicated to monitoring the outflow of an individual organ cavity (Fig. 8A,B) and three individual reservoirs to collect the spend medium from each organ cavity (Fig. 8A[5]). Microfluidic channels connect the relevant parts of each microbioreactor. The growth section diameter is less than 6 mm and provides organ growth space heights of nearly 500 μm. Thus, as the organ cavities are fabricated from microscopically transparent material, live tissue imaging can be carried out throughout the entire organ culture by means of, for example, two photon microscopy. Continuous feeding is possible for each growth section through a central inlet port that perfuses each of the three organ cavities simultaneously. The resulting metabolic products leave each organ growth section microfluidic channels, each dedicated to a single organ cavity. These outlet channels of dynamic microbioreactors are ideal tools for non-invasive pO_2, pH or cytokine measurements. The autonomous temperature of the OOC is ensured through a thermoregulating device at the base of the

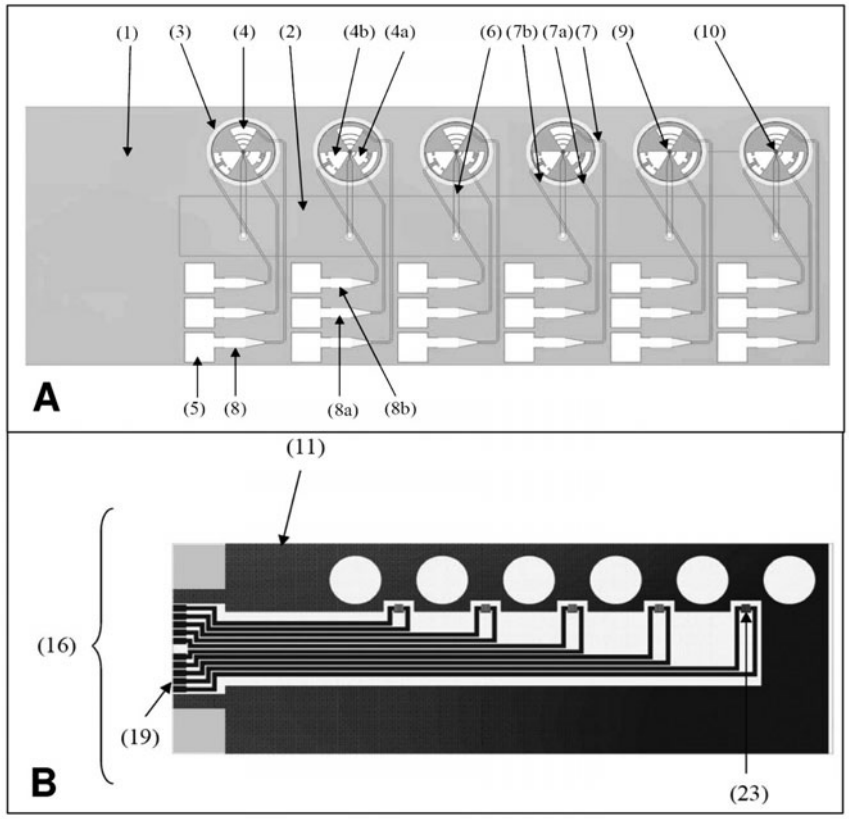

Figure 8. Technical drawing of an "organ-on-a-chip" (OOC) device. A) A top-down view of a section of a self-contained Organchip® (1), comprising six individual organ growth sections (3), each with three organ cavities (4,4a,4b). The medium flows through the microfluidic feed channel (6) to the center of an organ growth section (3), to permit the even distribution of the medium to the three organ cavities. The medium is fed into the organ growth section from an inlet (10) positioned opposite the central stem cell cavity (9). The organ cavities are microstructured to support the re-organization of cell populations or tissue slices into the desired sub-organoids. The outlet allowing medium to flow into the microfluidic waste channel (7,7a,7b) is located at a position opposite the inlet (10) of the microfluidic feed channel (6). The spent medium flows to sensors located in individual flow paths (8, 8a, 8b). Thus, the response to a given compound and/or environmental change can be assayed for individual sub-organoids within each organ cavity of a growth section. It is possible to withdraw a sample or the entire spent medium from the individual waste medium reservoirs (5), and to further analyze each waste medium from one organ and/or organoid individually. B) A top-down view of the upper side of the lower closing layer (16). The means of heating (11) is a temperature sensor composed of indium tin oxide (23). Electric connectors (19), made of gold, are depicted. The conductive paths are also made of gold. The lower closing layer (16) is made of glass.

OOC (Fig. 8B). This allows temperature maintenance and monitoring during the whole process time, including the time frames of online microscope-based, live tissue imaging.

The OOC is fabricated by MEMS from three microstructured thin glass layers (Fig. 9[13]), which are fluid-tight bonded together. A fourth microstructured upper closing layer made of polydimethylsiloxane (PDMS), comprising the central nutrient

reservoir (Fig. 9[2]) and the individual waste reservoirs of each organ cavity (Fig. 9[5]), is fluid-tight and connected to the glass layers. The individual reservoirs for spent medium are sized to support a continuous dynamic for at least a 10-day organ culture period. Both types of reservoirs and the nutrient and the waste reservoirs, are sterilely rechargeable to support organ maintenance over weeks and months, with little need for manual operator handling. The growth sections (Fig. 10) are designed to maintain a central stem cell niche (Fig. 10A[9]) and to support the self-organisation, maintenance and regeneration of various organ-specific sub-organoids in the individual organ cavities. Stem cell niches can be established in the cylindrical stem cell cavity by introducing feeder cells, semisolid media, appropriate scaffolds and components of the basal lamina relevant to the organ culture precursor stem cells. Due to its shape and geometry, a stem cell niche is provided with nutrients primarily by diffusion. Both organ slices and suspended cell populations can be directly loaded into organ cavities through openings in the upper closing layer (Fig. 10C[14]), by using microsyringes, micromanipulators

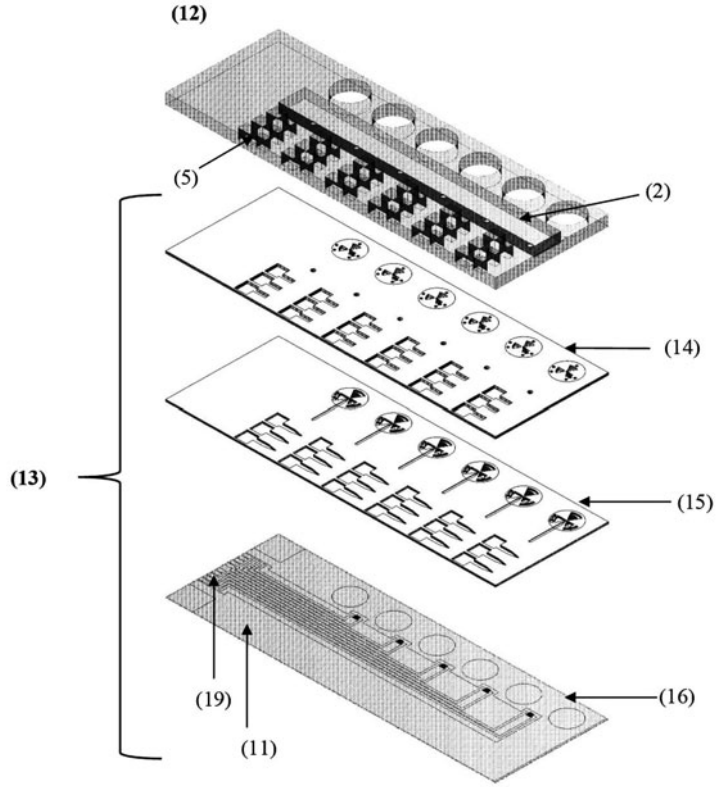

Figure 9. An expanded view of an OOC composed of the medium layer (12) and the organ growth section layer (13), comprised of an upper closing layer (14), the organ cavity layer (15) and the lower closing layer (16). The medium layer (12) has cut-outs to allow access to the organ growth sections, located in the organ growth section layer (15) and between the upper and lower closing layer. These cut-outs are commensurate in size with the cut-outs of the respective organ growth section located beneath, to allow access to each organ cavity within an organ growth section.

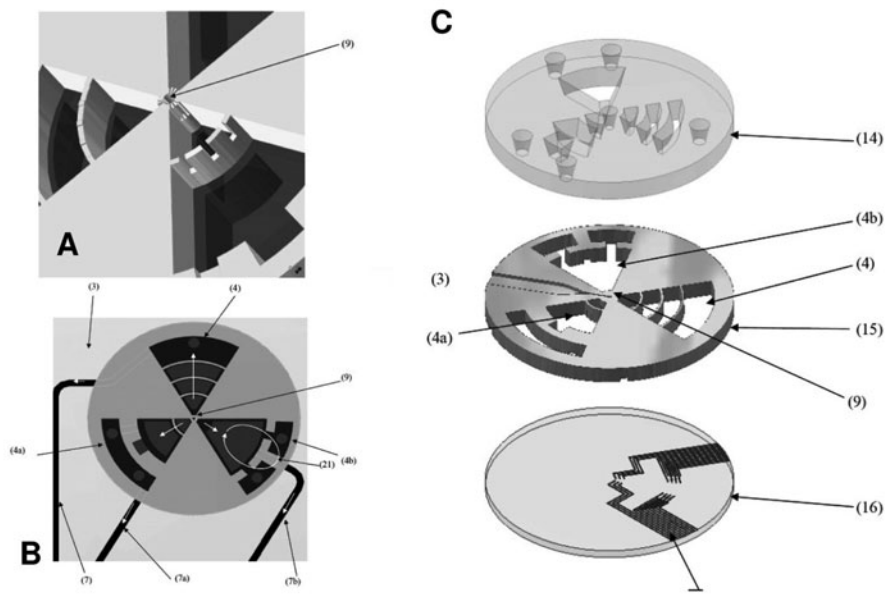

Figure 10. A growth section of an OOC device. A) A 3D view of part of a growth section composed of the three organ cavities, wherein a cavity of about 5 nL volume for formation of an adult stem cell niche (9) is positioned in the center of the three organ cavities. B) A top-down view of a section of the organ cavity layer (15) composed of an organ growth section (3) containing three differently structured organ cavities (4,4a,4b). The medium flow within the growth section (3) into the organ cavities (4,4a,4b) starts from the inlet of the microfluidic feed channel, which is juxtaposed to the stem cell cavity (9), into the organ cavities (4,4a,4b) and out through three separate microfluidic waste channels (7,7a,7b). The direction of the fluid flow is depicted by white arrows. In the growth cavity (4b) this provides an environment for the maintenance of vascularised liver sub-organoids. A secondary fluid flow (21) is imposed by pressurizing means located in the side chambers of this organ cavity (4b). C) An expanded view of a growth section (3) composed of the three organ cavities (4,4a,4b). The organ cavities, located in the organ cavity layer, are each partially closed on the upper side by the upper closing layer (14) and on the lower side by the lower closing layer (16), which provides, for example, a means of measuring impedance (22).

or automated spotters. The organ cavities each have an average capacity of 1.0-1.5 μL of cell suspension or organ tissue. The type of cells introduced into an individual organ cavity depends on the sub-organ micro-architecture and micro-environment the cavity.

The organ cavities of the OOC prototype presented in this publication are designed to allow for the simultaneous establishment and maintenance of brain tissue, sub-organoids of the bone-cartilage interface and vascularised liver (Figs. 10B,C[4,4a,4b] respectively). An organ cavity, for example, designed for the cultivation of central nerve tissue, is provided with four spaces to maintain the different layers of grey matter of the cerebellum (from periphery to the center: granular cell layer, molecular cell layer and Purkinje cell layer) and the white matter layer formed by nerves. The walls between the sections allow for dendrite and axonal projections. Axon-derived nerves have space to occupy the segment proximate to the stem cell cavity. Impedance measurements at the bottom of relevant segments serve as sensors for establishing a functional grey matter layer connection. During the operation of an OOC, the different sub-organoids, formed separately in each

of the three organ cavities, interact with each other through, for example, the outgrowth of nerves from the brain-specific cavity or microcapillaries from the vascularised liver sub-organoid cavity.

Once the whole system has reached homeostasis, test substances can be applied. An OOC device provides six identical dynamic multi-micro-organ systems, to satisfy the statistical requirements of high throughput testing. The OOC platform technology also allows for fast changes in microbioreactor design and rapid prototyping. Thus, it is intended that optimized OOC systems will be generated, which will perfectly match the requirements of systemic in vitro ADMET testing on vascularised microsystems of systemically connected multi-sub-organoid cultures. This may bridge the existing knowledge and technology gaps in xenobiotic testing.

CONCLUSION

Dynamic macro-scale bioreactor systems are the most recent breakthrough in cell culture technology. With more than seven commercially available liver bioreactor systems for acute liver failure, we are able to fully mimic the functions of a complex human organ in vitro. This has resulted in a spectacular long term performance over several weeks at a patient's bedside. This major achievement at the beginning of the 21st century coincides with the need for new approaches for the evaluation of chemicals, cosmetics, air particles or pharmaceuticals, in order to address the caveats which neither animal tests nor conventional human cell line or tissue testing have been able to eliminate. Tremendous efforts have already led to the development of a few miniaturized human in vitro systems that are able to provide research data on individual aspects of substance interaction with humans. Although testing on human liver models is the prime focus of such developments, other systems, such as perfused skin equivalents and the first dynamic human artificial lymph node system, also appear to be promising.

Another great leap forward was the recent proof-of-concept for the in vitro vascularization of human tissue cultures in dynamic bioreactors. This immediately opened up the possibility of evaluating the in vivo distribution of xenobiotics in vitro. A new milestone was reached with the dynamic vascularized human mucosa/gut and liver culture system.[15] This parallelized mini-system, where human mucosa and liver sub-organoids are loaded into the same vascularized culture segment, could address all aspects of ADMET within one bioreactor system.

The OOC technology integrates cell culture technologies with nanotechnologies at an autonomous microsystem level and thus provides a prime opportunity to cope with the challenging requirements of devices for predictive substance testing. The continuous monitoring of systemic parameters of homeostasis by means of systems biology and data processing by computational biology techniques, could lead to revolutionarily efficient, fully predictive procedures.

Outlook

Three types of dynamic microbioreactors, which match the requirements of predictive high throughput substance testing, as well as potential applications in research on the pathogenesis of human disease and to other fields of research, could appear on the scene within the next decade, namely:

1. dynamic human single organ/sub-organoid bioreactors, without self-regeneration potential, dedicated to the evaluation of toxicology, metabolism or immunogenicity of substances, primarily in single dose assays with conventional endpoint parameters;
2. dynamic human single organ/sub-organoid bioreactors, with integrated organ specific stem cell niches exhibiting regenerative potentials that could be used to examine the above endpoints during repeated-dose, long term assays with conventional endpoint parameters; and
3. dynamic human multi-micro-organ systems, providing a common microvasculature circuit with a central "blood" reservoir for several different organs/sub-organoids and their specific stem cell niches within a single microsystem for high content testing. These bioreactors would allow for the continuous monitoring of systemic parameters in addition to conventional endpoint measurements and would be suited to the evaluation of the complete ADMET profile of a substance and its efficacy in repeated-dose, long term test procedures over months.

REFERENCES

1. Clair EWS. The calm after the cytokine storm: lessons from the TGN1412 trial. J Clin Invest 2008; 118(4):1344-1347.
2. Carrel A. On the permanent life of tissue outside of the organism. J Exp Medicine 1912; 15:516-528.
3. Fell HB, Robison R. The growth, development and phosphatase activity of embryonic avian femora and limb buds cultivated in vitro. Biochem J 1929; 23:767-784.
4. McLimans WF, Crouse EJ, Tunnah KV et al. Kinetics of gas diffusion in mammalian cell culture systems. Biotech Bioeng 1968; 10:725-740.
5. Dexter TM, Lajtha LG. Proliferation of hemopoetic stem cells in vitro. J Haematol 1974; 28:525.
6. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol 2006; 7:211-224.
7. Gerlach JC, Encke J, Hole O et al. Hepatocyte culture between three dimensionally arranged biomatrix-coated independent artificial capillary systems and sinusoidal endothelial cell coculture compartments. Int J Artif Organs 1994; 17:301-306.
8. Sauer IM, Zeilinger K, Obermayer N et al. Primary human liver cells as source for modular extracorporeal liver support—a preliminary report. Int J Artif Organs 2002; 25:1001-1005.
9. Sauer IM, Kardassis D, Zeillinger K et al. Clinical extracorporeal hybrid liver support—phase I study with primary porcine liver cells. Xenotransplantation 2003; 10(5):460-9.
10. Sauer IM, Zeilinger K, Pless G et al. Extracorporeal liver support based on primary human liver cells and albumin dialysis—treatment of a patient with primary graft nonfunction. J Hepatol 2003; 39:649-653.
11. Powers MJ, Domansky K, Kaazempur-Mofrad MR et al. A microfabricated array bioreactor for perfused 3D liver culture. Biotechnol Bioeng 2002; 78(3):257-69.
12. Catapano G, Gerlach JC. Bioreactors for liver tissue engineering. Topics in Tissue Engineering 2007; 3:1-42.
13. Giese C, Demmler CD, Ammer R et al. A human lymph node in vitro—challenges and progress. Artif Organs 2006; 10:803-808.
14. Pörtner R, Giese C. Overview on bioreactor design, prototyping and in process controls for reproducible three-dimensional tissue culture. In: Marx U, Sandig V, eds. Drug Testing In Vitro—Breakthroughs and Trends in Cell Culture Technology. Weinheim: Wiley-VCH, 2007:53-70.
15. Mertsching H, Walles T, Hofmann M et al. Engineering of a vascularized scaffold for artificial tissue and organ generation. Biomaterials 2005; 26(33):6610-6617.
16. Linke K, Schanz J, Hansmann J et al. Engineered liver-like tissue on a capillarized matrix for applied research. Tissue Eng 2007; 13(11):2699-2707.
17. Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 2008; 9(1):11-21.

18. Kloepper JE, Tiede S, Brinckmann J et al. Immunophenotyping of the human bulge region: the quest to define useful in situ markers for human epithelial hair follicle stem cells and their niche. Exp Dermatol 2008; 17(7):592-609.
19. Walker MR, Stappenbeck TS. Deciphering the 'black box' of the intestinal stem cell niche: taking direction from other systems. Curr Opin Gastroenterol 2008; 24(2):115-120.
20. Kim CF. Paving the road for lung stem cell biology: bronchioalveolar stem cells and other putative distal lung stem cells. Am J Physiol Lung Cell Mol Physiol 2007; 293:1092-1098.
21. Can A. Haematopoietic stem cells niches: interrelations between structure and function. Transfus Apher Sci 2008; 38(3):261-268.
22. Martinez-Agosto JA, Mikkola HK, Hartenstein V et al. The hematopoietic stem cell and its niche: a comparative view. Genes Dev 2007; 21(23):3044-3060.
23. Yin T, Li L. The stem cell niches in bone. J Clin Invest 2006; 116(5):1195-1201.
24. Riquelme PA, Drapeau E, Doetsch F. Brain micro-ecologies: neural stem cell niches in the adult mammalian brain. Philos Trans R Soc Lond B Biol Sci 2008; 12; 363(1489):123-137.
25. Kloss D, Kurz R, Jahnke HG et al. Microcavity array (MCA)-based biosensor chip for functional drug screening of 3D tissue models. Biosensors Bioelectron 2008; 23:1473-1480.
26. Kobayashi A, Miyake H, Hattori H et al. In vitro formation of capillary networks using optical lithographic techniques. Biochem Biophys Res Commun 2007; 358(3):692-7.
27. Young EW, Wheeler AR, Simmons CA. Matrix-dependent adhesion of vascular and valvular endothelial cells in microfluidic channels. Lab Chip 2007; 7(12):1759-1766.
28. Barbulovic-Nad I, Yang H et al. Digital microfluidics for cell-based assays. Lab Chip 2008; 8:519-526.
29. Tanaka Y, Sato K, Shimizu T et al. Biological cells on microchips: new technologies and applications. Biosensors Bioelectron 2007; 23:449-458.
30. Powers MJ, Janigian DM, Wack KE et al. Functional behavior of primary rat liver cells in a 3D perfused microarray bioreactor. Tissue Eng 2002; 8:499-513.
31. Sivaraman A, Leach JK, Townsend S et al. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. Curr Drug Metab 2005; 6(6):569-591.
32. Schumacher K, Khong YM, Chang S et al. Perfusion culture improves the maintenance of cultured liver tissue slices. Tissue Eng 2007; 13(1):197-205.
33. Engel E, Michiardi A et al. Nanotechnology in regenerative medicine: the materials side. Trends Biotechnol 2007; 26(1):39-47.

CHAPTER 4

TISSUE ENGINEERING IN THE DEVELOPMENT OF REPLACEMENT TECHNOLOGIES

Kevin M. Shakesheff* and Felicity R.A.J. Rose

Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), Centre Biomolecular for Studies, School of Pharmacy, University of Nottingham, Nottingham, UK
**Corresponding Author: Kevin M. Shakesheff—Email: kevin.shakesheff@nottingham.ac.uk*

Abstract: The field of tissue engineering is generating new scaffolds, bioreactors and methods for stimulating cells within complex cultures, with the aim of recreating the conditions under which cells form functional tissues. Hitherto, the primary focus of this field has been on clinical applications. However, there are many methods of in vitro tissue engineering that represent new opportunities in 3D cell culture and could be the basis for new replacement methods that either replace the use of a tissue isolated from an animal or the use of a living animal. This chapter presents an overview of tissue engineering and provides tissue-specific examples of recent advances.

INTRODUCTION

The tissues that make up the mammalian organism are remarkable 3D structures that carry out complex functions for many years by self-organizing billions of cells into defined architectures. Within these architectures, cells are held within niches that define their environmental cues for proliferation and differentiation. Over the lifetime of an organism, these tissues will spontaneously develop, renew and, occasionally, repair themselves.

The field of tissue engineering aims to replicate cell niches in vitro and thereby control complex cell behaviors to a level not currently achieved by cell culture methods. The major motivation for the investment in tissue engineering over the last decade has been to generate clinical products or procedures that restore tissue and function within a patient. The technical challenge faced in developing therapies based on tissue engineering has driven the development of new methods of handling cells in three dimensions. A considerable spill-over benefit of the advances in clinically-driven tissue engineering could be the development of

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

replacement tissues that can be manufactured from small populations of expandable cells. Advances in stem cell technology can also be harnessed with tissue engineering, to generate differentiated models by using human cells, without immortalization.

This chapter provides an overview of the current state-of-the-art in tissue engineering and highlights the limited number of successes to date that have adopted tissue engineering principles in order to enhance replacement technologies.

OVERVIEW OF TISSUE ENGINEERING STRATEGIES

Scientific awareness of tissue engineering grew rapidly in the 1990s.[1-3] This awareness was fuelled by breakthroughs in the use of 3D polymer templates to host tissue formation and especially skin,[4] cartilage[5] and cardiovascular tissues.[3] In fact, most of the techniques required to engineer tissues had been under development for many decades before the term "tissue engineering" became widely recognized and used. The concepts of using cocultures, extracellular matrix (ECM) signals, bioreactors and 3D cell aggregates to enhance tissue functionality in cell populations were published in the 1970s.

However, the concept of combining such approaches in order to manufacture tissues of sufficient quality to implant into patients was realized by researchers in the 1990s and the potential of tissue engineering caught the imagination of scientists, clinicians and the general public.

An overarching principle of tissue engineering is the generation of in vitro environments for a cell population that mimic development, renewal or repair in in vivo environments. The key aspects of such environments are discussed below.

The ECM

Cell anchorage and motility require receptor-mediated interactions with the ECM.[6] Many tissue engineering and 3D cell culture methods employ natural ECM models to replicate integrin receptor-binding mechanisms. Alternatively, synthetic ECM mimics can be formed by the surface engineering of synthetic polymers to create surfaces that present short peptides that can bind to integrins.[7]

Soluble Growth Factors and Cytokines

The availability and concentration of growth factors represent a powerful switch in controlling cell behavior. Numerous growth factors and differentiation factors can be used to stimulate proliferation and differentiation. The simple addition of these factors to cell culture media is effective in many culture systems. However, the delivery of these factors is more sophisticated in vivo. For example, gradients and cascades of factors are used to pattern differentiation in stem cell populations. Furthermore, growth factor effects may be synergistic with ECM signalling.[8]

Biomechanical Conditioning

Many cell types show significant sensitivity to the local biomechanical environment.[9] The role of the biomechanical environment is clear for contractile or structural tissues. For many such tissue types, the restoration of appropriate physical forces has been demonstrated

to enhance tissue formation or maintenance in vitro. In other situations, such as in the liver, the direct role of mechanical forces in controlling cell niche is less clear. For these cases, it may be important to eliminate culture-induced forces (e.g., gravitational effects on cell suspensions). Biomechanical forces can be controlled through the use of bioreactors.

Cell-Cell Signaling

The functioning of many tissues is dependent on complex patterns of signaling between different cell types. The liver provides an excellent example, in which stellate cells and hepatocytes signal extensively through direct physical contacts.[10]

Architecture

The role of architecture in tissue functioning is observed at a number of different length scales. At the cell level, the simple organization of cells into 3D clusters can have a profound effect on the phenotype. This has been demonstrated by the work of Bissell et al.[11] Architectural effects are also evident in the structural components of tissues. For example, within the liver, the architecture of the liver lobule creates a unidirectional flow of blood from the periportal to the centrolobular regions. This flow pattern means that hepatocyte cells close to the periportal triads are exposed to blood that has just passed through the gut and has taken in any food or toxin-related molecules that have been absorbed. Hence, these cells bear the brunt of the liver's metabolic activity. In contrast, cells toward the centrolobular region have a phenotype that results in greater secretory activity. Thus, the architecture of a tissue can impose local fluid flow patterns, soluble molecule gradients, the polarity of cell-cell interactions and the location of cell-to-ECM binding.

REVIEW OF SPECIFIC TISSUE ENGINEERING ADVANCES BY TISSUE

In this section, examples of the application of the principles summarized in Overivew of Tissue Engineering Strategies will be provided.

Skin

The in vitro manufacture of skin is the most advanced area of tissue engineering and is the one example that has impacted on both clinical practice and replacement technologies. The success achieved in skin tissue engineering has been due to the following:

- The ability to expand primary cell cultures isolated from the dermal and epidermal layers.
- The limited thickness of the tissue, which permits good nutrition availability to all the cells without vascularization or sophisticated bioreactor design.
- The aspects of the skin required for some clinical and some replacement technologies are largely related to function as a barrier.
- There is a motivated user base that requires a replacement technology for product development.

Within the replacement field, two tissue engineered skin models have been fully validated and approved by regulators as functionally equivalent to the commonly performed in vivo methods.[12,13] The EpiDerm™ and the EpiSkin™ corrosivity tests can be used to determine the corrosive potential of a chemical by assessing the cytotoxic effect on reconstituted human epidermis, a test conventionally carried out in vivo by using the Draize rabbit skin test.[14] EpiDerm (MatTek, Ashland, MA, USA) comprises a reconstructed epidermis of human keratinocytes grown on inert polycarbonate filters, while EpiSkin (L'Oréal, Clichy, France) consists of a reconstructed epidermis grown on a Type I bovine collagen matrix, representing the dermis, surfaced with a film of Type IV human collagen.[15,16] Keratinocytes grown at the air-liquid interphase differentiate to form a stratified epithelium, which is similar to the stratum corneum of human skin and is suitable for use in an alternative assay system, effectively replacing the conventional in vivo model. Another reconstructed human epidermis product, Skin² ZK 1350™ (Advanced Tissue Sciences Inc., La Jolla, CA, USA), has also been discontinued, while a third, SkinEthic™ (SkinEthik, Nice, France), is of similar nature. The latter shows a similar behavior when subjected to the approved corrosivity protocols, but has not been fully validated nor been approved by regulators.[15,18,19] The European Center for the Validation of Alternative methods (ECVAM) International Validation Study on In Vitro Tests for Acute Skin Irritation has recently reported on the validity of the EpiSkin and EpiDerm assays and on the skin integrity function test.[46]

All of these reconstituted human skin models have similarity in terms of general structure, cellular composition and biochemistry and therefore provide a valuable tool in alternative toxicity testing, phototoxicity testing and drug transport studies. However, most of the current models are epidermis-only models. It is likely that a more-complex model, which includes an underlying dermis, will resemble the appearance of skin and its physiology more closely.

Other epithelia have also been reconstructed in vitro on artificial polymer matrices, as with human epidermis, as outlined above. MatTek has developed buccal (EpiOral™), corneal (EpiOcular™), ectocervico-vaginal (EpiVaginal™), gingival (EpiGingival™) and tracheal/bronchial (EpiAirway™) equivalents, while SkinEthik also offers alveolar, corneal, gingival, esophageal, oral and vaginal tissue equivalents. However, none of these reconstructed epithelia have thus far been used in the development of alternative assay systems for regulatory use.

Liver

There is a clear need for better in vitro models of the human liver. The challenge to engineer the liver is very significant and demonstrates a number of important tissue engineering principles. Within the body, the liver displays a remarkable regenerative capacity. For example, patients can spontaneously recover from major resections involving the removal of up to 80% of the liver mass. However, when hepatocytes are isolated in vitro, they display limited proliferative capacity and rapid changes in gene expression lead to dedifferentiation within hours and days. Hence, the use of human liver tissue in pharmacological and toxicological assays is inefficient and largely limited to very short-term studies involving acute metabolic events rather than chronic tissue-specific events. Therefore, if tissue engineering could restore the in vivo cues that permit liver regeneration and the long-term maintenance of liver functions, it would be possible to devise replacement technologies that increase the availability of in vitro tissue and permit chronic, repeat exposure studies.

A large body of literature, much of which predates the advent of tissue engineering as a defined field, involves investigations on the individual roles of the ECM, heterotypic cell-cell interactions and soluble factor effects. Applying each of these individual strategies can boost one or more liver-specific functions in vitro. However, in isolation, none of them can generate a liver tissue model that could profoundly affect replacement technologies.

The role of tissue engineering is to combine the influences of coculture, ECM and soluble factors and to contribute new ideas in the design of flow systems that mimic blood flow and the resulting chemical gradients within the liver. Excellent examples of this approach have been published by the team of Gerlach et al,[20] who demonstrated that oxygenation via gas-permeable hollow fibers that run through the 3D tissue space enhanced the metabolic activity of hepatocytes. More recently, they have built on their early work on oxygenation systems to evolve a new bioreactor that promotes the reorganization of hepatocytes and nonparenchymal cells into histiotypic structures.[21] This bioreactor system enabled cell metabolic activity to remain constant for at least 20 days. Both bile canaliculi and sinusoid-like structures developed spontaneously and increased proliferative activity was detected within the cell population. Proliferation combined with sustained liver-specific functions offer the potential to increase tissue mass from a primary cell source and thereby to reduce the tissue requirement for each clinical procedure. This type of bioreactor also shows significant potential as an in vitro model for drug metabolism and toxicology studies.[22]

Cima-Griffiths et al have also combined innovative bioreactor design and 3D cell culture. They have described a microarray bioreactor for the maintenance of heterotypic multicellular liver models.[45] The microarray system provides a thin transparent structure, within which a silicon scaffold provides square ports that host the liver cells. Fluid flow within the bioreactor occurs through the ports and hence, partially mimics liver blood flow dynamics. With this system, preformed spheroids were found to out-perform single cell suspensions as the format for the seeding of the bioreactor. When hepatocytes and nonparenchymal cells were formed into spheroids before addition to the bioreactor, histiotypic structures formed and albumin secretion was better maintained that when the same cell types were added directly, without the preformation of the spheroids.

Finally, Bhatia's group have recreated zonal patterns of hepatocyte function by controlling oxygen gradients within cocultures of hepatocytes and nonparenchymal cells.[23] Mathematical models of the oxygen gradients within the liver provided targets to be recreated in vitro. This team used a syringe pump to control the introduction of oxygenated medium at one end of flat-bed bioreactor. By culturing hepatocytes within this bioreactor, the in vivo-like zonation of expression of two key drug metabolizing enzymes was created. Furthermore, drug toxicity matched the zonation of the metabolizing enzymes.

Nerves

Tissue engineering approaches to enhance nerve regeneration provide examples of how molecular gradients and micron-scale patterns can be used to guide 2D and 3D pattern formation in tissues. Control over cell position within a regenerating tissue can be exerted by using location-specific ECM interactions or by using gradients of growth factors or trophic factors.

Patel et al[24] have demonstrated the ability to guide neurite extension by using peptides that mimic key ECM molecules. This provides an example of the guidance of neurites using a peptide containing the laminin-specific sequence, IKVAV. This peptide

was patterned on to a biodegradable polymer substrate by using microfluidic patterns to form stripes with widths of 12-70 μm and lengths of many millimeters. The PC12 cell line can respond to laminin surface cues by extending neurite-like projections and these projections follow the biochemical pattern.

Moore et al[25] have used the concept of patterning with a 3D scaffold with macroporous scaffolds and gradients of nerve growth factor (NGF) and neurotrophin-3 (NT-3). Gradients can be easily manufactured via the diffusion-limited mixing of the NGF/NT-3 with the polymer components that make up the scaffold, immediately prior to scaffold solidification. It was found that a NGF gradient of 310 ng/mL/mm was required to stimulate the guidance of neurite extension of chick dorsal root ganglion explants. However, this minimum concentration gradient was lowered if a graft of both NGF (200 ng/mL/mm) and NT-3 (200 ng/mL/mm) was prepared. This study provides an elegant demonstration of the ability to use synergistic effects between factors to elicit tissue-specific responses.

Cardiovascular Tissue

There are two major clinical drivers for cardiovascular tissue engineering, the regeneration of cardiac muscle to provide tissue repair after an infarction and the manufacture of small vessel conduits for vascular surgery.

The ability to regenerate cardiac muscle presents a major challenge, due to the lack of spontaneous repair or cardiomyocyte proliferation within the heart following ischemic damage. Clinical trials are under way to assess the therapeutic potential of cell therapies in which a cell population is injected in the heart wall. However, an alternative approach, whereby cardiac muscle is engineered in vitro, then surgically attached to the heart, offers greater potential in the search for tissue engineered models that could be used in toxicology.

The work of Radisic et al[26] provides a recent example of an approach to combining mechanical and biochemical cues to enhance the formation of cardiac muscle within scaffolds. A coculture of myocytes and cardiac fibroblasts was formed within an elastic porous scaffold. The scaffold was designed with an array of parallel channels running through its length, to promote fluid flow. This cell/scaffold construct was then transferred into a bioreactor, in which a novel culture medium flowed. The culture medium contained a perfluorocarbon (PFC) emulsion that carried high concentrations of oxygen (mimicking the role of hemoglobulin in the blood). Both the presence of the channels within the scaffold and the PFC carrying oxygen significantly improved the levels of cardiac muscle-specific markers such as troponin I and connexin-43. If this approach is combined with the current rapid progress in the derivation of cardiomyocytes from embryonic stem cells, and in vitro models of cardiac muscle, in which scale-up of manufacturing and scale-down of the size of the tissue under investigation, can be achieved.

Tissue engineered blood vessels (TEBV) are under intensive investigation as antithrombogenic and mechanically stable vessels that could be implanted into patients. L'Hereux et al[27] have formed layered tubular constructs of living adventitia, a decellularized internal membrane and an endothelium. TEBVs with internal diameters of 4.2 mm and wall thicknesses of approximately 409 μm displayed broadly similar burst pressures and compliance to saphenous veins.

Skeletal Muscle

Recent progress in the engineering of skeletal muscle has addressed a major shortcoming in many in vitro tissue engineering approaches, that is, the need for vascularization in order to generate thick tissues. Levenberg et al[28] have achieved early stages of in vitro vascularization using a 3D multicell construct. They combined myoblasts, embryonic fibroblasts and endothelial cells within porous scaffolds. The embryonic fibroblasts increased the levels of expression of vascular endothelial growth factor (VEGF) within the system and promoted the formation and stabilization of vessel networks. The myoblasts formed muscle tissue around the developing network of tubular endothelial cell constructs.

Gastrointestinal Tissue

Intestinal tissue engineering has, as yet, not been extensively studied and currently is limited to in vivo strategies where the body is used as the bioreactor.[29] The intestinal epithelium is a complex tissue, which lines the gastrointestinal tract and provides an interface between the contents of the gut lumen and the internal regions of the body. The epithelium is folded, so that it has a well-defined architecture of cavities, known as the crypts of Lieberkühn. Adult stem cells reside at the bases of these crypts and divide and migrate both up and down (in the case of the small intestine) the crypt, whilst differentiating to replenish the supply of mature functional cells. The differentiated cells perform their specific functions, undergo apoptosis and slough off into the lumen, having a total life-span of 5-7 days. The intestinal epithelium is an example of a rapidly renewing tissue, but the in vitro tissue engineering of this organ has so far eluded researchers, due to the difficulties associated with establishing a suitable cell source for this purpose; it is very difficult to culture primary intestinal progenitors in vitro.

Despite this, encouraging reports on oesophageal,[30,31] small bowel,[32] colonic[33] and stomach[33,34] tissue engineering have been published. All of these regeneration programs have been based on two common themes: (1) a material scaffold that is placed into an animal model following anastomosis (the removal of a section of the intestinal tube); or (2) epithelial organoids (mesenchymal cell cores surrounded by a polarized epithelium) that are obtained from a section of intestinal tissue and are seeded into a material scaffold before being transplanted into the animal. Such material scaffolds have included biodegradable poly(glycolic acid) (PGA) meshes, coated either with collagen or with poly(lactic acid) (PLA) and porcine small intestinal submucosa (SIS). All the seeded scaffolds were implanted in vivo, either into a region of the gastrointestinal tract of athymic mice following anastomosis or into the latissimus dorsi muscle of athymic mice. In all cases, post-implantation analysis demonstrated the presence of a functional and structural gastrointestinal epithelium, but which lacked the muscle component of the bowel. However, this research does demonstrate the feasibility of engineering a complex organ such as the gastrointestinal tract.

The Cornea

Diseases affecting the cornea and corneal trauma are a major cause of blindness worldwide.[35] For partial thickness defects, where only the surface of the cornea is affected, a novel treatment developed by the Okana group[36] involves “carrier free

cultivated corneal epithelial sheet transplantation". These bioengineered cell sheets are achieved by cultivating corneal limbal stem cells on temperature-sensitive culture dishes coated with the thermoresponsive polymer, poly-N-isopropylacrylamide (PIPAAm). Following culture, the stratified epithelial layers are simply released from the dish by the reduction of the temperature to the polymer's lower critical solution temperature (LCST) of 32°C.[37] Initial human trials revealed that the bio-engineered corneal epithelial cell sheet grafts remained intact and covered the entire cornea. Moreover, corneal transparency and visual acuity were restored. It is thought that by maintaining cell-cell interactions within the epithelial cell layer and between the epithelium and the underlying stroma, the long-term survival of the cultivated epithelial sheet is enhanced due to the maintenance of cell surface proteins and the ECM which is deposited. This technology has since been extended to the generation of corneal endothelial cell sheets, as a possible treatment for many pathological cases where the corneal endothelial layer is the only component requiring substitution.

The shortage of human corneal donors and the risk of immunorejection for the replacement of full thickness defects have been the driving force behind the generation of tissue-engineered corneal constructs for transplantation. Functional corneal reconstruction has largely focused on the culture of the three main layers of the cornea (epithelium, stroma and endothelium). The initial steps involve the isolation, culture and expansion of each cell type in vitro. The stroma is produced by mixing corneal keratocytes with a scaffolding material, usually collagen Type I and/or Type III, or a composite with glycosaminoglycans, which is subsequently remodeled by the keratocytes in the culture. The final stage involves culturing limbal epithelial cells (thought to include limbal stem cells) on the engineered stroma at an air-liquid interface.[38-41] These tissue engineered constructs have been shown to support neurite extension,[42] and to perform well when transplanted into an in vivo model.[43]

Airway Epithelium

A human airway epithelial wounding model have been developed by Wadsworth et al.[44] It has been used to study the pharmacological mechanism of the beneficial effects of glucocorticoids in the treatment of asthma. The model was constructed by using human bronchial epithelial cells (HBEC), which were cultured at an air-liquid interface and triggered to differentiate into the mucociliated phenotype. This model could be used to study repeated physical wounding and, hence, to study chronic mechanisms of drug action.

CONCLUSION

Tissue engineering has been a priority area for research across the world for the last decade and it is likely that it will remain a priority for many years to come. Scientifically, the field has advanced at a rapid rate and this chapter has highlighted the fact that many studies now involve the combined use of a number of engineering tools to recreate complex regenerative or repair environments.

Whilst the clinical and commercial impact of tissue engineering has been significant, there have been few examples of contributions toward the design of replacement technologies. The scientific case for using tissue engineering in replacement science is compelling since both fields require in vitro systems that accurately represent key

aspects of the structures, functions and reactions of tissues in vivo. One area of success has been the use of skin models, where the progress made in the use of skin models as replacement alternatives is possibly due to the relatively simple conditions required to create models that mimic the barrier properties in this relatively thin organ.

Looking to the future, there are a number of reasons for optimism about collaboration between tissue engineers and replacement alternative scientists. In the vast majority of cases, the clinical and commercial uptake of tissue-engineered products has been impeded by the complexity of the products and their manufacturing processes. For example, the first generation of tissue-engineered skin products has been clinically successful in terms of trial results, but disappointing in terms of commercial returns. Applying tissue engineering to tackle replacement offers a route to the early uptake of the new technology as the critical path to launching a successful replacement technology appears to be less arduous, although by no means simpler, than that involved in launching a living clinical product.

However, there are major barriers to the application of tissue engineering in the search for replacements. One barrier is the lack of communication between tissue engineers and replacement scientists. The clinical focus of tissue engineers does not provide many spontaneous opportunities for cross-talk between the communities. Organizations such as the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs), the Fund for Replacement of Animals in Medical Experiments (FRAME) and the Dr. Hadwen Trust, are promoting collaboration and discussion. Related to this problem, it can be difficult for tissue engineers, who lack a long background in replacement science, to identify the important replacement problems that their ideas can address.

Finally, there are at least two grounds for confidence that tissue engineering can make a major contribution to the future of replacement. Firstly, as shown in this chapter, the past few years have seen major advances in the engineering of complex tissues with embedded, albeit primitive, vascular networks and gradients of growth factors and oxygen, that begin to mimic a degree of the complexity of regenerating and developing tissues. Secondly, the rapid pace of development in stem cell technologies is generating populations of human cells that can be expanded to permit the scaling-up of in vitro models and that can be induced to differentiate and form most, if not all, the tissue types within the body.

REFERENCES

1. Freed LE, Vunjaknovakovic G, Langer R. Cultivation of cell-polymer cartilage implants in bioreactors. J Cell Biochem 1993; 51(3):257-264.
2. Langer R, Vacanti JP. Tissue engineering. Science 1993; 260(5110):920-926.
3. Ziegler T, Nerem RM. Tissue engineering a blood vessel—Regulation of vascular biology by mechanical stresses. J Cell Biochem 1994; 56(2):204-209.
4. Koller MR, Palsson BO. Tissue engineering—Reconstitution of human hematopoiesis ex-vivo. Biotechnol Bioeng 1993; 42(8):909-930.
5. Freed LE, Vunjaknovakovic G. Cultivation of cell-polymer tissue constructs in simulated microgravity. Biotechnol Bioeng 1995; 46(4):306-313.
6. Dipersio CM, Shah S, Hynes RO. Alpha-3a-beta-1 integrin localizes to focal contacts in response to diverse extracellular-matrix proteins. J Cell Sci 1995; 108:2321-2336.
7. Massia SP, Hubbell JA. Vascular endothelial cell adhesion and spreading promoted by the peptide Redv of the Iiics region of plasma fibronectin is mediated by integrin alpha-4-beta-1. J Biol Chem 1992; 267(20):14019-14026.

8. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005; 23(1):47-55.
9. Knight MM, Bomzon Z, Kimmel E et al. Chondrocyte deformation induces mitochondrial distortion and heterogeneous intracellular strain fields. Biomech Model Mechanobiol 2006; 5(2-3):180-191.
10. Nahmias Y, Berthiaume F, Yarmush ML. Integration of technologies for hepatic tissue engineering. Adv Biochem Eng Biotechnol 2007; 103:309-329.
11. Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds and signaling: Tissue architecture regulates development, homeostasis and cancer. Annu Rev Cell Dev Bi 2006; 22:287-309.
12. Anon. Commission Directive 2000/33/EC of 25 April 2000 adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Off J Eur Comm 2000; L136:90-107.
13. ICCVAM. ECVAM protocol for EPISKIN: an in vitro assay for assessing dermal corrosivity. Morrisville: NICEATM-ICCVAM, 2002. Available at: iccvam.niehs.nih.gov/docs/dermal_docs/cwgfinal02/07cdprot.pdf.
14. Draize JH, Woodard GK, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944; 82:377-390.
15. Netzlaff F, Lehr CM, Wertz PW et al. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity and substance transport. Eur J Pharm Biopharm 2005; 60(2):167-178.
16. Tinois E, Tiollier J, Gaucherand M et al. In vitro and posttransplantation differentiation of human keratinocytes grown on the human type IV collagen film of a bilayered dermal substitute. Exp Cell Res 1991; 193(2):310-319.
17. Poumay Y, Coquette A. Modelling the human epidermis in vitro: tools for basic and applied research. Arch Dermatol Res 2007; 298(8):361-369.
18. Kandarova H, Liebsch M, Schmidt E et al. Assessment of the skin irritation potential of chemicals by using the SkinEthic reconstructed human epidermal model and the common skin irritation protocol evaluated in the ECVAM skin irritation validation study. Altern Lab Anim—ATLA 2006; 34(4):393-406.
19. Rosdy M, Clauss LC. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. J Invest Dermatol 1990; 95(4):409-414.
20. Gerlach J, Kloppel K, Stoll P et al. Gas-supply across membranes in bioreactors for hepatocyte culture. Artif Organs 1990; 14(5):328-333.
21. Zeilinger K, Holland G, Sauer IM et al. Time course of primary liver cell reorganization in three-dimensional high-density bioreactors for extracorporeal liver support: An immunohistochemical and ultrastructural study. Tissue Eng 2004; 10(7-8):1113-1124.
22. Zeilinger K, Sauer IM, Pless G et al. Three-dimensional coculture of primary human liver cells in bioreactors for in vitro drug studies: Effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. Altern Lab Anim—ATLA 2002; 30(5):525-538.
23. Allen JW, Khetani SR, Bhatia SN. In vitro zonation and toxicity in a hepatocyte bioreactor. Toxicol Sci 2005; 84(1):110-119.
24. Patel N, Padera R, Sanders GHW et al. Spatially controlled cell engineering on biodegradable polymer surfaces. FASEB J 1998; 12(14):1447-1454.
25. Moore K, Macsween M, Shoichet M. Immobilized concentration gradients of neurotrophic factors guide neurite outgrowth of primary neurons in macroporous scaffolds. Tissue Eng 2006; 12(2):267-278.
26. Radisic M, Park H, Chen F et al. Bimirnetic approach to cardiac tissue engineering: Oxygen carriers and channeled scaffolds. Tissue Eng 2006; 12(8):2077-2091.
27. L'Heureux N, Dusserre N, Konig G et al. Human tissue-engineered blood vessels for adult arterial revascularization. Nat Med 2006; 12(3):361-365.
28. Levenberg S, Rouwkema J, Macdonald M et al. Engineering vascularized skeletal muscle tissue. Nat Biotechnol 2005; 23(7):879-884.
29. Vacanti JP. Tissue and organ engineering: Can we build intestine and vital organs? J Gastrointest Surg 2003; 7(7):831-835.
30. Fuchs JR, Nasseri BA, Vacanti JP. Tissue engineering: A 21st century solution to surgical reconstruction. Ann Thorac Surg 2001; 72(2):577-591.
31. Sato M, Ando N, Ozawa S et al. Artificial esophagus. Porous Materials for Tissue Engineering 1997; 250:105-114.
32. Choi RS, Riegler M, Pothoulakis C et al. Studies of brush border enzymes, basement membrane components and electrophysiology of tissue-engineered neointestine. J Pediatr Surg 1998; 33(7):991-996.
33. Grikscheit TC. Tissue engineering of the gastrointestinal tract for surgical replacement: a nutrition tool of the future? Proc Nutr Soc 2003; 62(3):739-743.

34. Hori Y, Nakamura T, Matsumoto K et al. Experimental study on in situ tissue engineering of the stomach by an acellular collagen sponge scaffold graft. ASAIO J 2001; 47(3):206-210.
35. Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. B World Health Organ 2001; 79(3):214-221.
36. Sumide T, Nishida K, Yamato M et al. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J 2005; 19(14):392.
37. Nishida K, Yamato M, Hayashida Y et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. New Engl J Med 2004; 351(12):1187-1196.
38. Doillon CJ, Watsky MA, Hakim M et al. A collagen-based scaffold for a tissue engineered human cornea: Physical and physiological properties. Int J Artif Organs 2003; 26(8):764-773.
39. Germain L, Carrier P, Auger FA et al. Can we produce a human corneal equivalent by tissue engineering? Prog Retin Eye Res 2000; 19(5):497-527.
40. Griffith M, Osborne R, Munger R et al. Functional human corneal equivalents constructed from cell lines. Science 1999; 286(5447):2169-2172.
41. Schneider AI, Maier-Reif K, Graeve T. Constructing an in vitro cornea from cultures of the three specific corneal cell types. In Vitro Cell Dev 1999; 35(9):515-526.
42. Liu Y, Griffith M, Watsky MA et al. Properties of porcine and recombinant human collagen matrices for optically clear tissue engineering applications. Biomacromolecules 2006; 7(6):1819-1828.
43. Li FF, Carlsson D, Lohmann C et al. Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation. Proc Natl Acad Sci USA 23 2003; 100(26):15346-15351.
44. Wadsworth SJ, Nijmeh HS, Hall IP. Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. J Clin Immunol 2006; 26(4):376-387.
45. Powers MJ, Janigian DM, Wack KE et al. Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. Tissue Eng 2002; 8:499-513.
46. Spielmann H, Hoffmann S, Liebsch M et al. The ECVAM International validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the skin integrity function test. Altern Lab Anim—ATLA 2007; 35(6):559-601.

CHAPTER 5

TOXICITY TESTING OF NANOMATERIALS

Amanda M. Schrand,*[,1,2] Liming Dai,[2] John J. Schlager[1] and Saber M. Hussain[1]

[1]Applied Biotechnology Branch,Human Effectiveness Directorate, Air Force Research Laboratory, Wright-Patterson AFB, Ohio, USA; [2]University of Dayton, Department of Chemical and Materials Engineering, Dayton, Ohio, USA

**Corresponding Author: Amanda M. Schrand—Email: amanda.schrand@udri.udayton.edu*

Abstract: The large-scale production and consumer exposure to a variety of nanotechnology innovations has stirred interest concerning the health consequences of human exposure to nanomaterials. In order to investigate these questions, in vitro systems are used to rapidly and inexpensively predict the effects of nanomaterials at the cellular level. Recent advances in the toxicity testing of nanomaterials are beginning to shed light on the characteristics, uptake and mechanisms of their toxicity in a variety of cell types. Once the nanomaterials have been satisfactorily characterized, the evaluation of their interactions with cells can be studied with microscopy and biochemical assays. The combination of viability testing, observation of morphology and the generation of oxidative stress provide clues to the mechanisms of nanomaterial toxicity. The results of these studies are used to better understand how the size, chemical composition, shape and functionalization may contribute to their toxicity. This chapter will introduce the reader to the impact of nanomaterials in the workplace and marketplace with an emphasis on carbon-based and metal-based nanomaterials, which are most commonly encountered. While most purified carbon nanomaterials were nontoxic to many cell lines, many metal nanoparticles (e.g., silver or manganese) were more toxic. Other side- effects of nanoparticle interactions with cells can also occur, such as increased branching and dopamine depletion. Further investigation into the characteristics, uptake and mechanisms of nanomaterial toxicity will continue to elucidate this fascinating and rapidly growing area of science.

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

INTRODUCTION

The emergence of the new field of nanotoxicity has spurred great interest in a wide variety of materials and their possible effects on living systems. In particular, carbon-based and metal-based nanomaterials are being pursued for novel applications in industry and healthcare and for military purposes. Due to the increasing risk of exposure, in vitro systems have been widely used to estimate nanomaterial toxicity or biocompatibility. This chapter will focus on the recent advances in the in vitro toxicity testing of nanomaterials through characterization, the monitoring of internalization and the elucidation of the cellular mechanisms at work.

Nanotechnology and the Health Risk

Nanotechnology involves the creation and manipulation of materials at the nanoscale, to create products that exhibit novel electrical, catalytic, magnetic, mechanical, thermal, or optical features. Engineered nanomaterials are defined as materials with features in the range of 1-100 nm (10^{-7} to 10^{-9}m) in length or diameter. A variety of nanomaterial innovations have infiltrated the market place, in products such as titanium oxide (TiO_2) and zinc oxide (ZnO) in sunscreens for enhanced product transparency, silver (Ag) in bandaids and cosmetics for antibacterial activity and carbon (C) nanomaterials in nanocomposites (Table 1).[1] Concomitantly, highly specialized industrial applications of nanomaterials are being developed, such as aluminum (Al) as a fuel additive, manganese (Mn) as a catalyst or in battery technology, carbon nanotubes (CNT) as drug, gene, or protein carriers,[2] quantum dots (QDs) and iron (Fe) nanoparticles as imaging probes, Ag as an antiviral agent[3] and polymers as novel scaffolds.[4]

There is a great concern about the health consequences of nanomaterials, due to the increased probability of contact with engineered nanomaterials in both commercial products and industrial settings. Therefore, determining the toxicity of nanomaterials is a fundamental question relating to their extremely small size, high surface area and increased surface reactivity (i.e., redox ability) as compared to larger materials.[5,6] The small size of nanomaterials permits their translocation across cell membranes into critical organelles

Table 1. Applications of commonly used nanoparticles

Nanoparticle Type	Abbreviation	Applications
Carbon nanotube (single or multiwalled)	CNT (SWNT, MWNT)	Cell delivery, biosensors
Silver	Ag	Antimicrobial
Quantum dot	QD	Fluorescent imaging
Aluminum	Al	Fuel additive
Iron	Fe	Magnetic imaging
Titanium dioxide	TiO_2	Paint, water treatment, food, cosmetics
Zinc oxide	ZnO	Transparent sunscreens
Manganese oxide	MnO	Catalysis, batteries

such as mitochondria.[7] This same small size may also allow the nanomaterials to evade the cells responsible for their clearance, leading to biopersistence. Studies have shown that ultra-fine particles (<100 nm) are more toxic than larger particles made of the same material, partly due to a decrease in their clearance functions and their persistence in tissues.[8]

In support of these studies, there is a great amount of evidence that some nontoxic, micron-sized particles become toxic once they are reduced in size to the nano-scale.[9-16] The physical and chemical properties which contribute to this can include changes in solubility and surface area, as well as novel surface chemistry. The small size of nanomaterials changes their physical behavior from classical physics to quantum physics with decreasing particle size, which affects solubility, transparency, color, absorption or emission wavelength, conductivity, melting point and catalytic behavior.[17] Studies have shown that combustion particles with very low metal content, such as carbon black, cause inflammation, purely due to their surface characteristics and not their solubility.[18,19] The small size also leads to greater interactions with individual cells and their bio-molecules, which are on a similar size scale to that of the nanomaterials. However, some studies show that not all nanomaterials are more toxic than fine-sized particles of similar chemical composition. For example, pulmonary exposure in rats to uncoated TiO_2 nanorods (200 nm × 30 nm) compared to TiO_2 nanodots (<30 nm) did not produce greater inflammation than fine-sized TiO_2 particles (270 nm), whereas naive TiO_2 appears less inflammatory than hydrophobic TiO_2.[20,21] A suggested set of nanomaterial characteristics considered valuable prior to toxicity testing include size distribution of primary particles, shape, surface area, composition, surface chemistry, surface contamination, surface charge, crystal structure, particle physicochemical structure, agglomeration state, porosity, method of production, heterogeneity, storage conditions, and concentration.[13]

Occupational Exposure to Nanomaterials

Occupational exposure is likely to occur through dermal contact, ingestion or inhalation. Therefore, nanomaterials could potentially have an impact on the deep regions of the lungs, such as the alveolar region, where the barrier between the alveolar wall and capillaries is only 500 nm thick.[22] For example, inhaled nanomaterials may become lodged in the lung and remain there, unable to be cleared, as with asbestos. Thus, some of the same properties that make nanomaterials useful, are also properties that may contribute to toxicity and make them a health hazard under certain conditions. Recently, low concentrations of CNTs and other nanomaterial particulates were found in the air during industrial processes, but a full assessment of exposure levels has yet to be made.[24] Aerosol release during the handling of unrefined single-walled nanotubes (SWNTs) suggests that concentrations released in laboratories are lower than 53 g/m^3 and glove deposit concentrations are between 0.2-6 mg/hand.[24] Current US Occupational Safety and Health Administration (OSHA) and National Institute for Occupational Safety and Health (NIOSH) standards limit the exposure of silver compounds (including solubles, metal dusts and fumes) to $0.01 mg/m^3$. This limit is intended to reduce the occurrence of argyria, a permanent discoloration of the skin, in the occupational setting.[25] Another common health effect of workers exposed to silver dust and particulates is upper and lower respiratory infection. Overall, the toxicity of silver heavily depends upon its form. For example, liquid silver nitrates and chlorides are found to be extremely toxic, but silver oxides are not. Drake and Hazelwoood also reported that exposure to large amounts of silver iodide does not lead to argyria.[25] Other studies involving humans have shown that elevated levels of manganese may increase

the risk of developing Parkinson's disease.[26] In environments where large amounts of manganese powder are being produced, such as steel, nonsteel alloy, battery, welding and fuel additive factories, the exposure risk is greatly increased.[27]

The Characterization of Nanomaterials

In order to understand the characteristics of nanomaterials that can contribute to toxicity, they are first assessed in the as-synthesized form, prior to use in in vitro systems and after dispersion in the appropriate aqueous media for cell dosing (Fig. 1). Some of the most common techniques for initially determining composition and concentration include energy dispersive X-ray analysis (EDS), atomic absorption spectroscopy (AAS), or inductively coupled plasma spectroscopy (ICPS). The Brunauer, Emmett and Teller (BET) technique and inverse gas chromatography (IGC) can be used to estimate size and surface area, while scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) are routinely used for evaluating size and morphology (http://kristall.uni-mki.gwdg.de/english/docs/BET.htm).[28-29] Other studies with environmental SEM or AFM can permit the examination of wet materials, while techniques such as dynamic light scattering (DLS), zeta potential and UV-visible spectroscopy are routinely performed in solution to provide size, charge and composition characteristics (http://www.malvern.com).[30]

After sufficient information is obtained on the characteristics of the nanomaterial, in vitro measurements commence, such as concentration-dependent effects on viability. In our studies, oxidative stress and apoptosis have been assessed in skin, lung and neuronal cell lines. The results of such preliminary studies can then be examined in animal systems

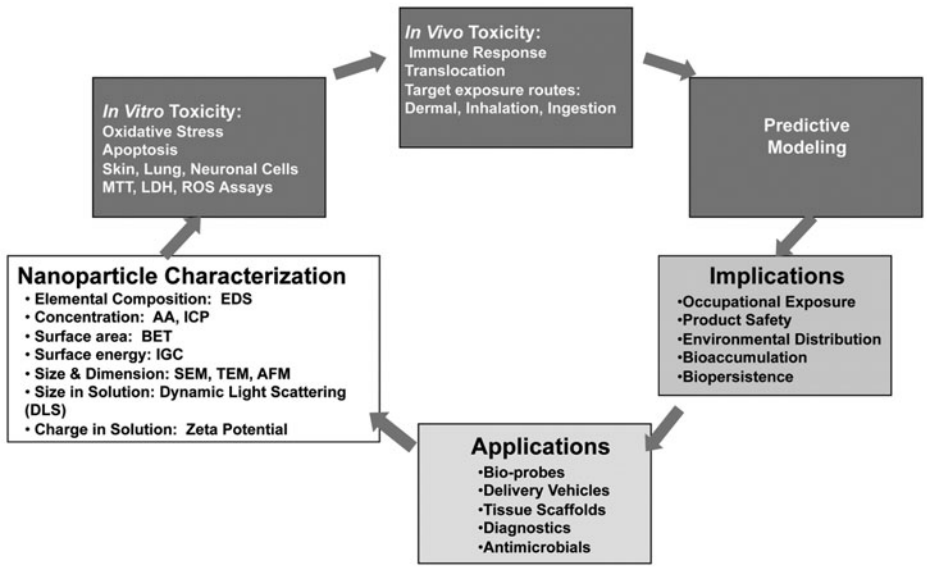

Figure 1. The integration of the areas studied in nanomaterials research.

(in vivo) for their effects on immune responses or on translocation to other areas after dermal, inhalation, or oral uptake. Once an adequate amount of data are collected, predictive modeling through computer-based approaches can be used to extrapolate the in vitro results to in vivo situations.[31] Toxicokinetic modeling describes the absorption, distribution, metabolism and elimination of xenobiotics (foreign materials) within an organism, as a function of dose and time. Toxicokinetic models can be divided into two main categories, namely, data-based compartmental models and physiologically-based compartmental models. Other quantitative structure–activity relationship (QSAR) models have been explored for structurally-related materials. However, many challenges still remain, including predicting the chronic effects that lead to conditions such as cancer, hematotoxicity, hepatotoxicity, lung fibrosis, nephrotoxicity and neurotoxicity, on the basis of in vitro studies. Additionally, most cellular responses are dependent upon dose and exposure time, where a low dose over a long period of time may result in an adaptive or even beneficial/protective effect.[31]

After careful consideration, the implications of the research outcome can be used to set safe limits for exposure in the work environment, in consumer products and in environmental waste. How the nanomaterials are distributed, accumulate and persist in the environment, are also matters of great concern. At the same time, these negative factors must be weighed against the benefits of using the nanoparticles, e.g., as antimicrobials, bio-probes, delivery vehicles, diagnostics and tissue scaffolds.

IN VITRO TOXICITY ASSESSMENT

Both in vitro cell culture and animal studies are being used to evaluate nanomaterials for their toxicity or potential to induce cell death.[31,32] In general, in vitro assays consist of subcellular systems (i.e., macromolecules, organelles), cellular systems (i.e., individual cells, coculture, barrier systems) and whole tissues (i.e., organs, slices, explants). Although in vitro data are not a substitute for whole animal studies, the use of relatively simple in vitro models with endpoints that reveal a general mechanism of toxicity can be a basis for further assessment of the potential risk of exposure to nanomaterials. For example, data reported on the toxicity testing of a series of high energy chemicals in an in vitro model,[27,33] were used to derive a baseline for extrapolation to a human health risk assessment.[34] The toxicity data obtained from such in vitro systems has been used to screen, rank and predict the acute hazards and mechanisms of compound interactions with animals or humans. This "basal toxicity" is defined as the ability of a compound to cause cell death as a consequence of damage to basic cellular functions. It can be used to define the concentration ranges of chemicals or nanomaterials which produce a toxic effect. The data obtained from basal toxicity studies have been found to be in good correlation with acute toxicity in animals and humans after studies involving diverse arrays of chemicals and assay systems.[35] However, kinetic factors and target organ specificity were parameters that weakened the correlation. Therefore, in vitro studies are conducted as a starting point and are very useful, because of their ability to rapidly and inexpensively produce results which may uncover the underlying toxic mechanisms of the selected chemicals, without the use of animals. The limitations of in vitro methods include: the transformation or immortalization of the cell lines, which may alter the properties and sensitivities of the cells; selective toxicity, in which some cell types are more sensitive than others; the isolation of the cells from their natural environment; and the difficulty encountered

in studying integrated groups of cells or organ systems. In our laboratory, rat alveolar macrophages were one of several cell lines selected for assessing the level of toxicity of nanomaterials and exploring possible mechanisms of toxicity after internalization that would be encountered after inhalation. Human lungs contain approximately 10^9 alveolar macrophages, which are found in the alveolar sacs, deep within the lungs.[36] Other cell lines which were tested for nanomaterial toxicity included murine neuroblastoma cells, PC-12 rat pheochromocytoma cells, which were derived from adrenal gland tumor cells and murine keratinocytes.

The Toxicity of Carbon-Based Nanomaterials

The interaction of carbon nanomaterials in a variety of cell types has been recently examined and reviewed, with uncertain conclusions. Many of these studies involve the use of established in vitro toxicity assays, based on the breakdown of the cellular permeability barrier, reduced mitochondrial function, changes in cell morphology, or changes in cell proliferation. The tests used in our laboratory involve microscopic examination and early biochemical endpoints, such as the MTT viability assay, lactase dehydrogenase (LDH) leakage and the production of reactive oxygen species (ROS). Because working with nanomaterials is different from that with other test materials, we have made minor modifications to the MTT assay, such as the incorporation of an additional centrifugation step to remove the nanomaterials from the solution, before the microplate reading step.[33,37,58]

Our recent viability results with nanodiamonds (NDs) ranging in size from 2-10 nm, showed that they are not cytotoxic to a variety of cell types, such as neuroblastoma, macrophage, keratinocyte and PC-12 cells.[37] Figure 2 shows the MTT viability results in neuroblastoma cells after a 24h exposure to acid or base functionalized NDs compared to those for 20 nm fine carbon black nanoparticles or the positive toxicity control, micron-sized cadmium oxide (CdO). Additional results showed that the cells did not produce significant ROS and they were able to grow on ND substrates with similar substrate growth observed by neurites grown on patterned carbon nanotubes.[23] These findings have stimulated the possible use of biocompatible carbon nanoparticles in advanced medical systems such as nanorobots or as fluorescent biolabels.[36,38]

While these NDs appear to be biocompatible, the parameters that are thought to influence the toxicity of carbon nanomaterials in general include their length, mass, functionalization and functionalization density.[42-44,46-48] Jia et al compared the relative cytotoxicities to macrophages of SWNTs, multi-walled carbon nanotubes (MWNTs) and fullerenes.[43] They found that the SWNTs significantly impaired the phagocytosis of macrophages at doses as low as 0.38 $\mu g/cm^2$, whereas the MWNTs and the fullerene, C60, induced injury only at the high dose of 3.06 $\mu g/cm^2$. The cytotoxicities appeared to follow a sequence on a mass basis: SWNTs > MWNTs > quartz > C60.

Investigations into the impact of surface chemical functionalization on toxicity have been performed in various cell types.[42,46,48] For example, human dermal fibroblasts incubated with water dispersible, functionalized SWNTs, showed that SWNT-phenyl-SO_3H, SWNT-phenyl-SO_3Na and SWNT-phenyl-$(COOH)_2$ were more biocompatible and had greater functionalization density compared to an unfunctionalized, surfactant-stabilized SWNT.[42] However, SWNT-phenyl-$(COOH)_2$ reduced cell viability to a greater extent than did SWNT-phenyl-SO_3H. In particular, the toxicity observed in cells incubated with SWNT-phenyl-$(COOH)_2$ was significantly higher than that in the controls, at concentrations ranging from 10-200 $\mu g/ml$ while SWNT-phenyl-SO_3H, with the highest

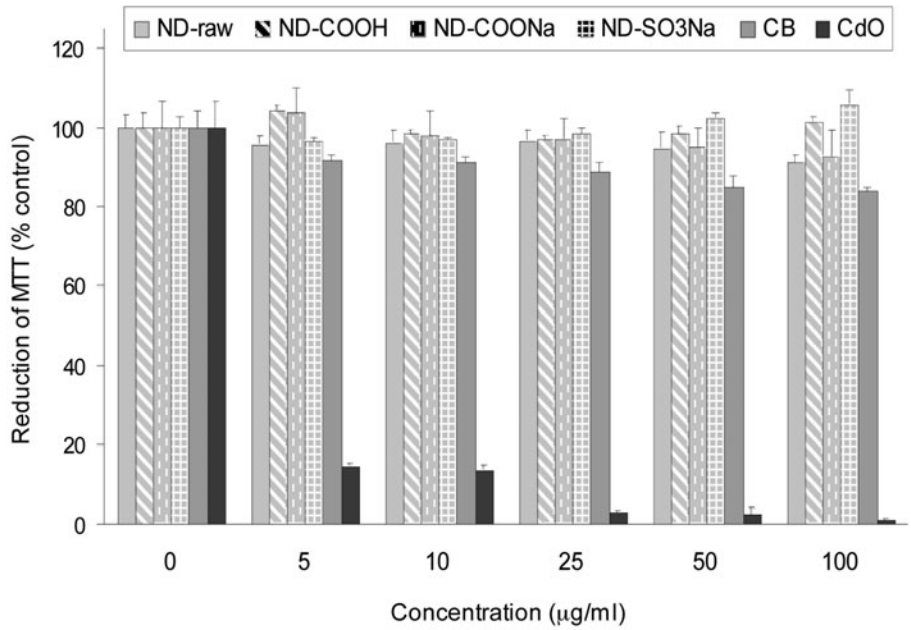

Figure 2. A cytotoxicity evaluation with neuroblastoma cells incubated with various nanoparticles for 24 h, as assessed with the MTT assay.[37] Reproduced with permission from Schrand et al. J Phys Chem Lett B 2007; 111(1):2-7.[37]

functionalization density, was not toxic up to the highest concentration tested, 2 mg/ml. Two other notable studies found that surface acid functionalization (the addition of carbonyl C = O, carboxyl (COOH) and/or hydroxyl [OH]) of carbon nanomaterials played a role in the cytotoxic effects in lung tumor cells and human acute monocytic leukemia cells, respectively.[46] After exposure to 0.02 μg/ml for 2 days, the viability of the lung tumor cells was reduced in the following order: carbon black (CB) > CNFs > MWNTs with MWNTs being the least toxic to the cells. The authors suggested that the MWNTs, having the highest aspect ratio out of the three materials, may have fewer dangling bonds, which preferentially occur at lattice defects or endcaps, compared to CB, where they are at high density. Cytopathological analysis showed that, after 1 day of incubation with 0.02 μg/ml of MWNTs, the cells lost their mutual attachments and retracted their cytoplasm, while their nuclei were more condensed, which was indicative of toxicity. Therefore, the conclusions to this study were that carbon nanomaterials are generally cytotoxic, with increasing toxicity after acid functionalization. Another research group found that the acid functionalization of hat-stacked carbon nanofibers, through the addition of carboxyl groups, led to only weak changes in toxicity to human acute monocytic leukemia cells and human embryonic kidney cells, while there was no difference between the toxicities to macrophages of MWNTs between 500 nm and 5 microns in length.[47-48]

While some studies have demonstrated the biocompatibility of unmodified carbon nanotubes with fibroblasts, osteoblasts and human umbilical vein endothelial cells, as well as murine and human macropages,[49,53-57] other studies with murine alveolar macrophages

have shown that carbon nanotubes are just as toxic as asbestos,[50,52] can inhibit cell growth or induce apoptosis in human embryonic kidney cells[39] and can generate an irritant and oxidative stress response with keratinocytes.[40,41,45] Therefore, based on the variety of carbon nanomaterials and cell types tested, these studies suggest that, to be least toxic to cells, an ideal material would be of small size, low mass and appropriately functionalized.

The Toxicity of Metal-Based Nanomaterials

The toxicity of silver nanoparticles in liver cells, germ-line stem cells and alveolar macrophages is greater than that of most carbon nanomaterials and many other metal nanomaterials. The toxicity of silver nanomaterials generally increases with decreasing size and with increasing concentration because of oxidative stress.[27,59-60] Research with C18-4 germ line stem cells showed that they were more sensitive to 15nm silver nanomaterials than were either BRL-3A liver cells or CRL-2192 alveolar macrophages. After 24 h of exposure to 15 nm Ag nanoparticles, the effective concentrations that decreased viability and increased LDH leakage respectively, by 50% were 8.75 μg/ml and 2.5 μg/ml for stem cells, 24 μg/ml and 50 μg/ml for BRL-3A liver cells and 28 μg/ml and 15 μg/ml for alveolar macrophages.[27,59-60] Other signs of toxicity included reductions in mitochondrial membrane potentials and in glutathione (GSH) levels and the activation of pro-inflammatory cytokines after exposure to 15nm silver nanoparticles.[27,60]

A variety of other metal nanomaterials have been screened for their toxicities through assays that measure LDH leakage through the plasma membrane. Because LDH is not typically found extracellularly in normal, healthy cell cultures, extracellular measures of LDH can be used as an indicator of membrane damage and subsequent cell viability. The LDH release of BRL 3A rat liver cells was measured after a 24h exposure to various nanoparticles (Fig. 3). The results demonstrated that exposure to micron-sized CdO dramatically increased membrane leakage (Fig. 3A) compared to silver nanoparticles (Fig. 3B) and also caused a dose-dependent increase in membrane leakage with concentration. Compared to other metal/metal oxide nanoparticles (Fig 3C), silver nanoparticles significantly increased LDH leakage at concentrations of 250 μg/ml.

The Uptake of Nanomaterials

The evaluation of nanomaterial uptake into cells answers many fundamental questions regarding toxicity by verifying the internalization of the nanomaterials, their location inside the cell and the amount that can be internalized over a certain period of time. The evaluation of nanomaterial uptake with in vitro cell culture has been monitored with fluorescent microscopy, flow cytometry, or fluorescent-activated cell sorting (FACS).[60-71] Other methods for quantitatively determining the uptake of nanomaterials include confocal microscopy,[49,73-74] ICPS,[52] radio-active labeling,[74-77] and AFM.[73] While newly-developed nanomaterials may be used purely as bio-labels for applications such as photodynamic therapy,[69] factors influencing their uptake can include size, concentration, temperature and surface properties, such as functionalization or charge. Additionally, it may be possible to distinguish active versus passive uptake by differences in kinetics. In our laboratory, we use advanced techniques in light and electron microscopy, such as ultrahigh resolution light microscopy and wet imaging under high vacuum conditions, in addition to observations on thin sections in TEM that can rapidly and accurately describe the uptake of nanomaterials into cells.

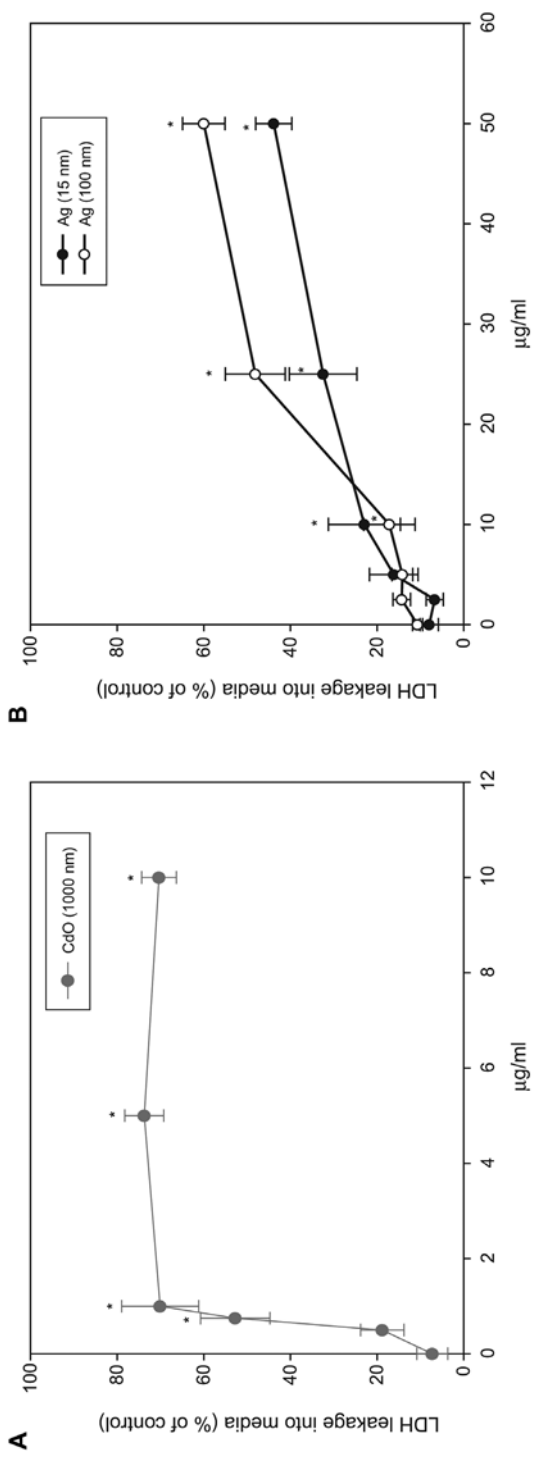

Figure 3. (A and B seen here, C on next page). The effects of nanoparticles on LDH leakage by BRL 3A rat liver cells after a 24 h exposure. The data are expressed as the mean ± SD of three independent experiments.[27] (*) indicates a statistically significant difference compared to the control ($p < 0.05$). Reproduced with permission from Hussain et al. Toxicol In Vitro 2005; 19:975-983.[27]

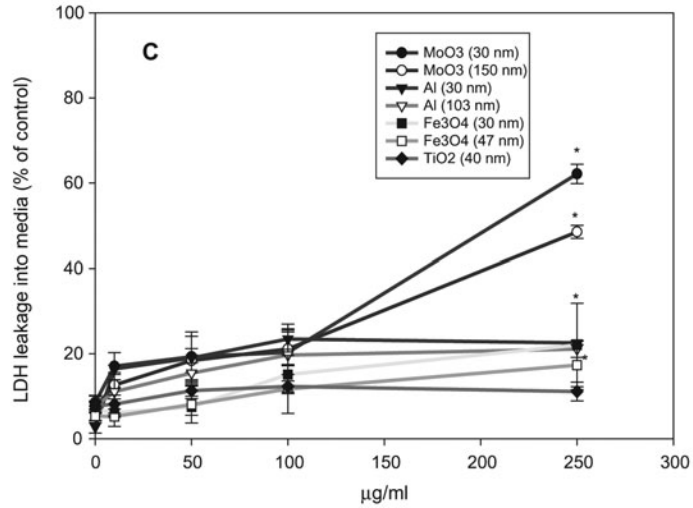

Figure 3, Continued. See legend on previous page

Morphological Observations

Because some nanomaterials exhibit pronounced toxicity while others appear biocompatible, light microscopy can be used to visualize overall cell morphology and nanomaterial interaction. In our studies, we used the CytoViva™ 150 Ultra Resolution Imaging (URI) system to observe changes in living cell morphology and uptake after exposure to nanomaterials.[78-79] This new technology surpasses the accepted 250 nm limit for light microscopy, by minimizing the spot size and stray light, which increases the efficiency of the metal halide lamp, resulting in resolution less than 150 nm.[80-81] Additionally, the unique light scattering capability brightly illuminates internal cell structures or agglomerates of metal nanoparticles inside living cells.[78-79,81] For example, PC-12 cells incubated for 24 hours with 50 µg/ml of MnO show distinct bright spots, which represent nanoparticle agglomerates (Fig. 4). This information complements the toxicological studies that showed that 40 nm MnO nanoparticles produced much greater levels of ROS than either Mn^{2+} or 15 nm silver nanoparticles.[78]

Another recent advance for imaging cells in a more natural state with minimal sample preparation, is the Quantomix capsule for wet SEM imaging under high vacuum conditions (http://www.quantomix.com/). This technology completely isolates the cell, tissue and other samples from the vacuum in the microscope chamber, making it possible to image and obtain X-ray composition data for fully-hydrated samples at a resolution as low as 10 nm.[82-85] The thin, yet electron and vacuum stable, transparent polyimide membrane permits the direct growth of cells and visualization into the interior of whole cells, whereas traditional secondary electron imaging in SEM only allows the visualization of surface topography. In order to gain greater contrast under the backscattered electron imaging mode, heavy metal staining can be performed directly in the capsule—without the additional steps that would be required for typical electron microscopy sample preparation, such as dehydration or critical point drying. Therefore, cell samples can be prepared quickly with preserved integrity in a hydrated form and labeled with nano-sized metal-based probes

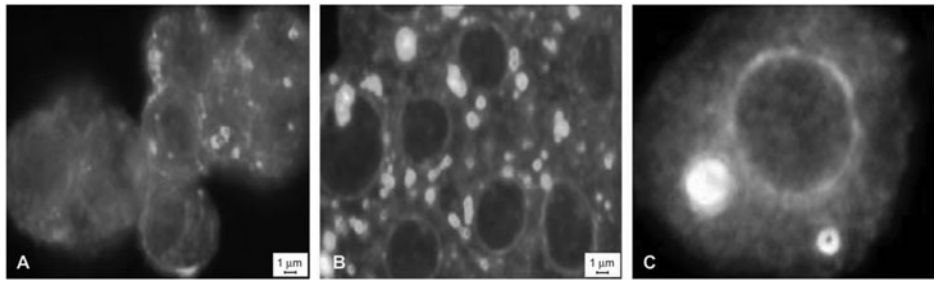

Figure 4. The uptake and distribution of manganese oxide nanoparticles associated with PC-12 cells after a 24 h incubation, visualized by high-illuminating inverted microscopy.[78] A) Control cells, B) Exposed cells and C) and enlargement of a single cell, showing bright areas that are agglomerated MnO nanoparticless. Reproduced with permission from J Toxicol Sci 2006; 92(2):456-463.[78]

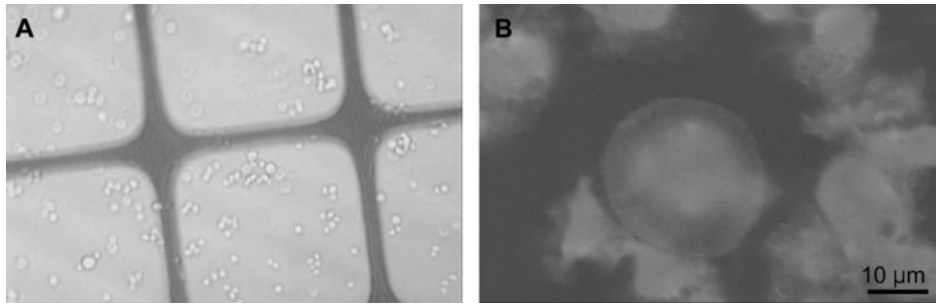

Figure 5. Demonstrations of (A) macrophage seeding and (B) SEM imaging after uranyl acetate and lead citrate staining in a Quantomix capsule for wet imaging under high vacuum conditions.

for the localization of antigen—antibody complexes or the uptake of nanoparticles. In our studies, we grew cells directly on a thin polymer membrane, which could be visualized with both light microscopy and SEM after uranyl acetate staining (Fig. 5).

By comparison, TEM preparation of cells consists of fixation with glutaraldehyde/paraformaldehyde, postfixation with osmium tetroxide, dehydration through a graded series of ethanols, embedding in resin, curing and thin sectioning. We have examined the presence of a variety of nanoparticles inside alveolar macrophages, neuroblastoma cells and keratinocytes. Although the cells may have different mechanisms of nanoparticle uptake, the nanoparticles still accumulate inside the cells, thereby reducing cell viability. Additionally, many of the nanoparticles are found inside cytoplasmic vesicles or freely in the cytoplasm, suggesting that mechanisms of uptake other than phagocytosis, such as endoycytosis or diffusion, are involved.

Fluorescence microscopy is another technique that can be used to examine the morphology of cells after incubation with nanoparticles. We recently observed increased branching of the actin cytoskeleton in ND-exposed cells (Fig. 6B) dual stained for actin and nuclei compared to the untreated controls (Fig. 6A), but there was no obvious uptake into the nuclei. It is not known whether the cells are merely responding to the presence of the nano-sized particles with increased neurite extension or whether the internalization of

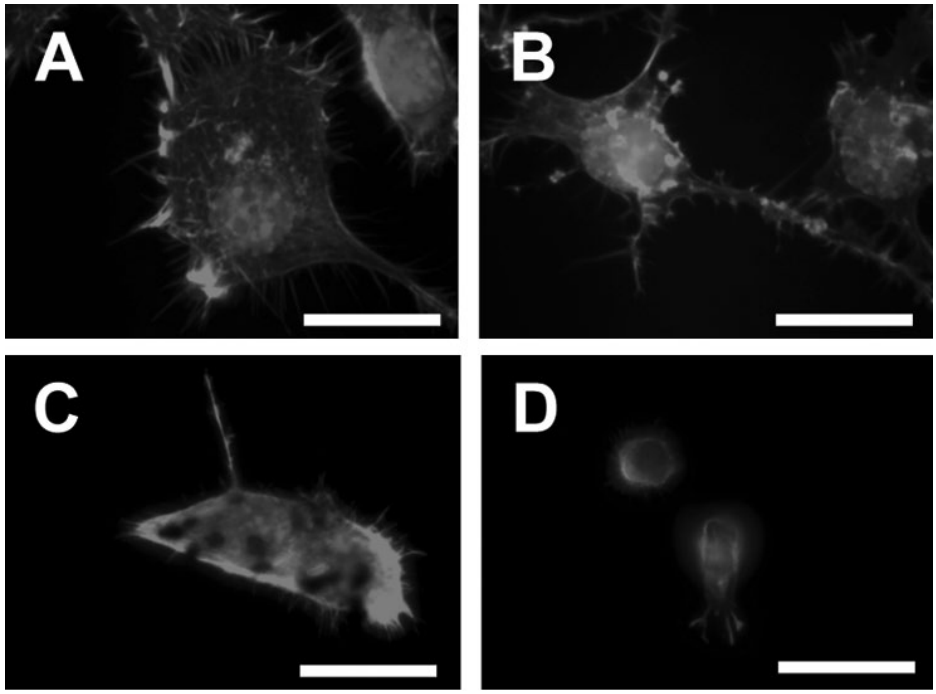

Figure 6. Fluorescence microscopy of neuroblastoma cells incubated with nanoparticles. Shown are cells incubated with nanoparticles for 24 h, then dual- stained for actin (red) and nuclei (blue) to reveal changes in cytoskeletal architecture. (A) Control, (B) 100 μg/ml ND-raw, (C) 100 μg/ml CB and (D) 2.5 μg/ml CdO. Scale bars are 10 um.[37] Reproduced with permission from Schrand et al. J Phys Chem Lett B 2007; 111(1):2-7.[37] A color version of this image is available at www.landesbioscience.com/madamecurie.

the NDs induces differentiation or other signaling pathways by an inside-out mechanism. Increased branching was not observed in cells incubated with fine CB nanoparticles (Fig. 6C), which suggests that there is a unique mechanism at work. By contrast, cells incubated with the positive control, CdO, showed reduced staining and cell shrinkage, which was indicative of toxicity (Fig. 6D).

MECHANISMS OF NANOMATERIAL TOXICITY

Collectively, the results of in vitro studies have identified oxidative stress-related changes in gene expression and cell signaling pathways as the underlying mechanisms of ultrafine particle effects, as well as roles for transition metals and certain organic compounds on combustion-generated ultrafine particles.[86] The interpretation of these studies is often difficult, due to differences in particle chemical composition, cell type, duration of exposure, endpoint and dosage. For example, 100 μg/ml, the highest concentration that we used in our studies, is not likely to be encountered in vivo, but provides information on the possible effects of high nanoparticles doses on mechanistic processes and modes of action.

The generation of intracellular ROS was determined by using dichlorofluorescin diacetate (DCFH-DA),[87] with minor modifications, as previously described by Hussain and Frazier.[33] In neuroblastoma cells incubated with carbon nanomaterials, there was no noticeable generation of ROS, except with CB nanoparticles. This suggests that, although carbon has generally been considered inert, it may react to produce some stress to the cells.[37] In BRL-3A cells and macrophages, there were significant increases in ROS generation after exposure to 10-50 μg/ml of Ag-15 nm for 6 h or 24 h, respectively, which suggests that the mechanism of toxicity is via an oxidative stress pathway. The dose-dependent increase in ROS generation following the exposure of BRL 3A cells for 6 h to 15 nm or 100 nm Ag at concentrations of 0, 5, 10, 25 and 50 μg/ml, is shown in Fig. 7A. Both sizes of silver nanoparticles significantly increased ROS generation compared to the untreated controls. The qualitative assessment of ROS generation was conducted by using fluorescence microscopy and showed increases in brightness after exposure to 25 μg/ml of Ag nanoparticles (Figs. 7B and 7C).

More-recent studies have shown that the chemical reactivity of the nanoparticles alone may be capable of generating ROS in the absence of a cellular environment. Therefore, the inherent reactivity of the nanomaterial must be taken into consideration as another characteristic which may be responsible for nanomaterial toxicity. Another factor to consider is the cell-specificity of effects, which may lead to particular diseases. The likely primary targets for nanomaterial exposure in the laboratory are the lung and skin, but cancerous conditions have not been linked to either organ after exposure and may be more related to the individual's health status.[17] The pathway of oxidative stress may be responsible for many conditions, such as Parkinson's disease, Alzheimer's disease and liver, heart and intestinal disease, but there have been no systematic studies which have directly linked exposure to nanomaterials to diseases in humans. Additionally, the dosing procedures and tumor-related effects following exposure of the lung may be limited to rats, as they have not been shown in other rodent species, which makes extrapolation to humans problematic.

CONCLUSION

Recent advances in the tools and techniques used for the toxicity testing of nanomaterials have permitted a more rapid and more thorough analysis of a variety of particles and cell types. Interdisciplinary technology, such as ultrahigh resolution light microscopy and capsules for wet imaging by SEM, combined with modifications to standard microscopic and biochemical techniques, has provided opportunities for collaboration on nanoparticle toxicology involving biologists, toxicologists and materials scientists. The characteristics of nanomaterials that can be used to predict their toxicity are still being elucidated, but our own work and that of many other groups, has shown that chemical composition, size, shape and functionalization are contributing factors. Further investigations into the characteristics, uptake and mechanisms of nanomaterials toxicity are expected to influence many fields of science for generations to come. However, at this point, no generalities have been identified, so nanomaterial toxicity should be evaluated on a case-by-case basis.

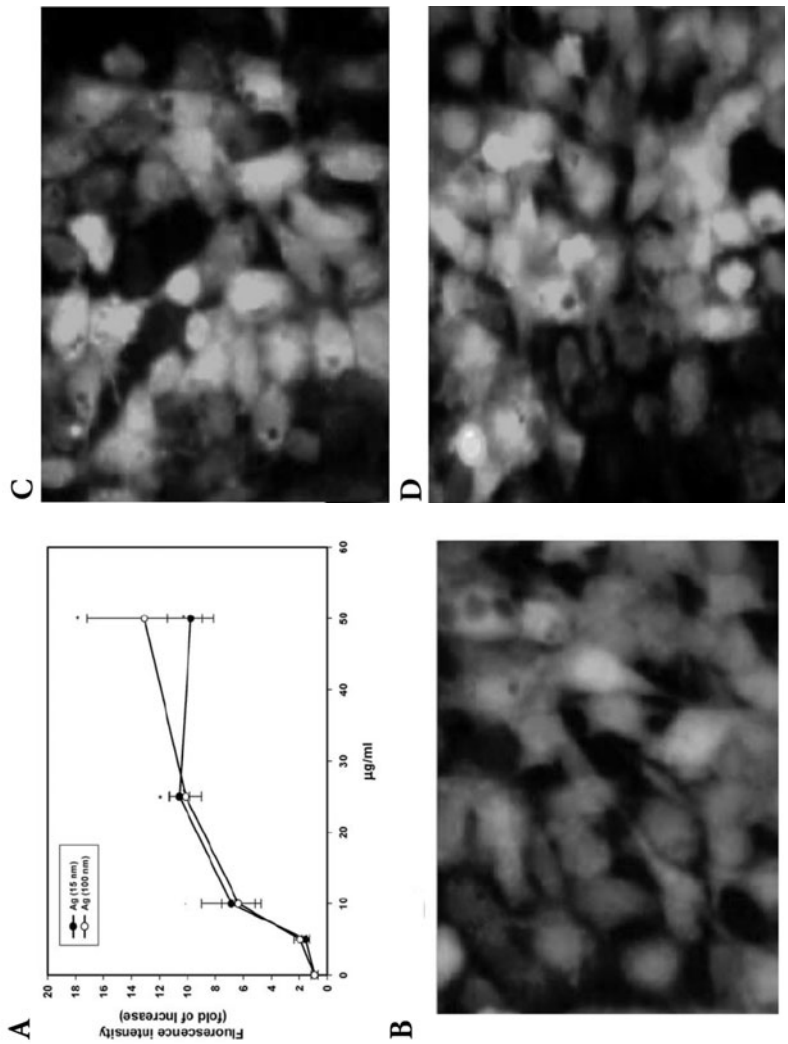

Figure 7. Effects of incubation for 6 h with 15 nm or 100 nm Ag nanoparticles on ROS generation in BRL- 3A rat liver cells. (A) The generation of ROS, assessed with DCFH-DA and expressed as fold increase relative to the untreated controls. The data are expressed as the mean ± SD of three independent experiments with (*) indicating a statistically significant difference compared to controls ($p < 0.05$). B-D) The qualitative characterization of ROS generation with DCFH-DA staining, by using fluorescence microscopy after a 6h-incubation with (B) Control, (C) 25 µg/ml Ag-15nm and (D) 25 µg/ml 100nm nanoparticles.[27] Reproduced with permission from Hussain et al. Toxicol In Vitro 2005; 19:975-983.[27]

ACKNOWLEDGEMENTS

The authors thank Col J. Riddle for his strong support and encouragement for this research and the Biological Interaction of Nanomaterials (BIN) group at AFRL/HEPB. Dr. Amanda Schrand received funding from the Biosciences and Protection Division, Air Force Research Laboratory, under the Oak Ridge Institute for Science and Education, Oak Ridge TN and the Dayton Area Graduate Studies Institute (DAGSI), Dayton OH.

REFERENCES

1. Dai L, ed. Carbon Nanotechnology: Recent Developments in Chemistry, Physics, Materials Science and Device Applications. Amsterdam: Elsevier, 2006:633-675.
2. Bianco A, Kostarelos K, Partidos C et al. Biomedical applications of functionalized carbon nanotubes. Chem Commun 2005; 5:571-577.
3. Elechiguerra J, Burt J, Morones J et al. Interaction of silver nanoparticles with HIV-1. J Nanobiotechnol 2005; 3(6):1-14.
4. Stupp S, Donners J, Li L et al. Expanding frontiers in biomaterials. MRS Bulletin 2005; 30:864.
5. Beck-Speier I, Dayal N, Karg E et al. Oxidative stress and lipid mediators induced in alveolar macrophages by ultrafine particles. Free Radic Biol Med 2005; 38:1080-1092.
6. Nel A, Xia T, Madler L et al. Toxic potential of materials at the nanolevel. Science 2006; 311:622-627.
7. Foley S, Crowley C, Smaihi M et al. Cellular localisation of a water-soluble fullerene derivative. Biochem Biophys Res Commun 2002; 294:116-119.
8. Zhang Q, Kusaka Y, Sato K et al. Toxicity of ultrafine nickel particles in lungs after intratracheal instillation. J Occup Health 1998; 40(3):171-176.
9. Jeng HA, Swanson J. Toxicity of metal oxide nanoparticles in mammalian cells. J Environ Sci Health A Tox Hazard Subst Environ Eng 2006; 41:2699-2711.
10. Oberdorster G, Ferin J, Gelein R et al. Role of the alveolar macrophage in lung injury-studies with ultrafine particles. Environ Health Perspect 1992; 97:193-199.
11. Ferin J, Oberdorster G, Penney DP. Pulmonary retention of ultrafine and fine particles in rats. Am J Respir Cell Mol Biol 1992; 6:535–542.
12. Kim JS, Yoon TJ, Yu KN et al. Toxicity and tissue distribution of magnetic nanoparticles in mice. Toxicol Sci 2006; 89:338-347.
13. Oberdorster G, Maynard A, Donaldson K et al. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2005; 2:8.
14. Oberdorster G, Yu CP. Lung dosimetry-considerations for noninhalation studies. Exp Lung Res 1999; 25:1-6.
15. Donaldson K, Li XY, MacNee W. Ultrafine (nanometer) particle mediated lung injury. J Aerosol Sci 1998; 29:553-560
16. Johnston CJ, Finkelstein JN, Mercer P et al. Pulmonary effects induced by ultrafine PTFE particles. Toxicol Appl Pharmacol 2000; 168:208-215.
17. Bergamaschi E, Bussolati O, Magrini A et al. Nanomaterials and lung toxicity: interactions with airways cells and relevance for occupational health risk assessment. Int J Immunopathol Pharmacol 2006; 19:3-10.
18. Duffin R, Gilmour PS, Schins RPF et al. Aluminium lactate treatment of DQ12 quartz inhibits its ability to cause inflammation, chemokine expression and Nuclear Factor-κB Activation. Toxicol Appl Pharmacol 2001; 176:10-17.
19. Brown DM, Stone V, Findlay P et al. Increased inflammation and intracellular calcium caused by ultrafine carbon black is independent of transition metals or other soluble components. Occup Environ Med 2000; 57:685-691.
20. Rehn B, Seiler F, Rehn S et al. Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: Untreated and surface treated. Toxicol Appl Pharmacol 2003; 189:84-95.
21. Chen HW, Su SF, Chien CT et al. Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. FASEB J 2006; 20:2393-2395.
22. Hoet P, Bruske-Hohlfeld I, Salata O. Nanoparticles: known and unknown health risks. J Nanobiotechnol 2004; 2:12.
23. Zhang X, Prasad S, Niyogi S et al. Guided neurite growth on patterned carbon nanotubes. Sensors and Acuators 2005; 106:843-850.
24. Maynard A, Baron P, Foley M et al. Exposure to carbon nanotube material: Aerosol release during the handling of unrefined single-walled carbon nanotube material. J Toxicol Environ Health 2004; 67:87-107.

25. Drake PL, Hazelwood KJ. Exposure-related health effects of silver and silver compounds: A review. Ann Occup Hyg 2005; 49:575-585.
26. Olanow CW. Manganese-induced parkinsonism and parkinson's disease. Ann NY Acad Sci 2004; 1012:209-223.
27. Hussain S, Hess K, Gearhart J et al. In vitro toxicity of nanoparticles in BRL-3A rat liver cells. Toxicol In Vitro 2005; 19:975-983.
28. Williams D, Carter B, eds. Transmission Electron Microscopy: A Textbook For Materials Science. New York: Plenum Press, 1996:621-635.
29. Goldstein J, Newbury D, Joy D et al, eds. Scanning Electron Microscopy and X-Ray Microanalysis. New York: Plenum Publishers, 2003:195-241.
30. McGown, E. MAXline microplate readers application note 32, 1999. Available at: http://www.moleculardevices.com/pages/instruments/190.html.
31. Eisenbrand G, Pool-Zobel B, Baker V et al. Methods of in vitro toxicology. Food Chem Toxicol 2002; 40:193-226.
32. Barile FA, ed. Introduction to In Vitro Cytotoxicology: Mechanisms and Methods, 1st ed. Boca Raton: CRC Press, 1994.
33. Hussain S, Frazier J. Cellular toxicity of hydrazine in primary hepatocytes. Toxicol Sci 2002; 69:424-432.
34. Trohalaki S, Zellmer R, Pachter R et al. Risk assessment of high-energy chemicals by in vitro toxicity screening and quantitative structure-activity relationships. Toxicol Sci 2002; 68:498-507.
35. Clemedson C, Barile F, Chesne C et al. MEIC Evaluation of acute systemic toxicity. Par VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further in vitro assays. Altern Lab Anim—ATLA 2000; 28:159-200.
36. Freitas RA Jr, ed. Nanomedicine, Vol IIA: Biocompatibility. Georgetown: Landes Bioscience, 2003: Available at: http://www.nanomedicine.com/NMIIA.htm.
37. Schrand A, Huang H, Carlson C et al. Are diamond nanoparticles cytotoxic? J Phys Chem Lett B 2007; 111(1):2-7.
38. Yu S, Kang M, Chang H et al. Bright fluorescent nanodiamonds: No photobleaching and low toxicity. J Am Chem Soc 2005; 127:17604-17605.
39. Cui D, Tian F, Ozkan C et al. Effect of single wall carbon nanotubes on human HEK293 cells. Toxicol Lett 2005; 155:73-85.
40. Monteiro-Riviere N, Nemanich R, Inman A et al. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol Lett 2005; 155:377-384.
41. Manna S, Sarkar S, Barr J et al. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-KB in human keratinocytes. Nano Lett 2005; 5(9):1676-1684.
42. Sayes C, Liang F, Hudson J et al. Functionalization density dependence of single-walled carbon nanotubes toxicity in vitro. Toxicol Lets 2005; 161(2):135-142.
43. Jia G, Wang H, Yan L et al. Toxicity of carbon nanomaterials: Single-wall nanotube, multi-wall nanotube and fullerene. Environ Sci Technol 2005; 39(5):1378-1383.
44. Cherukuri P, Bachilo S, Litovsky S et al. Near-infrared fluorescence microscopy of single-walled carbon nanotubes in phagocytic cells. J Am Chem Soc 2004; 126:15638-15639.
45. Monteiro-Riviere N, Inman A. Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon 2006; 44:1070-1078.
46. Magrez A, Kasas S, Salicio V et al. Cellular toxicity of carbon-based nanomaterials. Nano Lett 2006; 6(6):1121-1125.
47. Sato Y, Yokoyama A, Shibata K et al. Influence of length on toxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 in vitro and subcutaneous tissue of rats in vivo. Mol BioSyst 2005; 1:176-182.
48. Sato Y, Shibata KI, Kataoka H et al. Strict preparation and evaluation of water-soluble hat-stacked carbon nanofibers for biomedical application and their high biocompatibility: influence of nanofiber surface functional groups on toxicity. Mol BioSyst 2005; 1:142-145.
49. Fiorito S, Serafino A, Andreola F et al. Effects of fullerenes and single-wall carbon nanotubes on murine and human macrophages. Carbon 2006; 44:1100-1105.
50. Soto K, Carrasco A, Powell T et al. Comparative in vitro toxicity assessment of some manufactured nanoparticulate materials characterized by transmission electron microscopy. Nanoparticle Res 2005; 7:145–169.
51. Limbach LK, Li Y, Grass RN et al. Oxide nanoparticle uptake in human lung fibroblasts: Effects of particle size, agglomeration and diffusion at low concentrations. Environ Sci Technol 2005, 39:9370-9376.
52. Murr L, Garza K, Soto K et al. Toxicity assessment of some carbon nanotubes and related carbon nanoparticle aggregates and the implications for anthropogenic carbon nanotube aggregates in the environment. Int J Environ Res Public Health 2005; 2(1):31-42.
53. Smart S, Cassady A, Lu G et al. The biocompatibility of carbon nanotubes. Carbon 2006; 44:1034-1047.

54. Flahaut E, Durrieu M, Remy-Zolghadri M et al. Investigation of the toxicity of CCVD carbon nanotubes towards human umbilical vein endothelial cells. Carbon 2006; 44:1093-1099.
55. Chlopek J, Czajkowska B, Szaraniec B et al. In vitro studies of carbon nanotubes biocompatibility. Carbon 2006; 44:1106-1111.
56. Hurt RH, Monthioux M, Kane A. Toxicology of carbon nanomaterials: Status, trends and perspectives on the special issue. Carbon 2006; 44:1028-1033.
57. Donaldson K, Aitken R, Tran L et al. Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol Sci 2006; 92(1):5-22.
58. Carmichael J, DeGraff W, Gazdar A et al. Evaluation of a tetrazolium-based semi automated colorimetric assay: Assessment of chemo sensitivity testing. Cancer Res 1987; 47:936-942.
59. Braydich-Stolle L, Hussain S, Schlager J et al. In vitro toxicity of nanoparticles in mammalian germline stem cells. Toxicol Sci 2005; 88(2):412-419.
60. Carlson C. In vitro toxicity assessment of silver nanoparticles in rat alveolar macrophages. Masters Thesis. Dayton: Wright State University, 2006.
61. Lorenz MR, Holzapfel V, Musyanovych A et al. Uptake of functionalized, fluorescent-labeled polymeric particles in different cell lines and stem cells. Biomaterials 2006; 27:2820-2828.
62. Cherukuri A, Frye J, French T et al. FITC-poly-D-lysine conjugates as fluorescent probes to quantify hapten-specific macrophage receptor binding and uptake kinetics. Cytometry 1998; 31:110-124.
63. Patil NS, Wong DL, Collier KD et al. Fluorescent derivatization of a protease antigen to track antigen uptake and processing in human cell lines. BMC Immunology 2004; 5:12.
64. Huang M, Ma Z, Khor E et al. Uptake of FITC-chitosan nanoparticles by A549 cells. Pharmac Res 2002; 19(10):1488-1494.
65. Huang M, Khor E, Lim LY. Uptake and toxicity of chitosan molecules and nanoparticles: Effects of molecular weight and degree of deacetylation. Pharmac Res 2004; 21(2):344-353.
66. Ma Z, Lim LY. Uptake of chitosan and associated insulin in a Caco2 cell monolayers: A comparison between chitosan molecules and chitosan nanoparticles. Pharmac Res 2003; 20(11):1812-1819.
67. Maselli A, Laevsky G, Knecht DA. Kinetics of binding, uptake and degradation of live fluorescent (DsRed) bacteria by Dictyostelium. Microbiology 2002; 148:413-420.
68. Chenevier P, Veyret B, Roux D et al. Interaction of cationic colloids at the surface of J774 Cells: A kinetic analysis. Biophys J 2002; 79:1298-1309.
69. Saxena V, Sadoqi M. Tiny bubbles. SPIE's Magazine 2004; September 21-23.
70. Qaddoumi MG, Ueda H, Yang J et al. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. Pharmac Res 2004; 21(4):641-648.
71. Zhang Y, Huang N. Intracellular uptake of CdSe-ZnS/Polystyrene Nanobeads. J Biomed Mater Res Part B Appl Biomaterials 2005; 76B:161-168.
72. Schulze K, Koch A, Petri-Fink A et al. Uptake and biocompatibility of functionalized poly(vinylalcohol) coated superparamagnetic maghemite nanoparticles by synoviocytes in vitro. J Nanosci Nanotechnol 2006; 6(9-10):2829-2840.
73. Yang P, Sun X, Chiu J et al. Transferrin-mediated gold nanoparticle cellular uptake. Bioconjugate Chem 2005; 16:494-496.
74. John TA, Vogel SM, Tiruppathi C et al. Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. Am J Physiol. Lung Cell Mol Physiol 2003; L187-L196.
75. Bianco A, Hoebeke J, Godefroy S et al. Cationic carbon nanotubes bind to CpG Oligodeoxynucleotides and enhance their immunostimulatory properties. J Am Chem Soc 2004; 127:58-59.
76. Schubbe S, Kube M, Scheffel A et al. Characterization of a spontaneous nonmagnetic mutant of magnetospirillium gryphiswaldense reveals a large deletion comprising a putative magnetosome island. J Bacteriol 2003; 185(19):5779-5790.
77. Moore A, Marecos E, Bogdanov A et al. Tumoral distribution of long-circulating dextran-coated iron oxide nanoparticles in a rodent model. Radiol 2000; 214:568-574.
78. Hussain SM, Javorina AK, Schrand AM et al. The interaction of manganese nanoparticles with PC-12 cells induces dopamine depletion. J Toxicol Sci 2006; 92(2):456-463.
79. Skebo J, Grabinski C, Schrand A et al. Assessment of metal nanoparticle agglomeration, uptake and interaction using a high illuminating system. Int J Tox 2007; 26(2):135-141.
80. Foster B. Focus on microscopy: A technique for imaging live cell interactions and mechanisms. Am Lab 2004; 11:21-27.
81. Vodyanoy V. High resolution light microscopy of live cells. Microscopy Today 2005; 13:26-28.
82. Gileadi O, Sabban A. Squid sperm to clam eggs: Imaging wet samples in a scanning electron microscope. Biol Bull 2003; 205:177-179.
83. Thiberge S, Nechushtan A, Sprinzak D et al. Scanning electron microscopy of cells and tissues under fully hydrated conditions. Proc Natl Acad Sci USA 2004; 101(10):3346-3351.

84. Ruach-Nir I. An innovative method for imaging and chemical analysis of wet samples in scanning electron microscopes. Microscopy Today 2005:10-14.
85. Behar V. Applications of a novel SEM technique for the analysis of hydrated samples. Microsc Microanal 2005; 19(4).
86. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 2005; 113(7):823-839.
87. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999; 27:612-616.

CHAPTER 6

PHYSIOLOGICALLY-BASED PHARMACOKINETIC (PBPK) MODELS IN TOXICITY TESTING AND RISK ASSESSMENT

John C. Lipscomb,*[,1] Sami Haddad,[2] Torka Poet[3] and Kannan Krishnan[2]

[1]US Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Cincinnati, Ohio, USA; [2]Département de Santé Environnementale et Santé au Travail, IRSDUM, Université de Montréal, Montréal, Québec, Canada; [3]Center for Biological Monitoring and Modeling, Battelle Pacific Northwest Division, Richland, Washington, USA

**Corresponding Author: John C. Lipscomb— Email: lipscomb.john@epa.gov*

Abstract: Physiologically-based pharmacokinetic (PBPK) modeling offers a scientifically-sound framework for integrating mechanistic data on absorption, distribution, metabolism and elimination to predict the time-course of parent chemical, metabolite(s) or biomarkers in the exposed organism. A major advantage of PBPK models is their ability to forecast the impact of specific mechanistic processes and determinants on the tissue dose. In this regard, they facilitate integration of data obtained with in vitro and in silico methods, for making predictions of the tissue dosimetry in the whole animal, thus reducing and/or refining the use of animals in pharmacokinetic and toxicity studies. This chapter presents the principles and practice of PBPK modeling, as well as the application of these models in toxicity testing and health risk assessments.

INTRODUCTION

Toxicity tests and risk assessments improve our understanding of "how much chemical is too much", for human safety. Given the ethical considerations associated with human testing, animals have been employed as surrogates. With the highest level of emphasis placed on biologically relevant and cost-effective mammals, rodents are most often used in toxicity testing. While data from humans can be used in establishing safe exposure levels, human data are more frequently available for therapeutic and industrial compounds than for some classes of chemicals, such as pesticides (compounds developed

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

and marketed based on their ability to produce toxic, even lethal, responses) and other environmental contaminants. In many instances, estimates of acceptable human exposure limits are developed from the results of tests in animals.[1-4] Studies with laboratory animals can be conducted to identify the toxic responses observed and to estimate the potency of the chemical; their results are considered to be valuable both from a qualitative and a quantitative perspective for extrapolation to humans exposed to low doses.[5-7]

Initial studies conducted for the purpose of "Hazard Identification" facilitate the identification of the organs, tissues and systems that are adversely affected by the chemical.[8] For the dose–response assessment, data describing the responses are interpreted in the context of dose—most often in the context of the applied (external) dose.[8-9] This dose is typically reported as mg/m^3 in air for inhaled toxicants and in mg/kg/day for orally ingested toxicants. Because chemicals are subject to pharmacokinetic processes (such as absorption, distribution, metabolism and elimination (ADME)) differently in animals and humans, a detailed understanding of the interspecies differences in these processes is essential to confidently extrapolate biological response data from animals to humans.[10-12]

The biological response results from the interaction between the toxicant and the target tissue. For this reason, models that can predict the target tissue concentration of the toxicologically-active chemical species (parent compound or metabolite) are especially useful and have been applied in what is referred to as the "exposure–dose–response" paradigm (Fig. 1).[9,13] Here, the "dose" refers to the target tissue concentration of the putative toxic moiety of a chemical. This exposure–dose–response paradigm is critically important for establishing conditions where humans are at risk for adverse outcomes defined in animal models. Due to their strong biological underpinnings, biokinetic models have become the preferred approach for conducting extrapolations of potential internal dose surrogates associated with toxicity.[14-19] In essence, biokinetic modeling, when linked with dynamic biological responses, serves as a systems biology tool at the whole-organ/whole-body level. Once validated, model-predicted target tissue concentrations should be reliable for the extrapolation of dosimetry across dose, route, time and species. The ability of the biokinetic models, especially the physiologically-based pharmacokinetic (PBPK) or toxicokinetic models, to calculate target tissue dose contributes to addressing and/or reducing some sources of uncertainty in risk assessments.[15,18]

This chapter introduces the principles and practice of PBPK modeling as applied in toxicity testing and risk assessment.

MODEL DEVELOPMENT

PBPK modeling refers to the development of quantitative descriptions of the ADME of chemicals, on the basis of interrelationships among the critical determinants of these processes.[14,20-22] The critical determinants of ADME include tissue volumes, physiological flow rates, rates of absorption, diffusion across cell membranes, tissue:blood partition coefficients and rates and affinities for biochemical reactions. These models are more useful than the conventional data-based pharmacokinetic models, particularly for the conduct of various extrapolations central to predictive toxicology applications.[23-25] The biological and mechanistic basis of the PBPK models enables them to be used, with limited animal experimentation, for extrapolation of the kinetic behavior of chemicals from test animal species to humans, from one exposure route to another and from high dose to low dose.[21,26] Initial work on the development of PBPK models dates back to the research work of Haggard on volatile organics

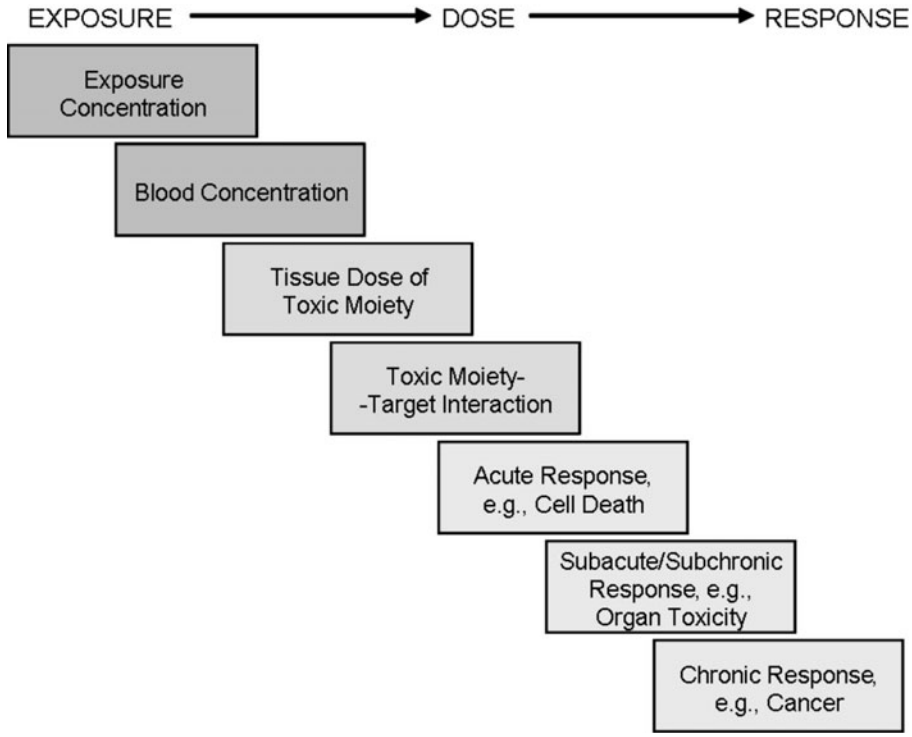

Figure 1. The exposure–dose–paradigm. Based on references 9 and 13.

and anaesthetics.[27] Further developments in the PBPK modeling of volatile chemicals, as well as pharmaceuticals, ensued.[28-35] Subsequently, the interest in the development of PBPK models has increased, due to their capacity to facilitate various extrapolations to enhance the scientific basis and efficiency of toxicity testing, as well as risk assessment.

At the most fundamental level, the PBPK model must be properly designed. Considerations include the biology of the animal species and the toxicity of the chemical. Failure to consider systematically the biology of the organism and the toxicity of the chemical of interest in guiding the model development process will prove detrimental. Flaws in the understanding of the key points of either will lead to incongruence and the failure of the developed model to meet expectations. Parsimony should be followed and the model should be only as complex as is necessary to address the key issues and tissues related to the toxicity of the chemical of interest.[36-37] Once the model structure has been established, values for physiological, physicochemical parameters and biochemical rate constants must be identified. Then, once the model has been structured and parameterized, the practitioner must determine its suitability through a process called evaluation or validation. This exercise demonstrates the fit between model predictions and data describing pharmacokinetic information (e.g., blood concentration–time-course data for the parent chemical, concentrations of metabolite in a given tissue). The success of this is critical to model application and is a function of the model structure, the appropriateness of the

parameter values and the reliability of the in vivo toxicokinetic data.[36-37] These various aspects are discussed in the following sections.

Model Structure

The structure of a PBPK model corresponds to a diagrammatic representation of the organism (i.e., species or individual) on the basis of the critical elements, in terms of tissues and ADME processes. Accordingly, the following aspects are considered to guide the selection of specific tissues for inclusion in the PBPK model:[37]

- Target organ or a surrogate compartment (e.g., blood)
- Portals of entry or uptake of chemicals (e.g., lungs, skin and gastrointestinal tract)
- Sites of significant metabolism (e.g., liver)
- Sites of significant storage capacity (e.g., adipose tissue, bone)

The tissue compartments are then interconnected via a systemic circulation (i.e., arterial and venous blood supplies), such that the mass balance of the cardiac output in the organism is maintained at all times in the model (Fig. 2). Tissues can be regrouped, if the concentration versus time-course of a chemical is comparable. Table 1 lists frequently used compartments in PBPK models, as well as the tissues/organs that are grouped together. The development of a reasonable model structure for a chemical then requires an understanding of the qualitative and quantitative determinants of ADME in the species of interest.

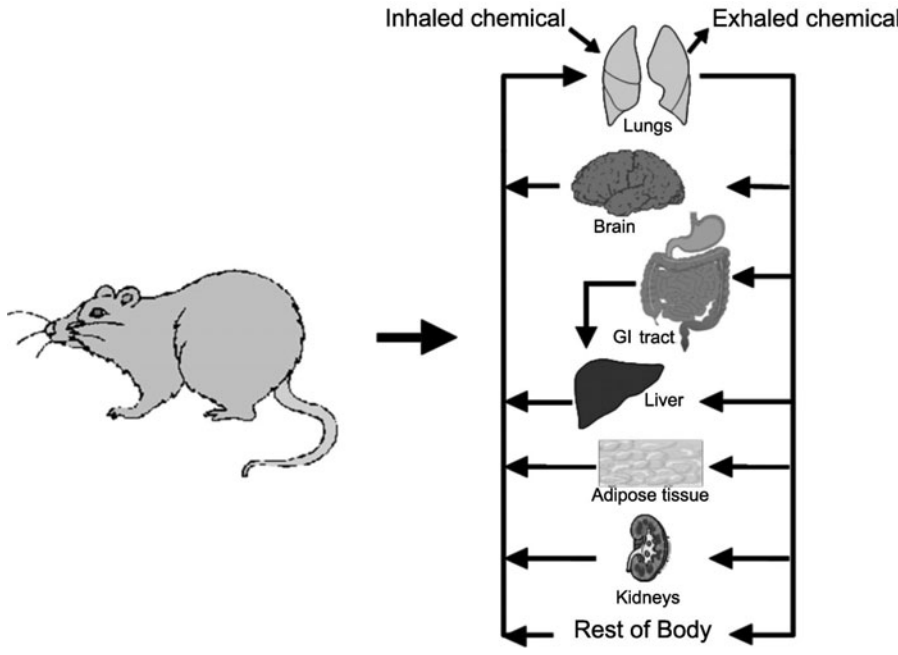

Figure 2. The structure of a PBPK model for a volatile organic chemical in the rat.

Table 1. Individual or groups of tissues frequently represented by compartments in PBPK models

Model Compartments	Tissues
Liver	Liver
Adipose tissue	Perirenal fat
	Epidymal fat
	Omental fat
	Subcutaneous fat
Slowly perfused tissues	Muscle
	Skin
Richly perfused tissues	Adrenal
	Kidney
	Thyroid
	Brain
	Lung
	Heart
	Testis
	Hepatoportal system

Model Equations

PBPK models consist of a set of differential equations based on physiological clearance (CL), in terms of L blood/hr. The various clearance terms represent the influx, efflux, metabolism and excretion processes. The rate of change in the amount of chemical during a given time interval (dA_t/dt) is then computed as follows:

$$\frac{dA_t}{dt} = (CL_{influx} \times C_a) - (CL_{efflux} \times C_{vt}) - (CL_{metabolic} \times C_a) - (CL_{renal} \times C_a) \quad (1)$$

where C_a = chemical concentration in arterial blood and C_{vt} = chemical concentraton in venous blood leaving, the concentrations of which are at equilibrium with concentrations in tissue t.

Equation (1) considers the tissue as a single homogenous compartment. Whereas this is adequate for low molecular weight compounds, it is often necessary to describe the uptake of high molecular substances via the vascular and intracellular compartments of the tissue separately.[38] Tissue distribution is typically modeled as flow-limited, where the concentration of agent in venous blood leaving the tissue is assumed to be in equilibrium with the concentration of agent in the tissue.

Table 2 presents the forms of equations frequently used in PBPK models for describing tissue influx, tissue efflux, renal clearance, as well as metabolic clearance.[37] Even though the venous equilibration model for hepatic metabolism has often been used in PBPK models, other types of physiological descriptions (i.e., parallel tube model, distributed sinusoidal perfusion model) may be used, depending on the intended use of the resulting PBPK model.[39-40]

Table 2. Examples of equations used in PBPK models for describing rate of change in tissues (i.e., influx-efflux), renal clearance (CL_r) and rate of hepatic metabolism ($\frac{dA_{met}}{dt}$)

Influx and efflux	$V_t \frac{dC_t}{dt} = Q_t \left(C_a - C_{vt} \right)$
Renal clearance	$CL_r = \frac{U_s \times V_u}{C_a}$
Metabolism	$\frac{dA_{met}}{dt} = \frac{V_{\max} C_{vt}}{K_m + C_{vt}}$
	$\frac{dA_{met}}{dt} = Q_t \times E \times C_a$

C_a : chemical concentration in arterial blood/plasma
C_t : concentration in tissue "t"
C_{vt} : concentration in venous blood/plasma leaving the tissue "t"
E : hepatic extraction ratio
K_m : Michaelis-Menten affinity constant
Q_t : flow rate to tissue
U_S : concentration of a substance in urine
V_{max} : maximal velocity of enzymatic reaction
V_t : volume of tissue "t"
V_u : urine flow rate

Parameter Estimation

PBPK models consist of a number of input parameters that can be conveniently categorized as physiological, physicochemical or biochemical in nature (Table 3). The physiological parameters frequently required for PBPK modeling include alveolar ventilation rate, cardiac output, tissue blood flow rates and tissue volumes. Table 4 provides reference values suggested by Arms and Travis[41] for adult rats and mice used in toxicity testing. Databases on animal and human physiological parameters in various age groups and strains/races are still evolving.[42-45]

The physicochemical parameters required for PBPK modeling are partition coefficients (PCs), which represent the relative distribution of a chemical between two matrices (i.e., blood and air or tissue and blood) at equilibrium. The blood:air and tissue:blood PCs for a number of chemicals have been determined by using in vivo pharmacokinetic data or in vitro techniques (equilibrium dialysis, ultrafiltration, vial equilibration).[37] Table 5 lists the various in silico methods that have become available for estimating the PCs for specific sub-groups of chemicals or drugs. A number of these animal-replacement methods use data on properties specific to chemicals, as well as characteristics specific to an individual or a population (examples are given in refs. 46-51). These in silico approaches account for the mechanistic determinants of tissue:blood PCs, which together with the volume

Table 3. Input parameters for a basic PBPK model

Type of Parameters	Specific Parameters
Physiological	Tissue volume Tissue blood flow Alveolar ventilation Cardiac output Glomerular filtration rate
Biochemical	Maximum velocity of metabolism Michaelis affinity constant Rate of absorption Binding affinity constant
Physicochemical	Blood:air partition coefficient Tissue:blood partition coefficient

Table 4. Reference physiological valuers for adult rats and mice. Based on Arms and Travis.[41]

	Weight (g)		Flow (mL/min)	
Compartments	Rats	Mice	Rats	Mice
Liver	10.0	1.4	20.8	4.3
Fat	17.5	2.5	7.5	1.5
Slowly perfused tissues	187.5	17.5	12.5	2.6
Richly perfused tissues	12.5	1.3	42.3	8.7
Whole body	250.0	25.0	83.0	17.0

of tissues and blood facilitate the computation of the volume of distribution (Vd), as shown below:[23]

$$Vd = V_b + \Sigma P_{tb} * V_t \quad (2)$$

where V_b = blood volume, V_t = volume of tissues and P_{tb} = tissue:blood PCs.

When uncomplicated by species differences in protein binding, simple allometric scaling of Vd determined in test species can produce reasonable estimates of Vd in humans. However, when such data are not available, or when interspecies difference in protein binding is significant, data on the fraction unbound would be essential to predict Vd, as well as PCs essential for PBPK modeling.[52-53]

The biochemical parameters required for PBPK modeling frequently include absorption rate constants, maximal velocity for metabolism (V_{max}), Michaelis constant (K_m), binding association constant and urinary/biliary excretion rate. These parameters have often been determined on the basis of time-course data collected in vivo or in vitro; data analysis to estimate specific parameter(s) is then conducted by using the portion of the time-course curve that is most sensitive to one or two dominant factors.[54-56]

The rate of oral absorption has been determined in vivo on the basis of kinetic data on the exhaled breath or blood concentrations of administered chemicals. Based on knowledge

Table 5. In silico approaches and their applicability to specific chemical classes for estimating partition coefficients

Chemical Class	Approach	References
Empirical Approaches		
Basic organic chemicals	Relationship of Pt:p with Log P	109
Weakly basic drugs	Relationship of Pt:p with Log P and phosphatidylserine tissue content	110
Volatile organic chemicals and drugs	QSAR relationships of PCs (Brain:air, brain:blood, blood:air, brain:air, brain:blood, muscle:air, muscle:blood, skin:plasma, skin:blood, liver:air, liver:blood, lung:air, lung:blood) using various molecular descriptors	111-117
Histamine receptor H 2 antagonists	Relationship between brain:blood and octanol:water, cyclohexane:water, molecular mass and water accessible volume	118
Histamine receptor H 2 antagonists	QSAR relationship between P brain:blood and free energy of salvation	119
Volatile organic chemicals	Relationship between Pt:b and log P using tissue and blood composition data.	48
Barbituric acids	Relationship Kpu with Log P.	120-121
Structurally diverse compounds	QSAR relationship between P brain:blood with several topological and constitutional descriptors of molecules.	122
Drugs	Use of muscle:plasma as surrogate for the estimation of Pt:p of other tissues except fat.	53
Acid and basic drugs	Use of muscle:plasma as surrogate for the estimation of Pt:p of other tissues except fat.	123

continued on following page

of the determinants (i.e., lipophilicity, pKa, solubility, particle size, permeability, as well as, if applicable, release kinetics and dissolution kinetics), mathematical models and algorithms have been developed to simulate the rate of absorption in animals and humans.[57-58] These types of models have more generally been used in pharmaceutical research, where estimation of rate of absorption is important in determining the passage from preclinical to clinical Phase 1 research. Often with environmental contaminants, the gastrointestinal absorption rates (i.e., first order rate constants) have been estimated on the basis of in vivo data,[37] whereas a number of in vitro systems (reconstituted enzyme preparations, subcellular fractions, postmitochondrial preparations, isolated cells, tissue slices and isolated perfused organs) have been used for the estimation of metabolic rate constants.[59-73] In this regard, several studies involving the use of microsomal protein, postmitochondrial fractions or freshly isolated hepatocytes, have demonstrated the feasibility of incorporating metabolic rate constants directly within PBPK models for low molecular weight organic chemicals.[13,74-76] In general, the K_m values obtained in vitro

Table 5. Continued

Chemical Class	Approach	References
Mechanistic Approaches		
Volatile organic chemicals	Estimation of Pt:b from Log P and tissue composition data (neutral lipids, phospholipids and water)	49
Volatile organic chemicals	Estimation of Pt:a and Pb:a from molecular structure and tissue composition data	50-51
Volatile organic chemicals	Estimation of Pb:a from Log P, tissue composition data and association binding constant for hemoglobin	50, 51, 99
Highly lipophilic chemicals	Estimation of adipose:plasma from tissue composition data only	124
Various Drugs	Estimation of Pt:p from log P, fraction unbound in plasma and tissue composition data	123
Various Drugs	Estimation of Pt:p from log P, fraction unbound in plasma and tissue composition data	125
Moderate to strong basic drugs	Estimation of Kpu from log P, pKa, fraction unbound in plasma and tissue composition and pH data and electrostatic interactions with acidic phospholipids	126
Acidic, very weak basic, neutral and zwitterionic drugs.	Estimation of Kpu from log P, pKa, fraction unbound in plasma, blood:plasma partitioning, tissue composition, pH, albumin and lipoprotein concentration data	127

Pt:p = tissue:plasma partition coefficient; Log P = n-octanol:water partition coefficient; Pt:b = tissue:blood partition coefficient; Kpu = tissue-to-plasma water partition coefficient

have been used directly, but V_{max} obtained in vitro has been scaled to the whole organism based on the mass recovery of the particular fraction, as follows:[37]

$$V_{max\ (in\ vivo)} = V_{max\ (in\ vitro)} \times C_{prot} \times F_{tiss} \quad (3)$$

where $V_{max\ (in\ vivo)}$ = maximal velocity of metabolism in vivo (mg/min per kg body weight), $V_{max\ (in\ vitro)}$ = maximal velocity of metabolism in vitro (mg/min/mg microsomal protein), C_{prot} = concentration of microsomal protein (mg/g tissue) and F_{tiss} refers to the fractional volume of the metabolizing tissue (e.g., g liver/kg body weight).

The generalizability of in vitro to in vivo extrapolation and animal-replacement algorithms is fairly limited, because the critical determinants in each of these cases are likely to vary as a function of the metabolic reactions (Phase I versus Phase II), metabolizing enzymes and physicochemical properties of the substrates. In fact, mechanistic animal-replacement approaches for predicting the numerical values of V_{max} and K_m of Phase I and Phase II metabolism of chemicals are not yet available. Some semi-empirical approaches relating the molecular structure information to metabolic rate constants have been developed.[77] A pragmatic animal-replacement approach focuses on the generation of "envelope" of simulations representing a plausible internal dose, by specifying complete or negligible hepatic extraction in PBPK models.[78] This approach is particularly useful for forecasting the possible internal dose of chemicals that are not rapidly cleared at the portal

of entry, thus making easier the construction of PBPK models to facilitate the planning of the exposure scenario (e.g., number of doses, dosing duration) for in vivo toxicology studies. Such screening level approaches to PBPK parameter estimation might help to determine the extent of improvement in model predictions that can be obtained while investing time and energy to refine or estimate specific input parameters for PBPK models.

The rate constants of chemical reaction with hemoglobin, tissue proteins, etc., determined in vitro or in vivo, have been incorporated into the PBPK model to make predictions of these phenomena in vivo.[79-80] The feasibility of incorporating in vitro data on receptor binding and DNA binding properties of chemicals within PBPK models for simulating in vivo behavior, has also been demonstrated.[81-82]

MODEL EVALUATION

Once the model is constructed, parameterized and written in a simulation/programming language, it is essential to evaluate the usefulness of the model for the intended applications. All mathematical models of complex reality have potentially built-in uncertainty or errors related to model structure and model parameters.[83] The adequacy of the model structure, as well as the parameter values, is often inferred by comparing the model simulations with experimental data that had not been used for estimating the parameters. This process has been referred to as "validation". even though the use of the term "evaluation" is being increasingly preferred by PBPK modelers.[84-85] Model evaluation is more global and consists not only of comparing model simulations with experimental data, but also conducting sensitivity, uncertainty and variability analyses for assessing the adequacy of the input parameters and structure.

Regardless of the terminology (i.e., validation versus evaluation), the intent is essentially to assess whether:

A. the major determinants of the system behavior are adequately captured by the model; and
B. the input parameters adequately represent the species or population and the chemical for specific exposure conditions.

The choice of method(s) for comparing model simulations with data (i.e., visual inspection, discrepancy indices, statistical tests including residual analysis) depends upon the purpose for which the model is to be used.[86-88] Even though quantitative tests of goodness-of-fit are useful, it is equally important to consider the ability of the model to provide an accurate prediction of the general trend of the time-course data (i.e., bumps, valleys).[21,89]

Following the satisfactory evaluation of a PBPK model, it is used for conducting extrapolations and computations of internal dose for improving the dose–response relationship in the context of toxicity testing and risk assessment.

MODEL APPLICATION

The principal application of PBPK models is to predict the target tissue dose of the toxic parent chemical or its metabolite. By using the tissue dose of the toxic moiety of

a chemical (or its surrogate) in risk assessment calculations, a better basis is provided for relating to the observed toxic effects than is the use of the external or exposure concentrations of the parent chemical.[9,15,90] A critical aspect in this regard relates to model selection, i.e., selecting a PBPK model that can adequately address a particular issue associated with an assessment. This process would require the consideration of the following aspects:[91-92]

- The species for which the model has been constructed versus the species used in toxicity tests or dose–response study chosen for an assessment
- The lifestage(s) for which the PBPK model has been parameterized and evaluated versus the lifestage for which the critical toxicity benchmark (e.g., the NOAEL) has been developed
- The exposure route used in the critical toxicity test versus exposure route(s) described in the model, as well as those that are of relevance to the assessment
- The exposure duration(s) for which the model has been tested versus the duration of the critical toxicity test
- The maximal dose for which the model performance has been evaluated versus the doses used in toxicity test or dose-response study
- The plausible measures of internal dose ("dose metric") based on the current state of knowledge on the mode of action of the chemical versus the capability of the PBPK model to simulate these various dose metrics
- The nature of parameter specification in the model (i.e., point estimate, distributions) versus the intended end-use of the model (e.g., estimation of an inter-individual variability factor)

Because PBPK models facilitate the prediction of target tissue dose for various exposure scenarios, routes, doses and species,[15,21] they can help reduce the uncertainty associated with the conventional extrapolation approaches and assessment factors employed in cancer and noncancer risk assessments, as well as improving the interpretation of the outcomes of toxicity tests.

Toxicity Testing

Animal tests generally focus on characterizing the pharmacokinetics, mode of action or toxicity associated with various dose levels, exposure routes and scenarios. Specifically, pharmacokinetic studies focus on determining the time-course of parent chemical, metabolite(s) or biomarkers in the exposed organism. In the design of such studies, it is critical to determine the time-points for sacrifice or sampling, so that animal use can be efficiently minimized. In this regard, one of the applications of PBPK models is to forecast the blood and tissue concentrations in the exposed animal as a function of time, such that appropriate sampling times can be chosen (Fig. 3). Such judicious use of PBPK models will facilitate the efficient determination of sacrifice/sampling times at which the chemical concentrations would still be above the limit of detection (LOD) of the analytical method, as well as be adequately representative of critical portions of the time-course curve to facilitate the calculation of dose metrics (e.g., AUC as a measure of internal exposure). When limited in vivo data are available, PBPK models can be particularly useful to predict kinetics in intact animals on the basis of in vitro data on metabolic rates and PCs.[93-97] Similarly, in silico approaches can also be used in generating initial estimates of chemical-specific parameters for constructing PBPK

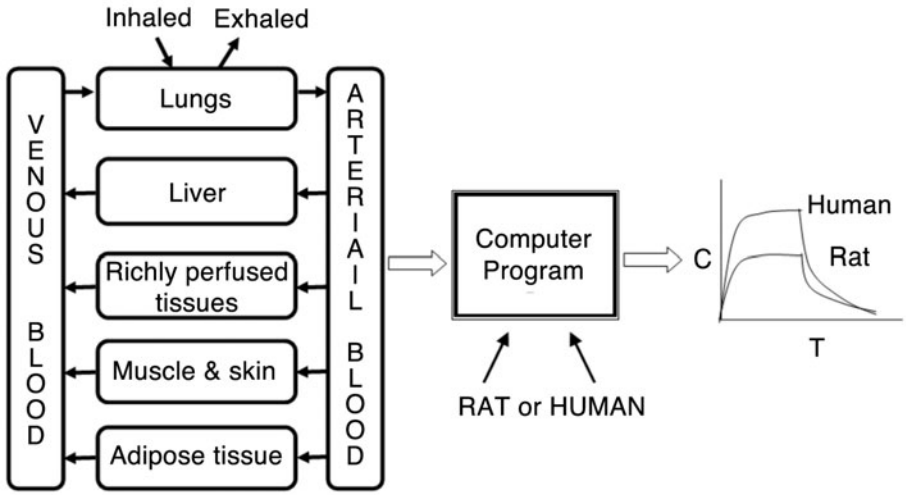

Figure 3. Illustration of the use of PBPK model for prediction of the time-course (C vs T) of tissue dose of a chemical in exposed animals and humans.

models to simulate the time-course of the blood or tissue concentrations of a chemical and its metabolite.[98-99]

In the context of toxicity tests focused on the characterization of the dose–reponse behavior of chemicals or identification of organ-specific effects, the PBPK models are of use in the study design and/or interpretation of results. Pharmacokinetic models and data are particularly useful for study design—specifically for determining the dose levels, as well as frequency, interval and duration of exposure. For example, a PBPK model can be used for determining the exposure conditions that are ideal for maintaining a certain level of internal dose (e.g., over a threshold level) and to choose dose levels that cover a range of conditions (e.g., first order, saturable). A PBPK model can also be used for determining the toxicologically-equivalent doses of systemically-acting chemicals for different exposure routes (Fig. 4). When PBPK models are integrated with biologically-based pharmacodynamic (PD) models, they allow not only the time-course of internal dose in exposed animals to be predicted, but also the toxicological responses, based on an understanding of the mode(s) of action.[14] The PBPK/PD models are also powerful tools for integrating the data on absorption, metabolism, protein binding, receptor interaction and other relevant mechanistic data obtained in vitro with animal physiology, for providing simulations of toxicity outcome in intact animals.[95,100] Even though there has been only limited progress in developing integrated PBPK/PD models for predicting toxicity profiles in silico, there are ample examples of the application of PBPK models in cancer and noncancer risk assessments.[101]

Cancer Risk Assessment

The risk assessment process for genotoxic and epigenetic carcinogens often requires the conduct of high-dose to low-dose, route to route and interspecies extrapolations. Instead

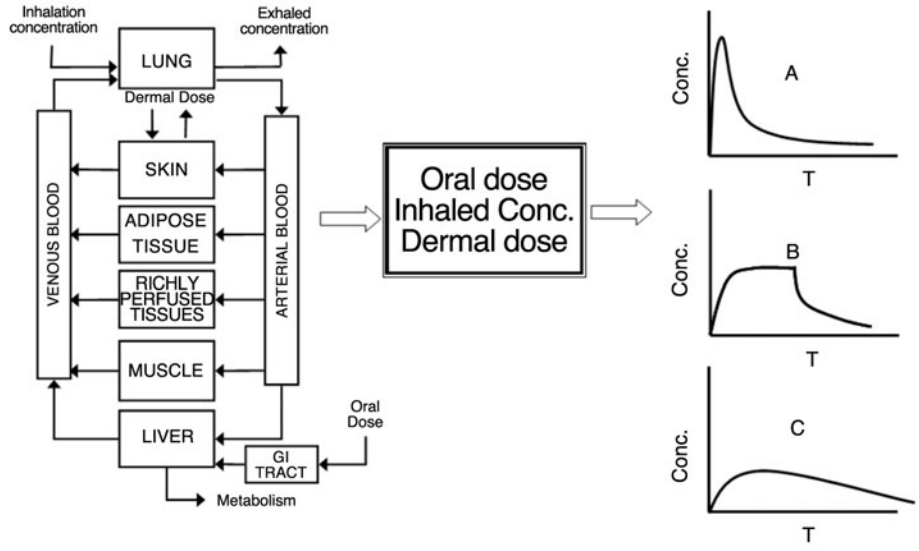

Figure 4. Illustration of the use of PBPK model to predict the concentration of the toxic moiety of chemical in animals exposed via the oral (A) or inhalation (B) routes or via dermal contact (C).

of relying on the conventional approaches based on body weight or body surface area, PBPK models are increasingly used to reduce the scientific uncertainty in the conduct of such extrapolations.[22] Due to their strong biological underpinnings, biokinetic modeling has become the preferred approach for conducting extrapolations of potential internal dose surrogates associated with carcinogenicity.[9,15,17-18] In this regard, extrapolation between laboratory animals and humans is achieved by using species-specific data on input parameters (Fig. 3). Accordingly, physiological parameters (breathing rate, cardiac output, tissue volumes, blood flows, glomerular filtration rate) are obtained for the species of interest or are computed on the basis of body surface scaling. The maximal velocity of metabolism is also scaled on the basis of body surface or body weight in data-poor situations, whereas tissue solubility and the Michaelis constant are most often considered to be species-invariant.[37] The ability of PBPK models to simulate the target tissue dose facilitates the enhancement of the scientific basis of cancer risk assessments. The initial application of PBPK models in cancer risk assessment was demonstrated with dichloromethane (DCM).[9,103] The PBPK model-based cancer risk assessment for this chemical predicted human low-dose risk, about 100- to 200-fold less than that predicted by the conventional approach based on linear extrapolation of high dose to low dose behavior and interspecies dose conversion based on body surface scaling.[104-105] Following the DCM example, there have been several reports of the use of PBPK models in the prediction of the dose metric for enhancing the scientific basis of cancer risk assessment for environmental agents (e.g., vinyl chloride, chloroform, methyl chloroform, 1,4-dioxane, trichloroethylene, acrylonitrile and methyl methacrylate). The vinyl chloride cancer assessment illustrates the unique usefulness of PBPK models, not only for the conduct of high dose to low and interspecies extrapolations, but also for the route-to-route extrapolation. Impressively, the PBPK model-based risk estimates

facilitated the demonstration of the similarity of the range of risk estimates obtained from epidemiological studies and animal bioassays.[106]

Noncancer Risk Assessment

Risk assessments for systemically-acting noncarcinogens have conventionally been based on the knowledge of the point of departure (e.g., NOAEL, LOAEL (lowest observed adverse effect level), BMD (benchmark dose) and the application of uncertainty factors. These factors account for interspecies differences and intraspecies variability in pharmacokinetics and pharmacodynamics, as well as address uncertainty associated with duration extrapolation, data base completeness and data quality.[107]

The application of PBPK models in noncancer risk assessment relies on the availability of sufficient information about the mode of action to define a reasonable internal dose surrogate that is relevant to toxicity. The adverse interaction between chemical agents and living systems is best addressed on a tissue basis, or even on a cellular or subcellular basis. This involves three equally important issues.

First, it requires a knowledge of the most sensitive endpoint, the species that demonstrates that endpoint and the exposure concentration or dose at which no toxicity is observed (NOAEL) in that species. The toxic endpoint of concern needs to be evaluated for relevance—for example, the importance of male rat-specific $\alpha 2\mu$ globulin-mediated nephrotoxicity to human risk assessment is likely to be minimal. In this scenario, the NOAEL will represent a point of departure (the dose–response point that marks the beginning of the low-dose no-effect level or the lower bound of the observed affect).

The second important issue for the use of PBPK models is an understanding of the dose metric, reflective of the effective (risk-relevant) internal dose of the parent chemical or metabolite that is associated with that most sensitive endpoint. The appropriate dose metric is then compared between humans and the most sensitive species by using a PBPK model, since human studies are rarely able to determine tissue-specific dose or toxicity due to ethical concerns.

The final aim is to come full circle and calculate a human equivalent exposure. This would be in the form of a human equivalent concentration (HEC) for inhaled toxicants and a human equivalent dose (HED) for orally-encountered toxicants. Humans encountering these concentrations would develop the same level of the dose metric (e.g., area under the curve [AUC] or maximal concentration [C_{MAX}]) as in the animals exposed to the dose or concentration representing the point of departure (the NOAEL or BMD_L. Generally, once a nonlethal exposure has reached a duration where systemic toxicity is observed, time-normalized dose metrics such as the AUC will represent a dose metric that is more representative of risk. C_{MAX} values are often useful in establishing the dose–response relationship for acute toxicities and are dependent upon dosing rate, such that the high concentration bolus doses commonly encountered in animal experiments will lead to higher peak concentrations than the multi-exposure (divided-dose) scenarios most often encountered by humans.

The role of PBPK models in noncancer risk assessments, particularly for characterizing the magnitude of the pharmacokinetic component of the interspecies uncertainty factor and the intraspecies variability factor, has been summarized by Dewoskin et al.[101] In internal dose-based assessments, the remaining uncertainty relates

to pharmacodynamics, i.e., the response of the tissues to the exposure.[17-18] An example of a noncancer risk assessment that serves to illustrate the use of PBPK models would be ethylene glycol monobutyl ether.[18] Here, the dose metric, $Cmax_{metabolite}$ associated with the point of departure (i.e., $LOAEL_{animal}$) in the animal study was determined by using an animal PBPK model. Subsequently, a human PBPK model was used to determine the oral dose associated with the same level of the dose metric. The resulting human-equivalent dose (7.6 mg/kg/d) was then divided by the appropriate uncertainty factors (10 for human interindividual differences in pharmacokinetics and pharmacodynamics; 3 to account for the LOAEL to NOAEL extrapolation) for deriving the reference dose for humans (0.3 mg/kg/d).[18,108]

CONCLUSION

PBPK modeling offers a scientifically-defensible framework for integrating mechanistic data relating to ADME for predicting dose to target tissues during toxicity tests in animals. A major advantage of these kinds of models relates to their ability to forecast the impact of specific mechanistic processes and determinants on the tissue dose. For example, one can conduct simulations of tissue dose to address the question of "what if …." with regard to variable factors such as the maximal rate of metabolism, the Michaelis constant, etc. In this regard, they provide a basis for integrating in vitro data and making predictions of the tissue dosimetry in the whole animal, thus reducing and/or refining the use of animals in pharmacokinetic and toxicity studies.

In vitro and in silico methods offer valuable alternatives to develop values for physicochemical parameters (e.g., tissue PCs) and biochemical rate constants for use in developing PBPK models. As opposed to in vivo methods, these alternatives offer the advantage that intact animals need not be exposed to test agents and they can be applied to human tissues obtained from organ donors. When the test agent is costly and/or potentially toxic, reducing animal use and avoiding human exposure can have obvious benefits. The reliability of risk values developed following advanced pharmacokinetic studies is largely determined by the choice of test system, so the practitioner should make well-informed choices among the various alternatives.

Effort should be made to assess confidence in the PBPK model for specific applications in toxicity testing and risk assessment. In this regard, PBPK models can support the choice of certain range of doses, such that they are within the linear phase of metabolism, or range of exposure scenarios that lead to steady-state conditions. Similarly, PBPK models can be used to guide dose selection for conducting toxicity test by different routes of exposure. In this case, the models would be used to determine the exposure dose for a new exposure route (e.g., dermal), based on information available for another route (i.e., inhalation) on the basis of equivalent tissue dose. These biologically-based models are dynamic constructs that can be adapted to reflect the exposure conditions of interest to the investigator(s) and updated as new information on mechanistic and molecular determinants becomes available.

In summary, the role of PBPK modeling in improving the exposure–dose–response relationship reflects the use of a systems approach to solving complex problems in experimental toxicology and risk assessment and as such it will be central to the success of the new toxicity paradigms.

DISCLAIMER

This manuscript presents the collective views of the authors. Views and opinions expressed do not necessarily reflect those of their respective employers. The views and opinions herein may not represent the views and policies of the U.S. Environmental Protection Agency.

NOTE ADDED AFTER PROOFS

Since this chapter was drafted, a valuable guidance document has been finalized by the World Health Organization's International Programme on Chemical Safety. *Principles of Characterizing and Applying PBPK Models in Risk Assessment* (WHO/IPCS, 2010)[128] offers the reader important insight into a careful evaluation process for PBPK models of potential use in health risk assessment. This document should be consulted by readers who are interested in more in-depth coverage of this topic.

REFERENCES

1. WHO. Assessing human health risks of chemicals: Derivation of guidance values for health-based exposure limits. Environmental Health Criteria 170, International Programme on Chemical Safety. Geneva: World Health Organization, 1994.
2. Health Canada. Human health risk assessment for priority substances. Ottawa: Environmental Health Directorate, Health Canada, 1994.
3. Younes M, Sonich-Mullin C, Meek ME et al. Risk: assessment and management. In: Herzstein JA, Bunn WB, Fleming LE, eds. International Occupational and Environmental Medicine. St Louis: Mosby, 1998:62-74.
4. Ritter L, Totman C, Krishnan K et al. Deriving incertainty factors for threshold chemical contaminants in drinking water. J Toxicol Environ Health B 2007; 10:527-557.
5. Sonich-Mullin C, Fielder R, Wiltse J et al. IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. Regul Toxicol Pharm 2001; 34:46-52.
6. Boobis AR, Cohen SM, Dellarco V et al. IPCS framework for analyzing the relevance of a cancer mode of action for humans. Crit Rev Toxicol 2006; 36:781-92.
7. Boobis AR, Doe JE, Heinrich-Hirsch B et al. IPCS framework for analysing the relevance of a noncancer mode of action for humans. Crit Rev Toxicol 2008; 38:87-96.
8. NRC Risk Assessment in the Federal Government: Managing the Process. Washington: National Academy Press, 1983.
9. Andersen ME, Clewell HJ, Gargas ML et al. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. Toxicol Appl Pharmacol 1987; 87:185-205.
10. Jarabek AM. Interspecies extrapolation based on mechanistic determinants of chemical disposition. Human Ecological Risk Assessment 1995; 1:641-662.
11. Jarabek AM. Inhalation RfC methodology: dosimetric adjusments and dose-response estimation of noncancer toxicity in the upper respiratory tract. Inhal Toxicol 1994; 6:301-325.
12. Monro A. Drug toxicokinetics: Scope and limitations that arise from species differences in pharmacodynamic and carcinogenic responses. J Pharm Biopharm 1994; 22:41-57.
13. Andersen ME, Krishnan K. Relating in vitro to in vivo exposures with physiologically based tissue dosimetry and tissue response models. In: Salem H, ed. Animal Test Alternatives: Refinement, Reduction, Replacement. New York: Marcel Dekker, 1995:9-26.
14. Andersen ME. Toxicokinetic modeling and its applications in chemical risk assessment. Toxicol Lett 2003; 138:9-27.
15. Clewell HJ, III, Andersen ME, Barton HA et al. A consistent approach for the application of pharmacokinetic modeling in cancer and noncancer risk assessment. Environ Health Persp 2002; 110:85-93.
16. Reddy MB, Yang RSH, Clewell HJ, Andersen ME, eds. Physiologically Based Pharmacokinetic Modeling: Science and Application. Hoboken: John Wiley and Sons, 2005:420.

17. EPA. Approaches for the Application of Physiologically Based Pharmacokinetic (PBPK) Models and Supporting Data in Risk Assessment (Final Report) EPA/600/R-05/043A. Washington, DC: US Environmental Protection Agency, 2006.
18. EPA. Toxicological review of ethylene glycol monobutyl ether. In support of summary information on the IRIS. Washington, D.C.: US Environmental Protection Agency, 2006: Available at: http://www.epa.gov/iris/toxreviews/0500tr.pdf.
19. Lipscomb JC, Ohanian EV, eds. Toxicokinetics and Risk Assessment. New York: Informa Healthcare, 2006:361.
20. Bischoff KB. Physiogically-based pharmacokinetic modeling. Drinking Water and Health 1987; 8:36-64.
21. Clewell HJ 3rd, Andersen ME. Dose, species and route extrapolation using physiologically-based pharmacokinetic models. Drinking Water and Health 1987; 8:159-182.
22. Krishnan K, Johanson G. Physiologically based pharmacokinetic and toxicokinetic models in cancer risk assessment. J Environ Sci Health 2005; 23(C):31-53.
23. Gibaldi M, Perrier D. Pharmacokinetics. New York: Marcel Dekker, 1982.
24. Wagner JG. Fundamentals of Clinical Pharmacokinetics. Hamilton, Illinois: Drug International, 1975
25. Renwick AG. Pharmacokinetics in toxicology. In: Hayes WA, ed. Principles and Methods of Toxicology. New York: Raven Press, 1994:101-147.
26. Clewell HJ 3rd, Andersen ME. Risk assessment extrapolations and physiological modeling. Toxicol Ind Health 1985; 1(4):111-131.
27. Haggard HW. The absorption, distribution and elimination of ethyl ether. Analysis of the mechanism of the absorption and elimination of such a gas or vapor as ethyl ether. J Biol Chem 1924; 59:753-770.
28. Kety SS. The theory and application of the exchange of inert gas at the lungs. Pharmacol Rev 1951; 3:1-41.
29. Fiserova-Bergerova V. Mathematical modeling of inhalation exposure. J Combust Toxicol 1975; 32:201-210.
30. Fiserova-Bergerova V. Toxicokinetics of organic solvents. Scand J Work Environ Health 1985; 11(Suppl 1-7):7-21.
31. Bischoff KB, Dedrick RL, Zakharo DS et al. Methotrexate pharmacokinetics. J Pharm Sci 1971; 60:1128-1133.
32. Mapleson WW. An electric analog for uptake and elimination in man. J Appl Physiol 1963; 18:197-204.
33. Riggs DS. The Mathematical Approach to Physiological Problems: A Critical Primer. Cambridge: MIT Press, 1970.
34. Teorell T. Kinetics of distribution of substances administered to the body. I. The extravascular modes of administration. Arch Int Pharmacodyn 1937; 57:205-225.
35. Teorell T. Kinetics of distribution of substances administered to the body. II. The intravascular modes of administration. Arch Int Pharmacodyn 1937; 57:226-240.
36. Clewell RA, Clewell HJ 3rd. Development and specification of physiologically based pharmacokinetic models for use in risk assessment. Reg Toxicol Pharmacol 2008; 50(1):129-43.
37. Krishnan K, Andersen ME. Physiologically-based pharmacokinetic and toxicokinetic modeling. In: Wallace Hayes A, ed. Principles and Methods in Toxicology. New York: Taylor and Francis Inc., 2007:232-291.
38. Gerlowski LE, Jain RK. Physiologically based pharmacokinetic modeling: Principles and applications. J Pharml Sci 1983; 72:1103-1127.
39. Robinson PJ. Effect of microcirculatory heterogeneity in the determination of pharmacokinetic parameters: implications for risk assessment. Drug Metab Rev 1991; 23:43-64.
40. Robinson PJ. Physiologically-based liver modeling and risk assessment. Risk Anal 1992; 12:139-148.
41. Arms AD, Travis CC. Reference Physiological Parameters in Pharmacokinetic Modeling. Office of Health and Environmental Assessment NTIS PB 88-196019. Washington D.C.: US Environmental Protection Agency, 1988.
42. Brown RP, Delp MD, Lindstedt SL et al. Physiological parameter values for physiologically based pharmacokinetic models. Toxicol Indust Health 1997; 13:407-484.
43. Davies B, Morris T. Physiological parameters in laboratory animals and humans. Pharmac Res 1993; 10:1093-1095.
44. Gentry PR, Haber LT, McDonald TB et al. Data for physiologically based pharmacokinetic modeling in neonatal animals: physiological parameters in mice and Sprague-Dawley rats. J Child Health 2004; 2(3-4):363-411.
45. Thompson C, Johns D, Sonawane B et al. Database for physiologically based pharmacokinetic modeling: physiological data for healthy and health-impaired elderly. J Toxicol Environ Health B 2009; 12:1-24.
46. Balaz S, Lukacova V. A model-based dependence of the human tissue/blood partition coefficients of chemicals on lipophilicity and tissue composition. QSAR 2000; 18:361-368.
47. Béliveau M, Lipscomb J, Tardif R et al. Quantitative structure-property relationships for interspecies extrapolation of the inhalation pharmaconkinetics of organic chemicals. Chem Res Toxicol 2005; 18:475-485.
48. DeJongh J, Verhaar HJM, Hermens JLM. A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-blood partition coefficients of organic chemical sin rats and humans. Arch Toxicol 1997; 72:17-25.

49. Poulin P, Krishnan K. A biologically-based algorithm for predicting human tissue:blood partition coefficients of organic chemicals. Human Exp Toxicol 1995; 14:273-280.
50. Poulin P, Krishnan K. A mechanistic algorithm for predicting blood: air partition coefficients of organic chemicals with the consideration of reversible binding in hemoglobin. Toxicol Appl Pharmacol 1996; 136:131-137.
51. Poulin P, Krishnan K. A tissue composition-based algorithm for predicting tissue:air partition coefficients of organic chemicals. Toxicol Appl Pharmacol 1996; 136:126-130.
52. Obach RS, Baxter JG, Liston TE et al. The prediction of human pharmacokinetic parameters from preclinical and in vitro metabolism data. J Pharmacol Exp Ther 1997; 283:46-58.
53. Poulin P, Theil FP. A priori prediction of tissue:plasma partition coefficients of drugs to facilitate the use of physiologically-based pharmacokinetic models in drug discovery. J Pharm Sci 2000; 89:16-35.
54. Gargas ML, Burgess RJ, Voisard DE et al. Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues. Toxicol Appl Pharmacol 1989; 98:87-99.
55. Gargas ML. An exhaled breath chamber system for assessing rates of metabolism and rates of gastrointestinal absorption with volatile chemicals. J Am Coll Toxicol 1990; 9:447-453.
56. Gargas ML, Clewell HJ, Andersen ME et al. Gas uptake inhalation techniques and the rates of metabolism of chloromethanes, chloroethanes and chloroethylenes in the rat. Inhalation Toxicol 1990; 2:295-319.
57. Dokoumetzidis A, Kalantzi L, Fotaki N et al. Predictive models for oral drug absorption: from in silico methods to integrated dynamical models. Expert Opin Drug Metab Toxicol 2007; 3(4):491-505.
58. Fagerholm U. Prediction of human pharmacokinetics—gastrointestinal absorption. J Pharm Pharmacol 2007; 59(7):905-16.
59. Dedrick RL, Forrester DD, Ho DHW et al. In vitro-in vivo correlation of drug metabolism: deamination of 1-β-D-arabinosyl cytosine. Biochem Pharmacol 1972; 21:1-16.
60. Nakajima T, Sato A. Enhanced activity of liver drug-metabolizing enzymes for aromatic and chlorinated hydrocarbons following food deprivation. Toxicol Appl Pharmacol 1979; 50:549-556.
61. Sato A, Nakajima T. A vial equilibration method to evaluate the drug metabolizing enzyme activity for volatile hydrocarbons. Toxicol Appl Pharmacol 1979; 47:41-46.
62. Hilderbrand RL, Andersen ME, Jensen LJ. Prediction of in vivo kinetic constants for metabolism of inhaled vapors from kinetic constants measured in vitro. Fundam Appl Toxicol 1981; 1:403-409.
63. Lin JH, Sugiyama Y, Awazu S et al. Physiological pharmacokinetics of ethoxybenzamine based on biochemical data obtained in vitro as well as on physiological data. J Pharmacokin Biopharm 1982; 10:649-661.
64. Mortensen B, Nilsen OG. Allometric species comparison of toluene and n-hexane metabolism: prediction of hepatic clearance in man from experiments with rodent liver S9 in headspace vial equilibration system. Pharmacol Toxicol 1988; 82:183-188.
65. Reitz RH, Mandrela AL, Guengerich FP et al. In vitro metabolism of methylene chloride in human and animal tissues: Use in physiologically-based pharmacokinetic models. Toxicol Appl Pharmacol 1989; 97:230-246.
66. Reitz RH, Mendrala AL, Park CN et al. Incorporation of in vitro enzyme data into the physiologically-based pharmacokinetic (PBPK) model for methylene chloride: implications for risk assessment. Toxicol Lett 1988; 43:97-116.
67. Gearheart JM, Jepson GW, Clewell HJ et al. A physiologically-based model for the in vivo inhibition of acetylcholinesterase by diisopropylfluorophosphate. Toxicol Appl Pharmacol 1990; 106:295-310.
68. DeJongh J, Blaauboer BJ. In vitro-based and in vivo-based simulations of benzene uptake and metabolism in rats. Altern Lab Anim—ATLA 1996; 24:179-190.
69. DeJongh J, Blaauboer BJ. Simulation of toluene kinetics in the rat by a physiologically based pharmacokinetic model with application of biotransformation parameters derived independently in vitro and in vivo. Fundam Appl Toxicol 1996; 32:260-268.
70. DeJongh J, Blaauboer BJ. Evaluation of in vitro-based simulations of toluene uptake and metabolism in rats. Toxicol In Vitro 1997; 11:485-489.
71. Iwatsubo T, Suzuki H, Sugiyama Y et al. Prediction of species differences (rats, dogs, humans) in the in vivo metabolic clearance of YM796 by the liver from in vitro data. J Pharmacol Exp Ther 1997; 283:462-469.
72. Mortensen B, Lokken T, Zahlsen K et al. Comparison and in vivo relevance of two different in vitro headspace metabolic systems: liver S9 and liver slices. Pharmacol Toxicol 1997; 81:35-41.
73. Lipscomb JC, Barton HA, Tornero-Velez R et al. The metabolic rate constants and specific activity of human and rat hepatic cytochrome P-450 2E1 toward toluene and chloroform. J Toxicol Environ Health 2004; 67(A):537-553.
74. Krishnan K, Gargas ML, Andersen ME et al. In vitro toxicology and risk assessment. Altern Meth Toxicol 1993; 9:185-203.
75. Lipscomb JC, Fisher JW, Confer PD et al. In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. Toxicol Appl Pharmacol 1998; 152:376-387.

76. Poet TS, Wu H, English JC et al. Metabolic rate constants for hydroquinone in F344 rat and human liver isolated hepatocytes: application to a PBPK model. Toxicol Sci 2004; 82:9-25.
77. Béliveau M, Krishnan K. In silico approaches for developing physiologically based pharmacokinetic (PBPK) models. In: Salem H, Katz S, eds. Alternative Toxicological Methods. Boca Raton: CRC press, 2003:479-532.
78. Poulin P, Krishnan K. Molecular structure based prediction of the toxicokinetics of inhaled vapors in humans. Int J Toxicol 1999; 18:7-18.
79. Frederick CB, Potter DW, Chang-Mateu MI et al. A physiologically-based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. Toxicol Appl Pharmacol 1992; 114:246-260.
80. Krishnan K, Gargas ML, Fennell TR et al. A physiologically-based description of ethylene oxide dosimetry in the rat. Toxicol Ind Health 1992; 8:121-140.
81. Farris FF, Dedrick RL, King FG et al. Cisplatin pharmacokinetics: applications of a physiological model. Toxicol Lett 43:117-137.
82. Terasaki T, Iga T, Sugiyama Y et al. Nuclear binding as a determinant of tissue distribution of adriomycin, daunomycin, adriamycinol, daunorubicinol and actinomycin D. J Pharmacodyn 1984; 7:269-277.
83. WHO. Principles of characterizing and applying human exposure models. International Programme on Chemical Safety. Geneva: World Health Organization, 2004.
84. Cobelli C, Carson ER, Finkelstein L et al. Validation of simple and complex models in physiology and medicine. Am J Physiol 1984; 246:R259-66.
85. Barton HA, Chiu WA, Setzer RW et al. Characterizing uncertainty and variability in physiologically-based pharmacokinetic models: state of the science and needs for research and implementation. Toxicol Sci 2007; 99(2):395-402.
86. Haddad S, Gad SC, Tardif R et al. Satistical approaches for the validation of physiologically-based pharmacokinetic (PBPK) model. Toxicologist 1995; 15:48.
87. Iyengar S, Rao M. Statistical techniques in modeling of complex systems: single and multiresponse models. IEEE Transactions on Systems, Man and Cybernetics 1983; SMC-13:175-188.
88. Krishnan K, Haddad S, Pelekis M et al. A simple index for representing the discrepancy between simulations of physiological pharmacokinetic models and experimental data. Toxicol Ind Health 1995; 11(4):413-22.
89. Anderson MW, Eling TE, Lutz RJ et al. The construction of a pharmacokinetic model for the disposition of PCBs in the rat. Clin Pharmacol Ther 1977; 22:765-773.
90. Benignus VA, Boyes WK, Bushnell PJ et al. A dosimetric analysis of behavioral effects of acute toluene exposure in rats and humans. Toxicol Sci 1998; 43:186-195.
91. Chiu WA, Barton HA, Dewoskin RS et al. Evaluation of physiologically based pharmacokinetic models for use in risk assessment. J Appl Toxicol 2007; 27:218-237.
92. Clark LH. Framework for evaluation of physiologically-based pharmacokinetic models for use in safety or risk assessment. Risk Anal 2004; 24(6):1697-1717.
93. Van Ommen B, de Jongh J, van de Sandt J et al. Computer-aided biokinetic modelling combined with in vitro data. Toxicol In Vitro 1995; 9:537-542.
94. Quick DJ, Shuler ML. Use of in vitro data for construction of a physiologically based pharmacokinetic model for naphthalene in rats and mice to probe species differences. Biotechnol Prog 2000; 15:540-555.
95. MacGregor JT, Collins JM, Sugiyama Y et al. In vitro human tissue models in risk assessment: report of a consensus-building workshop. Toxicol Sci 2001; 59(1):17-36.
96. Hissink EM, Bogaards JJP, Freidig AP et al. The use of in vitro metabolic parameters and physiologically based pharmacokinetic (PBPK) modeling to explore the risk assessment of trichloroethylene. Environ Toxicol Pharmacol 2002; 11:259-271.
97. Kamgang F, Peyret T, Krishnan K et al. An intergrated QSPR-PBPK modeling approach for the in vitro-in vivo extrapolation of pharmacokinetics in rats. SAR and QSAR in Environ Res 2008; 19(7-8):1-12.
98. Yamaguchi T, Yabuki M, Saito S et al. Research to develop a predicting system of mammalian subacute toxicity (3) construction of a predictive toxicokinetics model. Chemosphere 1996; 33:2441-2468.
99. Beliveau M, Krishnan K. Estimation of rat blood:air partition coefficients of volatile organic chemicals using reconstituted mixtures of blood components. Toxicol Lett 2000; 116:183-188.
100. Blaauboer BJ. The integration of data on physio-chemical properties, in vitro-derived toxicity data and physiologicaly based kinetic and dynamic as modelling a tool in hazard and risk assessment. A commentary. Toxicol Lett 2004; 138:161-171.
101. DeWoskin RS, Lipscomb JC, Thompson C et al. Pharmacokinetic/physiologically based pharmacokinetic models in integrated risk information system assessments. In: Lipscomb JC, Ohanian EV, eds. Toxicokinetics and Risk Assessment. New York: Informa Healthcare, 2007:301-348.
102. Leung HW, Paustenbach DJ. Physiologically based pharmacokinetic and pharmacodynamic modeling in health risk assessment and characterization of hazardous substances. Toxicol Lett 1995; 79(1-3):55-65.

103. Andersen ME, Clewell HJ, III, Gargas ML et al. Physiologically-based pharmacokinetic modeling with dichloromethane, its metabolite carbon monoxide and blood carboxyhemoglobin in rats and humans. Toxicol Appl Pharmacol 1991; 108:14-27.
104. Singh DV, Spitzer HL, White, PD et al. Addendum to the health risk assessment for dichloromethane. Updated carcinogenicity assessment for dichloromethane. EPA 600/8-82/004F. Washington, DC: US Environmental Protection Agency, 1987.
105. Krishnan K, Andersen ME. Physiological modeling and cancer risk assessment. In: Rescingo A, Thakkur A, eds. New Trends in Pharmacokinetics. New York: Plenum Press, 1991:335-354.
106. Clewell HJ, Gentry RP, Gearhart JM et al. Comparison of cancer risk estimates for vinyl chloride using animal and human data with a PBPK model. Sci Total Environ 2001; 274:37-66.
107. Dourson ML, Felter SP, Robinson D et al. Evolution of science-based uncertainty factors in noncancer risk assessment. Reg Toxicol Pharm 1996; 24:108-120.
108. Corley RA, Mandrela AL, Smith FA et al. Development of a physiologically-based pharmacokinetic model for chloroform. Toxicol Appl Pharmacol 1990; 103:512-527.
109. Yokogawa K, Nakashima E, Ishizaki J et al. Relationships in the structure-tissue distribution of basic drugs in the rabbit. Pharm Res 1990; 7:691-696.
110. Yata N, Toyoda T, Murakami T et al. Phosphatidylserine as a determinant for the tissue distribution of weakly basic drugs in rats. Pharm Res 1990; 7:1019-1025.
111. Abraham MH, Weathersby PK. Hydrogen bonding. 30. Solubility of gases and vapors in biological liquids and tissues. J Pharm Sci 1994; 83:1450-1456.
112. Abraham MH, Ibrahim A, Acree WE Jr et al. Air to blood distribution of volatile organic compounds: a linear free energy analysis. Chem Res Toxicol 2005; 18:904-911.
113. Abraham, MH, Ibrahim A, Acree WE Jr et al. Air to brain, blood to brain and plasma to brain distribution of volatile organic compounds: linear free energy analyses. Eur J Med Chem 2006; 41:494-502.
114. Abraham MH, Ibrahim A, Acree WE Jr et al. Air to muscle and blood/plasma to muscle distribution of volatile organic compounds and drugs: linear free energy analyses. Chem Res Toxicol 2006; 19:801-808.
115. Abraham MH, Ibrahim A. Blood or plasma to skin distribution of drugs: a linear free energy analysis. Int J Pharm 2007; 329:129-134.
116. Abraham MH, Ibrahim A, Acree WE Jr et al. Air to liver partition coefficients for volatile organic compounds and blood to liver partition coefficients for volatile organic compounds and drugs. Eur J Med Chem 2007; 42:743-751.
117. Abraham MH, Ibrahim A, Acree WE Jr et al. Air to lung partition coefficients for volatile organic compounds and blood to lung partition coefficients for volatile organic compounds and drugs. Eur J Med Chem 2008; 43:478-485.
118. Kaliszan R, Nasal A, Turowski M et al. Quantitative structure-retention relationships in the examination of the topography of the binding site of antihistamine drugs on alpha 1-acid glycoprotein. J Chromatogr A 1996; 722:25-32.
119. Lombardo F, Blake JF, Curatolo WJ et al. Computation of brain-blood partitioning of organic solutes via free energy calculations. J Med Chem 1996; 39:4750-4755.
120. Blakey GE, Nestorov IA, Arundel PA et al. Quantitative structure-pharmacokinetics relationships: I. Development of a whole-body physiologically based model to characterize changes in pharmacokinetics across a homologous series of barbiturates in the rat. J Pharmacokinet Biopharm 1997; 25:277-312.
121. Nestorov I, Aarons L, Rowland M et al. Quantitative structure-pharmacokinetics relationships: II. A mechanistically based model to evaluate the relationship between tissue distribution parameters and compound lipophilicity. J Pharmacokinet Biopharm 1998; 26:521-545.
122. Luco JM. Prediction of the brain-blood distribution of a large set of drugs from structurally derived descriptors using partial least-squares (PLS) modeling. J Chem Inf Comput Sci 1999; 39:396-404.
123. Bjorkman S. Prediction of drug disposition in infants and children by means of physiologically based pharmacokinetic (PBPK) modelling: theophylline and midazolam as model drugs. Br J Clin Pharmacol 2005; 59:691-704.
124. Haddad S, Poulin P, Krishnan K et al. Ratio of lipid content in adipose tissues and blood as the sole determinant of the adipose tissue: blood partition coefficients of highly lipophilic organic chemicals. Chemosphere 2000; 40:839-843.
125. Berezhkovskiy LM. Determination of volume of distribution at steady state with complete consideration of the kinetics of protein and tissue binding in linear pharmacokinetics. J Pharm Sci 2004; 93(2):364-74.
126. Rodgers T, Leahy D, Rowland M et al. Physiologically based pharmacokinetic modeling 1: predicting the tissue distribution of moderate-to-strong bases. J Pharm Sci 2005; 94:1259-1276.
127. Rodgers T, Rowland M. Physiologically based pharmacokinetic modelling 2: predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions. J Pharm Sci 2006; 95:1238-1257.
128. WHO/IPCS. Principles of Characterizing and Applying PBPK Models in Risk Assessment. Geneva: World Health Organization, 2010. Available at: http:www.who.int/ipcs/methods/harmonization/areas/pbpk_models.pdf.

CHAPTER 7

IN SILICO METHODS FOR TOXICITY PREDICTION

Robert D. Combes
Independent Consultant, Norwich, UK
Email: robert_combes3@yahoo.co.uk

Abstract: The principles and uses of (Q)SAR models and expert systems for predicting toxicity and the biotransformation of foreign chemicals (xenobiotics) are described and illustrated for some key toxicity endpoints, with examples from the published literature. The advantages and disadvantages of the methods and issues concerned with their validation, acceptance and use by regulatory bodies are also discussed. In addition, consideration is given to the potential application of these techniques in regulatory toxicity testing, both individually and as part of a chemically-based read-across approach, particularly for the risk assessment of chemicals within intelligent, integrated decision-tree testing schemes. It is concluded that, while there has been great progress in recent years in the development and application of in silico approaches, there is still much that has to be achieved to enable them to fulfill their potential for regulatory toxicity testing. In particular, there is a need for the wider availability of appropriate biological data and international agreement on how the systems should be validated. In addition, it is important that correlations between activity and physicochemical properties are based on a mechanistic basis to maximize the predictivity of models for novel chemicals.

INTRODUCTION

The fundamental basis of using in silico prediction methods is that the activity of a chemical in any given biological system is determined by its physicochemical properties.[1,2] In principle, it should be possible to develop computer prediction systems for any type of biological activity provided suitable data for developing models are available and the chemical structure of the test material is known. In 1991, the Second FRAME Toxicity Committee recommended: *'research into the development of QSARs [Quantitative*

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

Structure-Activity Relationships]……..should be continued and extended'.[3] While there has been much progress, as shown below, the full potential of in silico modeling has yet to be fulfilled. This chapter discusses the possibilities for using in silico methods to predict toxicity and considers some of the problems with developing, validating and using in silico methods.

QSAR MODELING

History

In QSAR modeling, equations are derived to predict biological activity based on physicochemical properties.[4] The origins of modeling structure activity go back to 1863 when Cros noticed that the mammalian toxicity of alcohols depended on their water solubility. Then, in 1868 and 1869, Crum-Brown and Fraser suggested that structure and biological activity of molecules could be described mathematically. In 1893, Richet found that the toxicity of a series of organic chemicals was inversely proportional to their water solubility. However, further progress had to await discoveries concerning atomic structure, and it was not until 1970 that Hammet first related biological activity of a molecule to its electronic state, in terms of a value he called the sigma constant (see ref. 5 for these early citations). The concept of QSAR modeling was developed further a decade or so later, most notably by Free and Wilson[6] and Hansch et al[7,8] who derived the first interpretable equations, based on linear free energy and the assumption that molecular fragments in similar chemical structures independently and additively contribute to activity, due to their physicochemical properties (molecular descriptors).

Developing Models

Main Steps

The main steps involved in developing a QSAR model are: (a) entry of structural information using codes such as SMILES[9] or a chemical drawing package like Isis/Draw, ChemDraw or CORINA (CoORdINAtes: http://www.molecular-networks.com/online_demos/corina_demo.html); (b) analysis of molecular descriptors; (c) processing of physicochemical and toxicological information; (d) model development (e) testing for goodness of fit; (f) validation; (g) defining an applicability domain (AD); and (h) application for toxicological assessment of specific endpoints (Fig. 1).

Training Set of Chemicals

Unlike other toxicity tests, in silico models must be developed by using a training set of chemicals, which consists of molecules that vary in structure and have well-characterized toxicities and mechanisms of action. The results obtained with the training set of chemicals will help to determine the applicability domain (AD) of the model (see below). Equations are then developed with defined variables and constants, which can then be solved for novel test chemicals to predict their toxicities.

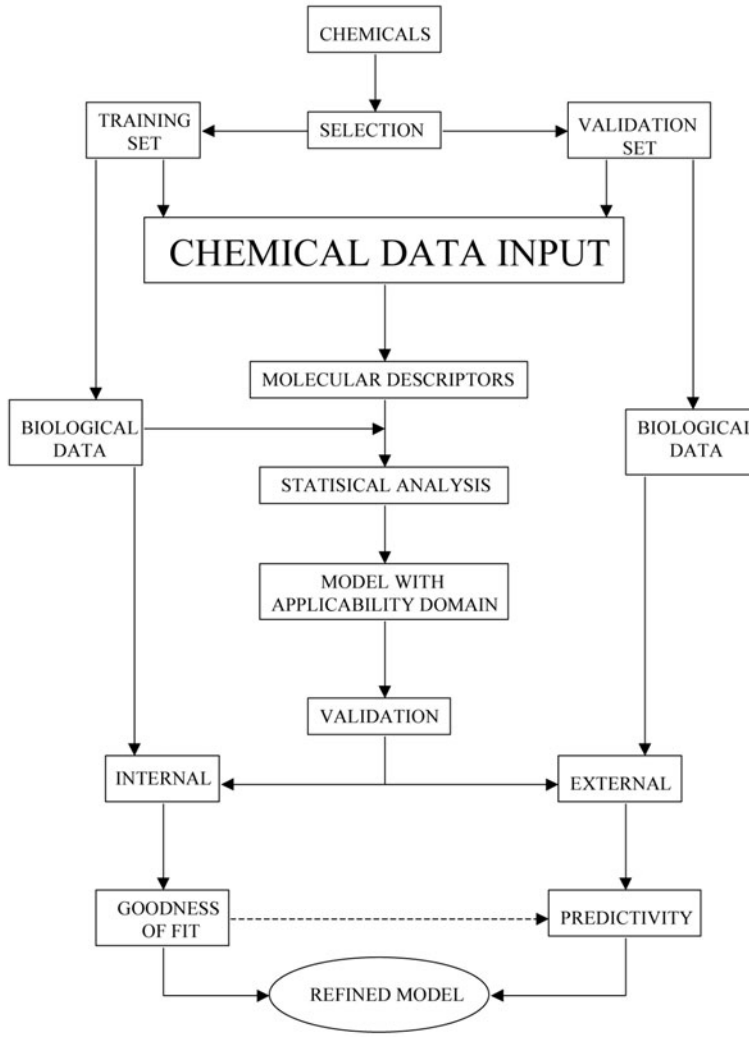

Figure 1. Overall scheme for developing and validating in silico methods for toxicity prediction.

Molecular Descriptors

More than 2,000 potential physicochemical properties (molecular descriptors), can be generated from molecular structures[10] using a variety of software packages (e.g., DRAGON, CDK, JOELib, TOPS-MODE and PowerMV).[11] The relevance of descriptors for predicting biological activity will depend on the endpoint, the biological system concerned and the class of chemicals involved. However, the following descriptors have frequently been found to be relevant: (a) molecular volume; (b) dipole moment; (c) molecular shape; (d) molecular connectivity (the manner in which different sub-structural fragments are connected to each other; (e) the overall number of atoms in the molecule; (f) steric effects

(e.g., the masking of a reactive site by another substituent); (g) molar refractivity; (h) acidic dissociation constant (pK_a); (i) electronegativity; (j) quantum chemistry, or electronic configuration states (intrinsic reactivity); and (k) the octanol/water partition coefficient, logP (lipophilicity or hydrophobicity—a measure of bioavailability).

An example of using a few selected descriptors is Lipinksi's rule of five[12,13] that is often, but not always, useful for predicting the oral activity of a drug on the basis of it having: (a) a molecular weight of < 500 g/mol; (b) < = 5 H- bond donors (OH and NH groups); (c) < = 10 H- bond acceptors (N and O); and d) a logP of < 5.

Overall three dimensional shape can be determined by X-ray crystallography of members of a functional or structural class of chemicals for which suitable crystal structures are available.[14] Another technique, called Comparative Molecular Field Analysis (CoMFA), can be used for comparing the molecular conformations of molecules.[15]

The reactivity of a molecule depends on the energy levels of the electrons in its various atoms, expressed as the delta E values for: (a) the lowest unoccupied molecular orbital (LUMO); and (b) the highest occupied molecular orbital (HOMO).[16] There are many examples of where QSAR models contain descriptor values for electronic properties. Zhang et al[17] found that the mutagenic potency of nitronaphthalenes and methylnitronaphthalenes depended on frontier orbital energy values of the molecules (the sum of the energies of the highest occupied molecular orbital and of the lowest unoccupied molecular orbital). Also, Contrera et al[18] recently developed eight models for screening chemicals for genotoxicity (mutagenicity in *Salmonella, Escherichia coli* and L5178Y mouse lymphoma cells and chromosomal damage in vivo), based on several molecular descriptors, including connectivity and frontier orbital energy states.

Biovavailability

Fundamentally, toxic chemicals are nonhazardous if they are not bioavailable and nontoxic chemicals are not hazardous even if they are bioavailable. Essentially, a molecule is more bioavailable if it can pass across lipid-rich biological membranes and into cells. The in silico prediction of aqueous solubility is, therefore, a key process when developing new chemicals,[19] as shown by the fact that only 15% of some 3,000 QSAR models were shown to lack terms for lipophilicity (logP) in one study.[20] LogP was also recently shown to be an important descriptor, together with molecular surface area, in determining the clastogenicity and aneugenicity of a group of 26 chemicals tested in the micronucleus test conducted in V79 Chinese Hamster cells in vitro.[21] A further example of the importance of logP comes from the work of Duchowicz and Ocsachoque[22] who analyzed 1,509 theoretical descriptors for a wide range of molecular parameters by using Dragon software in a dataset of more than 470 aliphatic organic chemicals. They found that hydrophobicity (high lipophilicity) was the most important parameter.

Toxicophore Concept

Sub-structural features associated with toxicity are called toxicophores. A chemical with a toxicophore can possess other toxicophores for the same or different toxicities and it might also contain a region that stops it from being toxic (sometimes referred to as a biophobe). Toxicophores determine whether chemicals can specifically interact with one or more of the following subcellular components: (a) receptors; (b) enzymes; or (c) macromolecules such as proteins and DNA, thereby perturbing their functions.

Statistical Relationship between Descriptors and Biological Activity

Analysis of molecular descriptors and their relative distributions in active and inactive molecules in a training set and in a query chemical in a test set is performed by several similarity searching, cluster analysis and statistical methods. Further mathematical and statistical methods, such as multiple linear regression (MLR), principal component analysis (PCA), discriminant analysis and artificial neural networks are used to search for associations between the presence and absence of molecular descriptors with biological activity.[16,23-27]

EXPERT SYSTEMS

Background

Computerized expert systems use rules developed from pre-existing information to predict biological activity.[28,29] Knowledge-based systems (KBS) use rules developed by human experts, which are programmed into software that then make predictions about likely activity based on the presence or absence of toxicophores using reasoning engines. Automated Rule Induction (ARI) systems (ARIs), develop rules using algorithms and are useful for analyzing complex data. The basic principles for developing rules for QSARs also apply to expert systems.

Examples of Expert Systems

Two examples of KBS for hazard prediction are HazardExpert[30,31] and DEREK for Windows (Deductive Estimation of Risk from Existing Knowledge[32,33]). KBS for biotransformation prediction also exist and include MetabolExpert and Meteor.[34] Examples of ARIs are CASE (Computer Automated Structure Evaluation) and MULTI-CASE,[35] which generate rules by statistically analyzing toxic and nontoxic molecules to identify and distinguish fragments associated with toxicity and those that are not. Once trained, the program produces quantitative predictions for novel chemicals. TopKat (Toxicity Prediction by Komputer Assisted Technology) identifies toxicophores by automatically applying QSAR modeling techniques.[36] TIMES[37] and METACASE[34] are two ARIs for predicting biotransformation. COMPACT is another ARI system, but this is based on analysing molecules to determine their ability to bind to the active site of CYP1A1 (and a few other cytochrome p450 (CYP450) isozymes) by modeling molecular shape and chemical reactivity.[38] COMPACT, however, is only useful for analysing molecules that are activated by these CYPs.

Predicting Metabolite Toxicity

Once the structures of major metabolites of a parent chemical have been identified, they can be input into an expert system and treated as the query chemical. This is most easily undertaken by using compatible software—METEOR with DEREK, HazardExpert with MetabolExpert and MetaCASE with MultiCASE. TIMES has a number of reactivity models for various toxicity endpoints (e.g., mutagenicity and sensitisation), simulating reactive metabolite formation by metabolising systems, like S9 (postmitochondrial

supernatant).[34,39] Some systems for biotransformation are part of integrated software that models absorption, distribution, metabolism and excretion (ADME).[40,41] Computational modeling of metabolic reactions is highly complex and in silico biotransformation prediction systems require further improvement.[42,43]

THE APPLICABILITY DOMAIN (AD)

It is crucial to determine the AD of any in silico model.[44] This is defined as 'the physicochemical, structural, or biological space, knowledge or information on which the training set of the model has been developed and for which it is applicable to make predictions for new compounds,' and methods for its determination have been reviewed.[45]

It is important that a model should only be used to predict the activities of chemicals that fall within its AD, as it cannot be expected to correctly predict the activity of molecules outside its AD. This important principle was not taken into account in a recent comparison of the performance of three expert systems DEREK, HazardExpert and TOPKAT for predicting the irritation potential of 116 chemicals.[46] It was claimed that only TopKat gave satisfactory results since it was able to correctly predict a high proportion of the chemicals. However, the comparison is invalid as only a few of the chemicals were within the ADs of the two other expert systems, while the majority were covered by TopKat.

VALIDATION AND REGULATORY ACCEPTANCE

Background

Validation of in silico methods is conducted by using internal and external methods.[47,48] There are several methods of internal validation: (a) Leave One Out Cross Validation (LOO-CV; Box 1); (b) Multiple Leave Many Out Cross Validation (LMO-CV; Box 1); (c) boot-strapping (Box 2); and (d) Y-scrambling (Box 3). Internal validation suffers from: (a) a need to use complex statistical methods to account for bias and to derive estimates

Box 1. LOO-CV and LMO-CV internal validation of QSAR models

LOO-CV involves the successive and random removal of individual chemicals from the training set of chemicals originally used for developing the model or the rule base. The model is then automatically regenerated by using information from the remaining training set of chemicals and this new model is used to predict the biological activity of the chemical that was removed, by treating it as a novel test chemical. This process is conducted iteratively, by successively removing other chemicals individually from the training set and then rederiving a new model or rulebase each time with which to make the next prediction. The results of the prediction exercises are then used to determine the validation status of the system overall. LMO-CV, on the other hand, involves leaving more than one chemical out at each stage (e.g., if n chemicals were omitted, this can be denoted as LnO) and is considered a more accurate method of internal validation for assessing predictivity as it corrects for the tendency of LMO-CV to overfit the data and thus to compromise the estimation of predictivity. The reader is referred to Golbraikh and Tropsha[1] for a more detailed explanation of the technique.

Box 2. Bootstrapping

Bootstrapping is an efficient statistical approach to resampling data,[2-4] as the entire data set is used for model development, although one disadvantage is that it tends to give over-optimistic results. The technique allows the error due to resampling to be determined.[2] In its simplest form, bootstrapping, repeatedly analyzes very small samples (sub-samples) of the data instead of repeatedly analyzing larger subsets, as undertaken with some other resampling methods. Each sub-sample is selected randomly from the full sample. Anywhere from 50 to 2000 sub-samples might be used in a typical analysis. Some authors favor bootstrapping over other methods of resampling.[5]

Box 3. Y-scrambling

Y-scrambling is a statistical sampling technique which guards against the possibility that molecular descriptors can be selected which seem to be important for biological activity due to a chance correlation with the biological data.[6,7] The procedure involves leaving unchanged the molecular descriptors for the training set of molecules that were used to develop the model. The toxicity data (parameter Y) are randomly shuffled to change their true correlation with the molecular descriptor data, thereby altering the descriptor/toxicity relationship. A new QSAR model is then derived from the re-arranged information and the correlation between descriptors and toxicity is calculated. These two steps in the overall process are repeated with correlations being calculated after each iteration (usually 50-100 such iterations and recalculations are performed). Correlation values obtained in the above fashion (i.e. being an association between toxicity and structure derived by chance) are compared with the true values obtained for the model fitted to the real data. These true values should lie well outside such a reference association (obtained by applying Y-scrambling) to indicate that there exists a real model based on the given data, and that it was not the same as one that could have been learnt by chance.

of predictivity for the overall model;[49] (b) a failure to provide a properly validated model developed with the full training set of chemicals; (c) lack of transparency (as in ref. 50); and (d) providing information of limited use on the predictivity of the model for new chemicals. However, internal validation is useful for estimating goodness of fit (a measure of how well a model predicts the training set of chemicals).[51]

External validation overcomes these drawbacks by challenging an in silico model with a completely new group of chemicals that the model has never been trained with.[49,52,53] It is important to avoid bias in the selection of chemicals in a test. The identities, biological activities and purities of the chemicals used in the training and test sets should always be given, as selection of chemicals can affect validation and model predictivity and applicability. However, sufficient numbers of appropriate chemicals for test sets can be difficult to obtain due to: (a) lack of information; and (b) the ease with which models can be retrained with new chemical structures. These problems can, in part, be addressed by dividing suitable chemicals into separate training and test sets *before* model development. Certain, specialized techniques, such as k-means clustering, a sphere exclusion algorithm and a novel support vector machine method, have been shown to be useful for this purpose as they also avoid selection bias.[54,55] In addition, a model could also be challenged with a test set of chemicals that have been custom-synthesized once their toxicity has been determined.[5,52] For example, one study compared the respective predictive performances

for rodent carcinogenicity of several expert systems (DEREK, TopKat, MultiCASE, Compact and HazardExpert), which had previously been trained on chemicals in the National Toxicology Program database, using new data for a further set of chemicals once the carcinogenicity results for them had become available.[56]

An Example of Validation

A recent validation study where sufficient chemicals were available to use for external validation was described by Zhu et al who assembled a database of 983 chemicals that had been tested for toxicity to *Tetrahymena pyriformis*.[57] From this set, 644 chemicals were randomly selected and used to independently develop 15 different QSAR models in a number of laboratories. The remaining 339 chemicals in the original set (external set 1) and 110 additional chemicals (external set 2) were used as two test sets of chemicals for validation. It has also been suggested that a second set of chemicals could be used to calibrate a model before subjecting it to an external validation (Box 4).

Guidance on Developing and Validating Models

Several recommendations and guidance documents have emerged for validating in silico models.[58] The Setubal Principles[59] state that models should have: (a) a well-defined endpoint; (b) unambiguous and transparent structural descriptors; (c) an appropriate AD; (d) goodness of fit together with any external validation results; (e) a plausible physicochemical or biological basis and a toxicological pathway; and (f) full access to all appropriate supporting information. These criteria form the basis of the latest OECD requirements[60] although both sets of guidance only imply that external validation is desirable, rather than mandatory.

Validation of in silico models is controversial: some believe that they need not be validated, in view of their high inherent reliability and the difficulties of assessing relevance due to lack of novel chemicals to test them with.[61] Others consider that models should always be validated externally.[52] The regulatory acceptance of in silico systems is likely to depend on a resolution of this conflict.

Box 4. Using a calibration set of chemicals

Once a model has been developed by using a training set of chemicals it could be further refined by using a different group of chemicals (a calibration set) whose structures, biological activities and predictions in the model can be used to refine the model, before it is assessed for predictivity by using a test set of chemicals—all of the chemicals being unique to each set. This approach was adopted by Toropov et al[8] for a QSAR model of acute oral toxicity in rats (LD50) for 61 benzene derivatives. These were divided into a training set (27 chemicals), a calibration set (24 chemicals) and a test set (10 chemicals). They found that electronegativity and the presence of amino groups in the molecule were key determinants of toxicity and that improved predictivity was obtained for the training set of chemicals if the calibration set of chemicals had first been used to refine it. In their assessment of several models for ecotoxicity in a project called DEMETRA (Development of Environmental Modules for Evaluation of Toxicity of pesticide Residues in Agriculture), Porcelli et al[9] devised a new index of model uncertainty which takes into account chemicals that behave as outliers in correlations between structure and activity.

APPLICATIONS OF QSAR MODELING AND EXPERT SYSTEMS

Background

In silico modeling can be suitable for predicting the toxicities of large numbers of closely related chemicals (congeneric (homogenous) molecules), for example, in high throughput screening of combinatorial libraries of drug candidates.[62] Thus, in silico approaches are widely used as part of Computer Aided Drug Design (CADD) approaches.[63,64] In silico modeling of other endpoints relevant to pharmaceuticals, like hERG K^+ channel affinity QT prolongation interval, has also been undertaken[65] and DEREK has a rulebase for this endpoint.[66]

Toxicity End-Point Coverage

As the development of in silico models for toxicity is dependent on the availability of data and defined mechanisms of action, it is not surprising that they are more prevalent for endpoints where there are large databases (Table 1). Such examples include models for ecotoxicity,[31,67] mutagenicity and carcinogenicity,[68-71] skin sensitisation[72,73] and endocrine disruption via ER receptor binding and ER reporter gene activation.[74] The in silico prediction of some other endpoints, like eye and skin irritation and corrosion, is being improved as more data are being accumulated,[75] but few models for general mammalian toxicity, particularly long-term effects, are available due to lack of knowledge about many of the mechanisms involved, even though much data are available.[76,77] Now that more data are available, attempts are also being made to develop QSAR models for predicting the inhalation toxicity of nanoparticles by using descriptors such as the ratio of size to surface area, surface charge and surface derivatization.[78]

In Silico Methods and REACH

Background

The European Commission REACH (Registration, Evaluation and Authorisation of Chemicals) system, which was adopted into legislation in June 2007, necessitates re-evaluating the risks to humans and wild life of exposure to >30,000 existing chemicals.[79,80] For logistical and animal welfare reasons, testing under REACH should make maximum use of pre-existing information and use in vitro methods in exposure-based intelligent integrated testing strategies to prioritize chemicals for testing.[81,82]

Prioritizing Chemicals for Further Testing

Persistence (recalcitrance, or resistance to bio-degradation), bioaccumulation and evidence of some toxicity (PBT) have been identified as particularly important properties of chemicals and confer on them a high testing priority. Various proprietary software packages have been designed for this purpose. PBT Profiler (Table 1) and a set of QSAR models developed by using the Danish Environmental Protection Agency's (EPA's) extensive database on chemicals (www.mst.dk/English/Chemicals/Substances_and_materials/QSAR/) are examples of such models. PBT Profiler incorporates the US EPA's BCFWIN and ECOSAR software for determining environmental persistence, bio-concentration

Table 1. Some expert systems and related computerized systems and databases for predicting toxicity and related endpoints

System (Type)*	Supplier	Website	Examples of Endpoint Predicted
ASTER (DTS)	US EPA	www.epa.gov/med/databases/aster.html	7
BCFWIN (DTS)	US EPA	www.epa.gov	Fish bioconcentration factor
BIOEPISTEME (ARI)	Prous Institute for Biomedical Research	www.prousresearch.com	2, 12, 13, multiple toxicity endpoints and therapeutic activity for various targets
CASE/MCASE/CASETOX (ARI)	MultiCase Inc	www.multicase.com	1, 2, 4, 5, 7, 17, 19, 20
COMPACT (ARI)	University of Surrey	www.surrey.ac.uk	3
DEREK for Windows (KBS)	LHASA Ltd	www.lhasalimited.org	2, 4, 5, 9, 10, 15, 19
ECOSAR (Ecological Structure Activity Relationships) (DTS)	US EPA OPPT	www.epa.gov/oppt/newchems/tools/21ecosar.htm	7: Acute and chronic toxicity
HazardExpert (KBS)	CompuDrug Ltd	www.compudrug.com	1, 2, 4, 5, 8, 11, 13, 14, 16
MetabolExpert (KBS)	ComuDrug Ltd	www.compudrug.com	12
METACASE (ARI)	MultiCase Inc	www.multicase.com	12
METEOR (KBS)	LHASA Ltd	www.lhasalimited.org	12
TOPKAT (ARI)	Accelrys Inc.	www.accelrys.com	1, 2, 4, 5, 6, 7, 9, 17, 19, 22, 23, 24
LAZAR (ARI)	Christoph Helma, University of Freiburg	http://echeminfo.colayer.net/COMTY_mehelmac	2, 5
MDL-QSAR (ARI)	Elsevier MDL Information Systems Ltd	www.symyx.com/products/pdfs/qsar_ds.pdf	2: Multiple endpoints

continued on following page

Table 1. Continued

System (Type)*	Supplier	Website	Examples of Endpoint Predicted
OASIS/TIMES (ARI)	Laboratory of Mathematical Chemistry, Bourgas, Bulgaria	http://omega.btu.bg	7: Skin and liver toxicity models (12)
ONCOLOGIC (ARI)	US EPA	www.epa.gov/oppt/cahp/actlocal/can.html	2
PASS (Prediction Activity Spectra for Substances) (ARI)	VN Orechovich Institute of Biomedical Chemistry, Russia	http://195.178.207.233/PASS/	2, 5 (and some 2,500 different biological activities for a wide range of toxicity endpoints)
TerraQSAR (DTS)	TerraBase Inc.	www.terrabase-inc.com	1, 7, 13, 15, 20, 26
Toxscope (ARI)	Leadscope Inc.	www.leadscope.com	Database of physicochemical information of large number of structures together with a wide range of toxicity endpoints, including especially 2, 4, 11, 21, 24, 29
Toxmatch (KBS/DTS)	European Chemicals Bureau	http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c = TOXMATCH	11, 13, 75
Toxtree (KBS/DTS)	European Chemicals Bureau	http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c = TOXTREE	87, 90
PBT (Persistent Bioaccumative and Toxic) profiler (ARI)	US EPA OPPT (P2 Assessment Framework)	www.pbtprofiler.net	2, 7, 14, 27, 28

*ARI, Automated Rule Induction System; KBS, Knowledge Base System; DTS, Decision Tree System.
(1) acute mammalian toxicity; (2) rodent carcinogenicity; (3) carcinogenicity via CYP 1A1 and 2E biotransformation; (4) developmental toxicity; (5) mutagenicity; (6) chronic toxicity; (7) aquatic toxicity; (8) membrane irritancy/sensitivity; (9) skin sensitisation; (10) respiratory sensitisation; (11) neurotoxicity; (12) biotransformation; (13) bioavailability; (14) bioaccumulation; (15) skin irritation; (16) immunotoxicity; (17) eye irritation; (18) biodegradability; 19) chronic toxicity; (20) endocrine disruption; 21) hepatotoxicity; 22) systemic toxicity; (23) subchronic toxicity; (24) reproductive toxicity; 25) ecotoxicity; 26) acute aquatic toxicity; (27) environmental fate; (28) population exposure; (29) urinary tract toxicity.

potential and aquatic toxicity. Zachary and Greenway[83] recently compared the results obtained from using PBT profiler with the Danish EPA's QSAR models for the same set of chemicals. Discrepancies between the predictions were found and it was recommended that both methods should be used for chemical prioritization. However, ECOSAR has recently been shown by Reuschenbach et al to categorize 1,000 randomly-chosen chemicals correctly according to the EU ecotoxicity classification scheme (either not harmful, harmful, toxic or very toxic) with only 65% accuracy at best.[84]

Zvinavashe et al found that QSAR models exist for determining the acute toxicities of 57% of the substances in the EINECS (European Inventory of Existing Commercial Substances) database, which is a major source of information on chemicals for which data-gaps exist.[85] In many cases, those substances that were not covered were found to be chemical mixtures e.g. food extracts, botanical and animal extracts which call for more complex in silico prediction approaches for mixtures of chemicals based on the need to identify the component chemicals and their structures, their relative amounts and to obtain information on their possible interactions.

Decision-Tree Schemes

Various decision-tree schemes have been devised for categorizing chemicals according to their potential hazards. One of the earliest was developed by Cramer and Ford for mammalian acute toxicity.[86] This classifies organic chemicals according to structure and/or biochemical and physiological chemistry into low priority substances (low human exposure and toxicity); high priority substances (high human exposure and toxicity); and intermediate priority substances (possible human exposure and toxicity). This scheme has been recommended for priority setting of chemicals in REACH[81] and was recently computerized. It is now one of four methods originally forming the basis of a software package released by the European Chemicals Bureau (ECB) called Toxtree (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c = TOXTREE).

The three other decision-tree schemes initially involved in Toxtree are one for aquatic toxicity,[87] a rule-base for skin and eye irritation and corrosion[88] and a rule-base for mutagenicity and carcinogenicity.[89] Toxtree now has additional modules, for biodegradation and persistence, as well as chromosomal damage (the in vivo micronucleus assay). A decision-tree scheme for oestrogenicity has also been developed that could be updated and computerised for inclusion in Toxtree.[90]

Read-Across

The idea of read-across is to avoid the need to test every chemical for every toxicity endpoint to fill in data gaps, since it facilitates prediction of the toxicity of a chemical by adopting the hazard profile of another chemical of sufficiently similar physicochemical properties to categorize both together in the same group.[91,92] A chemical category is, therefore, a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (Fig. 2; Box 5). It is assumed that all the chemicals in the same category share a common mechanism of toxicity for the endpoint in question, which may not be true.

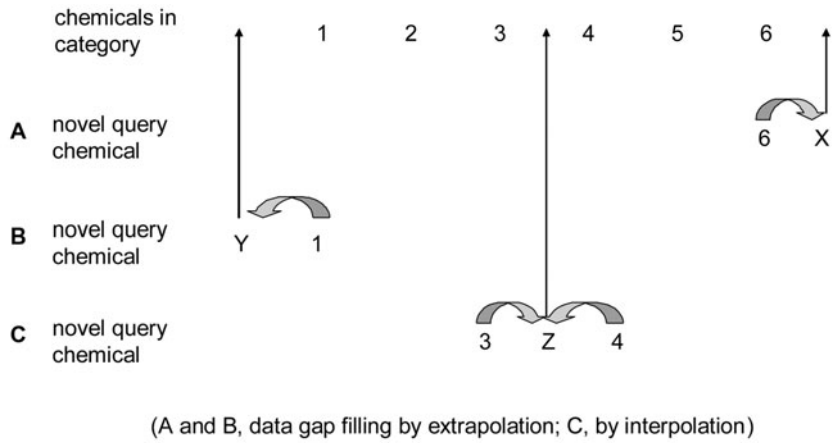

Figure 2. Two ways for filling data gaps for three query chemicals, X, Y and Z, by applying read-across (see Box 5).

Box 5. Applying read-across by using chemical categories

In Figure 3, for cases A and B, the query chemical (X or Y) lies at either extreme of the spectrum of physicochemical properties of chemicals of a category and the data gap is extrapolated from the properties of chemical 1 in case A and chemical 6 in case B. In case C, the properties of chemical Z are between those of chemicals 3 and 4 and the toxicity of Z is interpolated from the toxicities of chemicals 3 and 4, without having to test Z for the toxicity endpoint in question for purposes of prioritization. Guidelines on applying read-across are given on the European Chemicals Agency (ECA) website (http://echa.europa.eu/). Categorization involves the characterization of the chemical structures to be compared in terms of relevant molecular descriptors encoding their physicochemical properties, followed by a quantitative comparison of the information by using similarity and dissimilarity indices,[10] an overall process very similar to how QSAR modeling is conducted and one that has also been computerized and is available as another open-source software package released by the ECB, called Toxmatch[11] (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c = TOXMATCH). Toxmatch is based on two similarity computational methods, atom environments and molecular fingerprints and other related methods, namely k-nearest neighbor grouping and clustering. Toxmatch has been shown to be able to define categories of chemicals specifically for aquatic toxicity, bio-accumulation and teratogenicity by using information from a number of different databases.[12,13] In some cases, it was also possible to make predictions about the toxicity of several query chemicals (i.e., perform read-across) Read-across might be feasible from one salt of a substance to another salt and from chemicals with, for example, alkyl chains of one length (e.g., C7), to molecules with a chain of near similar length (e.g., C6 or C8),[14] particularly when toxicity data for an homologous series of substances vary little (e.g., toxicity due to a functional group conserved between molecules). However, read-across should be based on a QSAR model for the query chemical and the chemicals with which it is to be categorised.[15] A recent OECD guidance document for read-across[16] emphasises the need for a mechanistic basis for grouping chemicals into a category and suggests categorisation criteria.

OECD QSAR Applications Toolbox

The above approaches to prioritizing chemicals for testing have been incorporated in a software package and released by the OECD as the (Q)SAR Applications Toolbox (www.oecd.org/document/54/0,3343,en_2649_34379_42923638_1_1_1_1,00.html). The objective of the toolbox is to provide a means for making QSAR technology readily accessible, transparent and less demanding in terms of infrastructure costs. The first version of the software was released in March 2008, with an updated version released in December 2008. The toolbox presents the process of in silico prediction of chemical toxicity as a logical workflow comprising six sequential steps: (a) chemical input; (b) chemical profiling; (c) selection of SIDS (Screening Information Data Sets; www.librariesanddirectories.com/index.php?page = resources & t = 2 & rid = rm6435) toxicity endpoints; (d) categorization of chemicals; (e) data gap filling; and (f) use of templates for reporting.[93]

Intelligent Testing Strategies

REACH requires much toxicity testing, the extent of which is difficult to predict due to lack of information on: (a) the number of data gaps; and (b) the consequences of testing for further hazard assessment. The use of in silico methods in REACH has been reviewed.[94] Risk assessment requires both safety and exposure information.[95] For logistical and animal welfare reasons, exposure-based intelligent integrated testing strategies, involving pre-existing information, in silico modeling and read-across to prioritize chemicals for further testing, as well as in vitro and in vivo animal tests, are needed (Fig. 3).

DISCUSSION

The advantages and disadvantages of using in silico models are summarized in Table 2. In silico models crucially depend on the quality of the information used to develop and validate them. However, although this is acknowledged to be true, the issue is easy to ignore and, therefore, universally-agreed criteria for quality control (QC) for using data are urgently needed. As a minimum requirement, quantitative data from published original papers involving chemicals of stated high purity should be used. This would help to improve the quality of the biological information and reduce the chance that toxicity, or lack of activity, is due to the presence of an impurity.

Computational modelers and expert toxicologists must work together to develop new models. This is because it is rare for any one individual to have sufficient knowledge about a chemical, statistics, modeling and toxicology. In addition, training sets that comprise a high proportion of chemicals with positive toxicity are often used, resulting in complex data interpretation issues. Sensitivity of the model (ability to correctly predict positive toxicity) can then be much higher than specificity (ability to correctly predict lack of toxicity).[96] Collaboration between several groups of modelers can also be advantageous. This process has been called combinatorial modeling by Zhu et al[57] who participated in a large study which involved developing 15 different QSAR models for toxicity to *Tetrahymena pyriformis.* The data were used to develop a consensus model with a predictivity and an AD that exceeded each of the individual models.

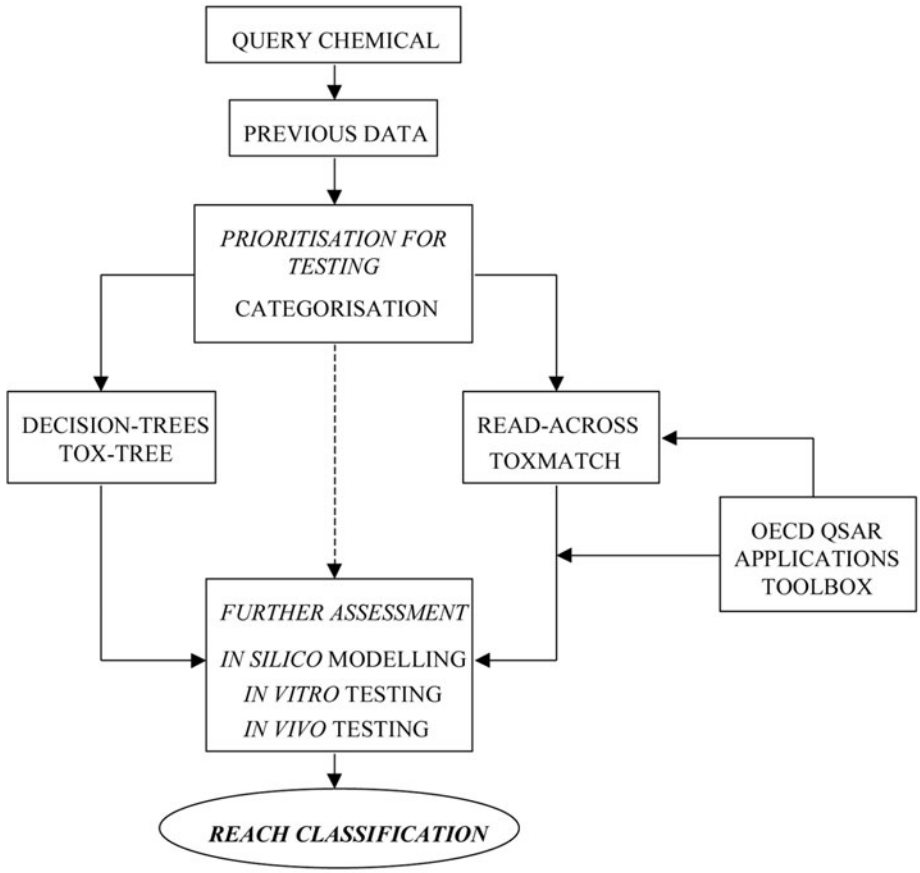

Figure 3. Integrated scheme for use of Toxmatch, Toxtree and the OECD QSAR Applications Toolbox for chemicals testing under REACH. Assessment can be terminated at any stage if a classification is possible and the decision-tree and read-across stages can be ignored (indicated by the dashed line) depending on indications from any prior information available; further assessment can be delayed if a chemical is assigned a low priority.

While in silico toxicity prediction methods have been greatly improved since the publication of the report of the Second FRAME Toxicity Committee, it is clear that they have not become widely accepted by toxicologists and regulatory agencies for purposes other than for very early screening. There are three main problems with using in silico modeling. One is as an over-reliance on complex methods of analysis and the second is the possibility that subtle changes in molecular structure have dramatic effects on activity (rather confusingly referred to as the presence of 'activity cliffs' by Maggiore[97]). The third reason is that correlations may not be mechanistically relevant. These issues have also been discussed by Dowyeko[5] and Johnson,[98] who warn against the indiscriminate use of molecular descriptors, which can be generated rapidly and in large numbers. Descriptors can be shown to be highly correlated with biological activity without a mechanistic relationship existing between the two. Such a correlation can

Table 2. Some advantages and disadvantages of in silico methods for toxicity prediction

Advantages	Disadvantages
Relatively inexpensive	Crucially dependent on pre-existing data
Can be developed rapidly and can readily be updated and improved (though this can encourage the inappropriate use of data)	Data have not always been obtained from properly conducted studies with test samples of high purity (no data-no model; poor data-poor model)
Few problems with reproducibility and interlaboratory transferability)	Some models have been developed with information that is not in the public domain and that cannot be verified
Expensive laboratory equipment and facilities are not required	Training sets of chemicals are often biased toward toxic chemicals since negative data are difficult to interpret and/or to obtain
Can readily be used for high-throughput screening and for testing large numbers of chemicals	Models often have limited applicability domains and there is a lack of consensus on how such domains should be defined
Very useful for compound prioritization	Involve the use of complex terminology as well as mathematical and statistical procedures that are not readily understood by non-experts
Can be incorporated into decision-trees and expert systems with the capability of predicting a wide range of endpoints and properties, including bioavailability, biodegradability and toxicity	The validation of models is controversial and no models have been validated to internationally-agreed criteria in a fully independent and transparent way
Usually based on a mechanism of action related to a toxicity endpoint	It is difficult to find suitable sets of test chemicals for external validation, since new chemicals are easily and continually being added to the training set iteratively, in order to refine the model
Readily amenable to being incorporated into test batteries comprising models with complementary and overlapping applicability domains	Modelling the effects of mixtures of chemicals (highly relevant with respect to environmental hazard) is extremely complicated

lead to a model with low predictivity for novel chemicals that are seemingly within its AD. Of course, this situation is exacerbated further by the occurrence of 'activity cliffs'. The combined use of models that have been developed by using different algorithms and molecular descriptors for the same toxicity endpoint should help to alleviate this problem. Such an approach was adopted by Mathews et al[69] who used four different ARIs (MultiCASE, MDL-QSAR, BioEpisteme and Leadscope PDM). These software systems use different QSAR models developed for predicting rodent

carcinogenicity, based on similar biological data. It was found that the four models were complementary, each detecting different profiles of carcinogens. Accepting the combined positive predictions from any two of the programs showed better overall performance than when any program was used alone.

CONCLUSION

During the development of in silico methods for toxicity prediction, modelers should: (a) use toxicity data from primary literature sources; (b) provide full details of training and test sets of chemicals; (c) validate their models externally as well as internally; (d) not accept a correlation between toxicity and the presence/absence of molecular descriptors without a mechanistic basis; and (e) seek the advice of expert physical chemists and toxicologists, as necessary.

The fact that in silico models, unlike other types of toxicity tests, are based *solely* on correlations creates the serious dilemma that the causes of poor predictivity, due to lack of causal relationships between descriptors and activity, as well as the existence of 'activity cliffs', are only going to be apparent *after* the QSAR model has been refined to cater for a chemical with an 'activity cliff'—i.e., after such a chemical has been used to further train the model. However, it is of no use for a toxicity model to be able to only predict the activities of the chemicals that were used to train it.

Resolution of the above dilemma might have to wait until more data become available on mechanisms of toxicity and how they are caused by the many different physicochemical properties of molecules. Such information will also help in the development of models for a wider range toxicity endpoints. In the meantime, in silico models for toxicity prediction should be applied judiciously, with the focus on compound prioritization and early screening.

REFERENCES

Since the completion of the manuscript, the use of in silico modeling for toxicity prediction was discussed in a series of papers published in Alternatives to Laboratory Animals – ATLA 2009 37 (issue 5); also, several papers have appeared showing how structure-activity landscapes can be mapped (discussed in Combes RD. Is computational toxicology withering on the vine? Archives of Toxicology 2010; 84:333-336; and also in Combes, RD. Challenges for computational structure-activity modelling for predicting chemical toxicity: future improvements? Expert Opinion on Drug Metabolism and Toxicity 2011; 7:1129-40).

1. Barratt MD. Prediction of toxicity from chemical structure. Cell Biol Toxicol 2008; 16:1-13.
2. Judson PN, Combes RD. Artificial intelligence systems for predicting toxicity. Pesticide Outlook 1996; 7:11-15.
3. Combes R, Balls M, Bansil L et al. An assessment of progress in the use of alternatives in toxicity testing since the publication of the report of the Second FRAME Toxicity Committee (1991). Altern Lab Anim—ATLA 2002; 30(4):365-406.
4. Tichý M Experimental toxicology in silico. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2005; 149:217-219.
5. Doweyko AM. QSAR: dead or alive? J Comput Aid Mol Des 2008; 22:81-89.
6. Free SM, Wilson JW. A mathematical contribution to structure-activity studies. J Med Chem 1964; 7:395-399.

7. Hansch C. Quantitative approach to biochemical structure-activity relationships. Accounts Chem Res 1969; 2:232-239.
8. Hansch C, Fujita T. ρ, -σ, -π analysis. A method for the correlation of biological activity and chemical structure. J Am Chem Soc 1964; 86:1616-1626.
9. Toropov AA, Benfenati E. SMILES in QSPR/QSAR modeling: results and perspectives. Curr Drug Disc Technol 2007; 4:77-116.
10. Hansch C. Quantitative structure-activity relationships and the unnamed science. Accounts Chem Res 1993; 26:147-153.
11. Todeschini R, Consonni V. Handbook of Molecular Descriptors. New York: J Wiley, 2000:667.
12. Helguera AM, Cordeiro MNDS, González MP et al. Applications of 2D descriptors in drug design: A DRAGON Tale. Curr Top Med Chem 2008; 8:1628-1655.
13. Lipinski CA, Lombardo F, Dominy BW et al. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliver Rev 1997; 23:3-25.
14. Ghose AK, Viswanadhan VN, Wendoloski JJ. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery, J Comb Chem 1999; 1:55-68.
15. Smyth MS, Martin JHJ. X-Ray crystallography. J Clin Pathol-Mol Pa 2000; 53:8-4.
16. Rathi LG, Kashaw SK, Agrawal RK et al. Comparative molecular field analysis (COMFA): a modern approach towards drug design. Indian J Pharmacol Sci 2001; 63:367-370.
17. Livingstone DJ. Building QSAR models: a practical guide. In: Cronin M, Livingstone D, eds. Predicting Chemical Toxicity and Fate. Boca Raton: CRC Press, 2004:151-170.
18. Zhang Z, Niu J, Zhi X. A QSAR model for predicting mutagenicity of nitronaphthalenes and methylnitronaphthalenes. B Environ Contam Tox 2008; 81:498-502.
19. Contrera JF, Matthews EJ, Kruhlak NL et al. In silico screening of chemicals for genetic toxicity using MDL-QSAR, nonparametric discriminant analysis, E-state, connectivity and molecular property descriptors. Toxicol Mech Meth 2008; 18:207-216.
20. Dearden JC. In silico prediction of aqueous solubility. Exp Opin Drug Dis 2006; 1:31-52.
21. Delaney JS. Predicting aqueous solubility from structure. Drug Discov Today 2004; 10:289-295.
22. Dorn SB, Degen GH, Bolt HM et al. Some molecular descriptors for nonspecific chromosomal genotoxicity based on hydrophobic interactions Arch Toxicol 2008; 82:333-338.
23. Duchowicz PR, Ocsachoque MA. Quantitative structure-toxicity models for heterogeneous aliphatic compounds. QSAR Comb Sci 2009; 28:281-295.
24. Wold S, Albano C, Dunn WJ III et al. Modeling data tables by principal components and PLS: class patterns and quantitative predictive relations. Analysis 1984; 12:477-485.
25. Benigni R, Guiliani A. Multivariate analyses in genetic toxicology. In: Devillers J, Karcher W, eds. Applied Multivariate Analysis. SAR and Environmental Studies. Dordecht: Kluwer Academic Publishers, 1991:347-375.
26. Eriksson L, Jaworska J, Worth AP et al. Methods for reliability and uncertainty assessment and for applicability evaluations of classification- and regression-based QSARs—Mini-Monograph. Environ Health Persp 2003; 111:1361-1375.
27. Maran U, Sild S. QSAR modeling of mutagenicity of noncongeneric sets of organic compounds. In: Dubitzky W, Azuaje F, eds. Artificial Intelligence Methods and Tools for Systems Biology. Dordecht: Springer, 2004:19-35.
28. Votano JR, Parham M, Hall LM et al. QSAR modeling of human serum protein binding with several modeling techniques utilizing structure-information representation. J Med Chem 2006; 49:7169 -7181.
29. Dearden JC, Barratt MD, Benigni R et al. The development and validation of expert systems for predicting toxicity. The report and recommendations of an ECVAM/ECB workshop (ECVAM workshop 24). Altern Lab Anim—ATLA 1997; 25(3):223-252.
30. Combes RD, Rodford R. The use of expert systems for toxicity prediction—illustrated with reference to the DEREK program. In: Cronin M, Livingstone D, eds. Predicting Chemical Toxicity and Fate. Boca Raton: CRC Press, 2004:193-229.
31. Cronin MTD, Dearden JC. QSAR in toxicology. 4. Prediction of nonlethal mammalian toxicological endpoints and expert systems for toxicity prediction. QSARs 1995; 14: 518-523.
32. Sanderson D M, Earnshaw C G, Judson PN. Computer prediction of possible toxic action from chemical structure; the DEREK System. Hum Exp Toxicol 1991; 10:261-273.
33. Greene N. Computer prediction systems for toxicity; an update. Adv Drug Deliver Rev 2002; 54:417-431.
34. Coecke S, Ahr H, Blaauboer BJ et al. Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM Workshop 54. Altern Lab Anim—ATLA 2006; 34(1):49-84.
35. Saiakhov RD, Klopman G. MultiCASE expert systems and the REACH Initiative. Toxicol Mech Method 2008; 18:159-175.

36. Gombar VK, Enslein K, Hart JB et al. Estimation of maximum tolerated dose for long-term bioassays from acute lethal dose and structure by QSAR. Risk Anal 1991; 11:509-517.
37. Patlewicz G, Dimitrov SD, Low LK et al. TIMES-SS- A promising tool for the assessment of skin sensitization hazard. A characterization with respect to the OECD validation principles for (Q)SARs and an external evaluation for predictivity. Regul Toxicol Pharm 2007; 47:225-239.
38. Lewis DVF. Computer-assisted methods in the evaluation of chemical toxicity. In: Lipkowitz KB, Boyd DB, eds. Reviews in Computational Chemistry, Vol 3. NewYork: VCH Publishers, 1992:173-222.
39. Mekenyan O, Dimitrov S, Schmeider P et al. In silico modeling of hazard endpoints: current problems and perspectives. SAR and QSAR Environ Res 2003; 14:361-371.
40. Jacob A, Pratuangdejkul J, Buffet S et al. In silico platform for xenobiotics ADME-T pharmacological properties modeling and prediction. Part II: the body in a Hilbertian space. Drug Discov Today 2009; 14:406-441.
41. Boobis A, Gudert-Remy U, Kremers P et al. In silico prediction of ADME and pharmacokinetics: report of an expert meeting organised by COST B 15. J Pharm Sci 2002; 17:183-193.
42. Payne MP. Computer-based methods for the prediction of chemical metabolism and biotransformation within biological organisms. In: Cronin M, Livingstone D, eds. Predicting Chemical Toxicity and Fate. Boca Raton: CRC Press, 2004:205-227.
43. Zvinavashe E, Murk AJ, Rietjens IMCM. Promises and pitfalls of quantitative structure-activity relationship approaches for predicting metabolism and toxicity. Chem Res Toxicol 2008; 21:2229-2236.
44. Weaver S, Gleeson MP. The importance of the domain of applicability in QSAR modeling. J Mol Graphics Model 2008; 26:1315-1326.
45. Netzeva TI, Worth AP, Aldenberg T et al. Current status of methods for defining the applicability domain of (Quantitative) Structure–Activity Relationships. The report and recommendations of ECVAM Workshop 52. Altern Lab Anim—ATLA 2005; 33:155-173.
46. Mombelli E. An evaluation of the predictive ability of the QSAR software packages, DEREK, HAZARDEXPERT and TOPKAT, to describe chemically-induced skin irritation. Altern Lab Anim—ATLA. 2008, 36:15-24.
47. Wold S. Validation of QSAR's. Quant Struct-Act Rel 2001; 10:191-193.
48. Tichy M, Rucki M. Validation of QSAR models for legislative purposes. Interdiscip Toxicol 2009; 2(3):184-186.
49. Bolboacă SD, Jäntschi L. Modeling the property of compounds from structure: statistical methods for models validation. Environ Chem Lett 2008; 6:175-181.
50. Loonen H, Lindgren F, Hansen B et al. Prediction of biodegradability from chemical structure: modeling of ready biodegradation test data. Environ Toxicol Chem 1999; 18:1763-1768.
51. Gramatica P. Evaluation of different statistical approaches to the validation of Quantitative Structure-Activity Relationships. Ispra: ECVAM, 2004:177. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/information-sources/qsar-document-area/Report_on_QSAR_validation_methods.pdf.
52. Combes R, Balls M. Intelligent testing strategies for chemicals testing—A case of more haste, less speed? Altern Lab Anim—ATLA 2005; 33(3):289-297.
53. Tropsha A, Gramatica P, Gombar VK. The importance of being earnest: validation is the absolute essential for successful application and interpretation of QSPR Models. QSAR Comb Sci 2003; 22:69-77.
54. Roy PP, Leonard JT, Roy K. Exploring the impact of size of training sets for the development of predictive QSAR models. Chemometr Intell Lab Systems 2008; 31-42.
55. Massarelli I, Imbriani M, Coi A et al. Development of QSAR models for predicting hepatocarcinogenic toxicity of chemicals. Eur J Med Chem 2009; 44:1-7.
56. Parry JM. Detecting and predicting the activity of rodent carcinogens. Mutagenesis 1994; 9:3-5.
57. Zhu H, Tropsha A, Fourches D et al. Combinatorial QSAR modeling of chemical toxicants tested against Tetrahymena pyriformis. J Chem Inf Model 2008; 48:766-784.
58. Worth AP, Cronin MTD. Report of the workshop on the validation of QSARs and other computational prediction models. Altern Lab Anim—ATLA 2004; 32(Suppl 1):703-706.
59. Zvinavashe E, Murk AJ, Rietjens IMCM. Promises and pitfalls of quantitative structure-activity relationship approaches for predicting metabolism and toxicity. Chem Res Toxicol 2008; 21:2229-2236.
60. Anon. The Report from the Expert Group on (Quantitative) Structure-Activity Relationships on the Principles for the Validation of (Q)SARs. OECD Series on Testing and Assessment Number 49. Paris: Organisation of Economic Co-operation and Development, 2001:206.
61. Mekenyan O, Dimitrov S, Schmeider P et al. In silico modeling of hazard endpoints: current problems and perspectives. SAR and QSAR in Environ Res 2003; 14:361-371.
62. Bhogal N, Combes R, Balls M. Preclinical drug development planning. In: Gad S, ed. Preclinical Development Handbook: Toxicology. Hoboken: John Wiley & Sons, 2008:1-63.
63. Kapetanovic IM. Computer-aided drug discovery and development (CADDD): In silico chemico biological approach. Chem-Biol Interacts 2008; 171:165-176.

64. Muster W, Breidenbach A, Fischer H et al. Computational toxicology in drug development. Drug Discov Today 2008; 13:303-310.
65. Thaia K-M, Ecker GF. A binary QSAR model for classification of hERG potassium channel blockers. Bioorg Med Chem 2008; 16:4107-4119.
66. Gibson L, Naven RT, Patel ML et al. Recent advances in the prediction of HERG channel inhibition using Derek for Windows. Poster presented at the International Congress of Toxicology (ICT). 2007, Montreal.
67. Grindon C, Combes R, Cronin MT et al. Integrated decision-tree testing strategies for environmental toxicity with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2006; 34:651-664.
68. Contrera JF, Matthews EJ, Kruhlak NL et al. In silico screening of chemicals for genetic toxicity using MDL-QSAR, nonparametric discriminant analysis, E-state, connectivity and molecular property descriptors. Toxicol Mech Method 2008; 18:207-216.
69. Matthews EJ, Kruhlak NL, Benz RD et al. Combined Use of MC4PC, MDL-QSAR, BioEpisteme, Leadscope PDM and Derek for Windows software to achieve high-performance, high-confidence, mode of action–based predictions of chemical carcinogenesis in rodents. Toxicol Mech Method 2008; 18:189-206.
70. Benigni R, Worth A, Netzeva T et al. Structural motifs modulating the carcinogenic risk of aromatic amines. Envir Mol Mutagen 2009; 50:152-161.
71. Combes R, Grindon C, Cronin MT et al. Integrated decision-tree testing strategies for mutagenicity and carcinogenicity with respect to the requirements of the EU REACH legislation. Alterm Lab Anim—ATLA 2008; 36:Suppl 1:65-74.
72. Grindon C, Combes R, Cronin MTD et al. Integrated decision-tree testing strategy for skin sensitisation with respect to the requirements of the EU REACH Legislation. Altern Lab Anim—ATLA 2007; 35:683-697.
73. Fabjana E, Hulzebos E. An evaluation of selected valid and mechanistically based SARs for skin sensitisation. Toxicol In Vitro 2008; 22:468-490.
74. Liu H, Papa E, Walker JD et al. In silico screening of estrogen-like chemicals based on different nonlinear classification models. J Mol Graph Model 2007; 26:135-144.
75. Gallegos-Salinera A, Poater A, Jeliazkovad N et al. Toxmatch — A chemical classification and activity prediction tool based on similarity measures. Reg Toxicol Pharmacol 2008; 52:77-84.
76. Tsakovska I, Lessigiarska I, Netzeva T et al. A mini review of mammalian toxicity (Q)SAR models. QSAR Comb.Sci 2008; 27:41-48.
77. Grindon C, Combes R, Cronin MTD et al. An integrated decision-tree testing strategy for repeat dose toxicity with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36:93-101.
78. BéruBé K, Aufderheide M, Breheny D et al. In vitro models of inhalation toxicity and disease. The report of a FRAME workshop. Altern Lab Anim—ATLA 2009; 37:89-141.
79. Combes R, Dandrea J, Balls M. A critical assessment of the European Commission's proposals for the risk assessment and registration of chemical substances in the European Union. Altern Lab Anim—ATLA 2003; 31:353-364.
80. Grindon C. The new EU REACH regulation has finally been adopted: Is this the end of the campaign trail… or just the beginning? Altern Lab Anim—ATLA 2007; 35:239-242.
81. Combes R, Barratt M, Balls M. An overall strategy for the testing of chemicals for human hazard and risk assessment under the EU REACH system. Altern Lab Anim—ATLA 2003; 31(1):7-19.
82. van Leeuwen CJ, Patlewicz GY, Worth AP. Intelligent testing strategies. In: van Leeuwen CJ, Vermiere TG, eds. Risk Assessment of Chemicals, An Introduction, 2nd ed. Dordrecht: Springer, 2007:467-509.
83. Zachary M, Greenway GM. Comparative PBT screening using (Q)SAR tools within REACH legislation. SAR QSAR Environ Res 2009; 20:145-157.
84. Reuschenbach P, Silvani M, Dammann M et al. ECOSAR model performance with a large test set of industrial chemicals. Chemosphere 2008; 71:1986-95, 200.
85. Zvinavashe E, Murk AJ, Rietjens IMCM. On the number of EINECS compounds that can be covered by (Q)SAR models for acute toxicity. Toxicol Letts 2008; 184:67-72.
86. Cramer GM, Ford RA, Hall RL. Estimation of toxic hazard: a decision-tree approach. Food Cosmet Toxicol 1978; 16:255-276.
87. Verhaar HJM, Solbe J, Speksnijder J et al. Classifying environmental pollutants: Part 3. External validation of the classification system. Chemosphere 2000; 40:875-883.
88. Gerner I, Graetschel G, Kahl J et al. Development of a decision support system for the introduction of alternative methods into local irritancy/corrosivity testing strategies. Development of a relational database. Altern Lab Anim—ATLA 2000; 28:11-28.
89. Benigni R, Bossa C. Structure alerts for carcinogenicity and the Salmonella assay system: A novel insight through the chemical relational databases technology. Mutat Res 2008; 659:248-261.
90. Hong H, Tong W, Fang H et al. Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of tree-based model with structural alerts. Environ Health Persp 2002; 110:29-36.

91. Barratt MD. Principles of toxicity prediction from structure. In: Balls M, van Zeller AM, Halder ME, eds. Progress in Reduction, Refinement and Replacement in Animal Experimentation. Amsterdam: Elsevier, 2002, 449-456.
92. Anon. Guidance on Grouping of Chemicals. OECD Environment Health and Safety Publications series on testing and assessment Number 80. Paris: Organisation for Economic Cooperation and Development, 2007:99.
93. Matthews EJ, Contrera JF. In silico approaches to explore toxicity end points: issues and concerns for estimating human health effects. Expert Opin Drug Metab Toxicol 2007; 3:125-134.
94. Anon. Technical Guidance Document to Industry on the Information Requirements for REACH PART 1 (of 4). General Issues REACH Implementation Project (RIP) 3.3 Phase 2. Helsinki: European Chemical Agency, 2007.
95. Combes R, Balls M, Illing P et al. Possibilities for a new approach to chemicals risk assessment—the report of a FRAME workshop. Altern Lab Anim—ATLA 2006; 34:621-649.
96. Helguera AM, Pérez MAC, Combes R et al. The prediction of carcinogenicity from molecular structure. Curr Comp-Aided Drug Des 2005; 1:65-72.
97. Maggiora GM. On outliers and activity cliffs—why QSAR often disappoints. J Chem Inf Model 2006; 46:1535-1535.
98. Johnson SR. The trouble with QSAR (or how I learned to stop worrying and embrace fallacy). J Chem Inf Model 2008; 48:25-26.

REFERENCES IN BOXES

1. Golbraikh A, Tropsha A. Beware of q^2! J Mol Graphic Model 2002; 20:269-276.
2. Anon. What are cross-validation and bootstrapping? 2007. Available at: http://www.faqs.org/faqs/ai-faq/neural-nets/part3/section-12.html.
3. Anon. Sarchitect: Theory—Statistical Methods. 2006. Available at: http://www.strandls.com/sarchitect/documents/manual_html/stattheory.html.
4. Anon. Sarchitect: Building Models in Sarchitect. 2006. Available at: http://www.strandls.com/sarchitect/documents/manual_html/modelbuild.html.
5. Efron B. Estimating the error rate of a prediction rule: Improvement on cross-validation. J Am Stat Assoc 1983; 78:316-331.
6. Grammatica P. Different approaches to QSAR models' validation. 2007. Available at: http://echeminfo.colayer.net/html/abstracts/paolaabs.htm.
7. Faber NM, Rajkó R. An evergreen problem in multivariate calibration. Spectroscopy Europe 2006; 18:24-28.
8. Toropov AA, Rasulev BF, Leszczynski J. QSAR modeling of acute toxicity by balance of correlations. Bioorg Med Chem 2008; 16:5999-6008.
9. Porcelli C, Roncaglioni A, Chana A et al. A comparison of DEMETRA individual QSARs with an index for evaluation of uncertainty. Chemosphere 2008; 71:1845-1852.
10. Enoch SJ, Cronin MTD, Madden JC et al. Formation of structural categories to allow for read-across for teratogenicity. QSAR Comb Sci 2009; 00:1-13.
11. Patlewicz G, Jeliazkova N, Gallegos-Salinera A et al. Toxmatch—a new software tool to aid in the development and evaluation of chemically similar groups. SAR and QSAR Environ Res 2008; 19:397-412.
12. Jaworska J, Nikolova-Jeliazkova N. How can structural similarity analysis help in category formation? SAR QSAR Environ Res 2007; 18:195-207.
13. Gallegos-Salinera A, Poater A, Jeliazkovad N et al. Toxmatch — A chemical classification and activity prediction tool based on similarity measures. Regul Toxicol Pharm 2008; 52:77-84.
14. Thatcher M, Payne G. Moving Towards Substance-based Toxicity Testing to Meet New OSPAR Requirements. Aberdeen: European Oilfield Speciality Chemicals Association, 2003: Available at: www.eosca.com/Docs/RSC%20CIO8.pdf.
15. Combes R, Balls M. Intelligent testing strategies for chemicals testing—A case of more haste, less speed? Altern Lab Anim—ATLA 2005; 33(3):289-297.
16. Anon. Guidance on Grouping of Chemicals. OECD Environment Health and Safety. Publications Series on Testing and Assessment Number 80. Paris: Organisation for Economic Cooperation and Development, 2007:99.

CHAPTER 8

LUMINESCENT QUANTUM DOTS FOR MOLECULAR TOXICOLOGY

Shivang R. Dave,[1] Collin C. White,[2] Terrance J. Kavanagh*,[2] and Xiaohu Gao*,[1]

[1]Department of Bioengineering, University of Washington, Seattle, Washington USA; [2]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, Washington, USA
**Corresponding Authors: Xiaohu Gao—Email: xgao@u.washington;*
and Terry Kavanagh—Email: tjkav@u.washington.edu

Abstract: Recent developments in nanotechnology have made available a host of new approaches for the improved quantitative detection of biomarkers due to the enhanced sensitivity of nanoparticle-based assays. The majority of molecular toxicology studies revolve around sensitive measurement of cell-death (apoptosis) and cell-health biomarkers present in living cells or formalin-fixed and paraffin embedded (FFPE) tissue samples. In this regard, semi-conductor quantum dots (QDs) which exhibit high brightness, photo-stability and degree of multiplexing, are predicted to have a significant impact on research in molecular toxicology. Due to these superior photophysical properties of QDs as compared to traditional fluorophores and the unsurpassed versatility of QDs as enabling components for new assays, these nanoparticles promise to facilitate new discoveries in molecular toxicology. Indeed, multiplexed QD-based assays have been incorporated into cell imaging, flow cytometry and other homogenized sample-based assays for detecting multiple biomarkers including those associated with cell injury and apoptosis.

INTRODUCTION

The field of molecular toxicology focuses on the application of technology to the discipline of molecular and cellular biology, in order to understand how the harmful effects of drugs, chemicals and toxins are manifested at the molecular, biochemical and cellular levels. This type of detailed information not only falls within the general scope of toxicology, but also aids in the development of more-sensitive and more-accurate assays

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

to detect and determine the harmful effects of toxicants. Molecular toxicology employs a variety of modern technologies, the detection sensitivity and high-throughput of which permit the more accurate determination of factors that are fundamental to toxicology, including dose—response relationships and risk estimation. Molecular toxicologists also strive to elucidate the relationship between the molecular effects of toxicants and the development of disease. Cancer, for instance, has been linked to the mutagenic effects of a variety of chemicals and the disease has its origins at the molecular level in the loss of tumor suppressors, the activation of oncogenes and the dysregulation of cell cycle regulating factors such as checkpoint proteins. Studies in molecular toxicology rely heavily on fluorescence techniques, such as dye-labeled biomolecules (peptides, oligonucleotides, antibodies), for the investigation of molecular events. Unfortunately, the traditional organic dyes exhibit optical properties that are unfavorable for the multiplexed and sensitive detection of features such as broad emission profiles, lack of photostability and small Stokes shifts.

In this chapter, we will briefly discuss the prominent biomarkers and research techniques employed in molecular toxicology and then focus on recent advancements in nanotechnology, with particular reference to fluorescent quantum dots, which not only promise to improve detection sensitivity and throughput, but also permit discoveries useful to molecular toxicology.

BIOMARKERS RELEVANT TO MOLECULAR TOXICOLOGY

The key to understanding the nature of chemically-induced cytotoxicity is to properly distinguish between the two prominent modes of cell death, necrosis and apoptosis. Necrosis is "accidental" cell death, often caused by external injury or trauma. Apoptosis, on the other hand, is commonly referred to as "programmed" cell death or cell suicide, resulting from the activation of a complex sequence of events within the cell.[1] Regardless of the mode of death, changes in cellular structure, morphology and biochemistry occur rapidly in dying cells.[2] As necrosis and apoptosis involve some distinct characteristic biochemical and morphological events at early and late stages of cell death, such biomarkers can aid in accurately distinguishing the mode of cell death. Furthermore, the type of toxicant can affect the onset and rate of apoptosis.[3] A commonly employed assay for cytotoxicity is to qualitatively assess the permeability of the plasma membrane or mitochondrial membrane, as an indicator of membrane integrity. Information regarding membrane integrity is useful in indicating cytotoxicity, but the general nature of the assay precludes precise determination of the mode of cell death. For example, during apoptosis, the cellular membrane does not lose integrity early in the process. This demonstrates that generalized assays that function on the basis of the uptake of vital dyes as an indicator of membrane integrity, although useful, may be deficient, because of the time required for cells to undergo apoptosis. Whether a cell undergoes necrosis or apoptosis depends on the nature of the toxic agent. A number of distinct biomarkers which show high specificity for changes associated with cell death, can be used to distinguish between necrosis and apoptosis and thus can result in better understanding of the molecular and cellular effects of a given toxin.

REGULATORY CELL DEATH GENES AND APOPTOTIC BIOMARKERS

The roles of oncogenes, tumor-suppressor genes and their protein products in cell death modulation have been well documented, in addition to those of stress and acute phase proteins.[2] These genes and proteins can have positive or negative impacts on apoptosis and necrosis and can be used to distinguish between the two modes of cell death.

Myc gene family members, including the closely related *c-myc*, *v-myc*, *n-myc* and *b-myc* genes, are known to be highly active in the regulation of cell proliferation.[4] *C-myc*, in particular, is a short-lived nuclear protein the over expression of which has been demonstrated to induce apoptosis, which can be blocked in T-cell hybridomas by the administration of a *c-myc* antisense oligonucleotide.[5] The p53 gene is the most widely known tumor-suppressor gene.[6] Its fundamental role in tumor-suppression is demonstrated by the loss of p53 function in over half of all human tumors. At the cellular level, the p53 gene maintains genomic stability through mediating the response to DNA damage, including the suppression of cell growth via the regulation of cell-cycle arrest and the induction of apoptosis.[7] The members of the *Bcl-2* gene family are also important regulators of apoptosis. Some family members (including *Bcl-2*) function as apoptosis inhibitors, whereas other members, such as *Bax*, accelerate the rate of apoptosis.[8-10] Bcl-2 proteins are associated with and are located on or near, mitochondria, the endoplasmic reticulum and nuclear membranes.[11] Ras protein family members are essential for controlling normal cellular growth and transformation and play an important role in preventing apoptosis.[12-15] Many receptor-ligand signaling pathways, such as epidermal-, fibroblast- and nerve growth factors, in addition to T-cell receptor CD3 and CD95 complexes, converge on Ras, illustrating its key role in transmembrane signaling. Heat-shock proteins can protect cells from a number of stressors, via their ability to act as chaperones for protein refolding, and are involved in the ubiquitin system of protein disposal and have been implicated in resistance to anticancer drugs.[16] Many toxicants are known to induce oxidative and or nitrosative stress and antioxidant genes (such as those coding for superoxide dismutases, catalase, glutathione peroxidases, glutathione S-transferases, peroxiredoxins, thioredoxin/thioredoxin reductases, glutamate cysteine ligase and glutathione synthetase) are known to be important in cellular defenses against such toxicants. Mitogen/stress activated protein kinases (MAPK/SAPK), such as p38, Erk and JNK, have also been implicated in cell survival and cell death in a large number of studies.

The onset and progression through apoptosis are associated with enzymatic activation and alteration in the distribution of biomarkers. Caspases are proteolytic proteins that are transcribed and/or activated from their proenzyme state during apoptosis and are responsible for a variety of changes, such as the degradation of the cytoskeleton and other macromolecular structures and the activation of nucleases responsible for DNA cleavage, which result in the morphological changes observed in apoptosis.[17-19] Various assays employ dyes which, when cleaved by a caspase, result in a colorimetric or fluorescent signal. Phosphatidylserine (PS) is a plasma membrane phospholipid that is normally asymmetrically distributed, being found only in the inner leaflet of the plasma membrane. However, during apoptosis, this phospholipid asymmetry is lost, resulting in the externalization of PS to the outer leaflet of the plasma membrane.[20] Annexin-V, a small 35kDa protein, can bind PS in a calcium-dependent manner, making dye-labeled Annexin-V a useful tool for detecting

apoptosis in its early stages.[21-22] The key genetic biomarkers, protein products and their effects on cellular markers, if incorporated into molecular toxicology studies, can provide a wealth of information with regard to mechanisms of toxicant-induced cell death.

FLUORESCENCE-BASED TECHNIQUES IN MOLECULAR TOXICOLOGY

The in vitro assays used to assess the effects of toxicants on cell growth, viability and metabolism, often employ fluorescent probes. The specificity of some probes is achieved by combining a fluorophore with a targeting moiety, such as an antibody, nucleic acid, peptide, or other small molecule. Other probes report on the concentration of cellular ions (e.g., calcium), membrane potential, redox status (lipid peroxidation, thiol status) and cell cycle distribution (DNA stains). The variety of dyes and the assays used to detect and distinguish cellular indicators of cell death are too vast to be considered in detail. Nonetheless, the following four main techniques employed in molecular toxicology studies, all of which typically employ fluorescence as the detection signal, will be discussed.

Fluorescence In Situ Hybridization and Molecular Beacons

Fluorescence in situ hybridization (FISH) is a useful technique for analyzing specific DNA and mRNA sequences within cultured cells or tissue preparations that are frozen and cryostat-sectioned or formalin-fixed and paraffin-embedded (FFPE).[23] The localization and quantitative assessment are performed by using fluorescently labeled oligonucleotide probes. The method is based on the hybridization of the dye-labeled single-stranded DNA or RNA oligonucleotide probe to the target single-stranded DNA or RNA sequence. The high sensitivity of this technique permits the assessment of transcription/expression of mRNA in response to a stimulus. Such probes are typically 20-50 nucleotides in length; the longer probes having higher sensitivity, while the small probes are easier to synthesize.

A related method is based on molecular beacons, which are single-stranded oligonucleotide probes that contain complementary ends to enable self-hybridization and a hairpin-loop structure. The opposing ends of the strand are conjugated to either a dye or a quencher. In the closed state (unbound to target), the dye fluorescence is suppressed by the quencher in close proximity, through a nonradiative fluorescence resonance energy transfer (FRET) mechanism.[24-25] Upon the binding of a complementary target mRNA, the hairpin loop is linearized and emits fluorescence due to the inactivation of the distance-dependent quenching mechanism. Similar to FISH probes, molecular beacons can be used to quantitatively detect intracellular mRNA sequences. Molecular beacons have the advantage of a lower background signal and have been used with living cells.[26]

Immunohistochemistry and Immunofluorescence

In immunohistochemistry (IHC), immunofluorescence is an important technique, by which proteins (antigens) are localized and visualized from the fluorescence of the appropriate dye-labeled antibodies within a frozen or FFPE tissue section. The antibodies employed can be polyclonal or monoclonal, but monoclonal antibodies tend to exhibit greater specificity. There are two strategies for IHC, direct (one-step) and indirect (two-step). Direct IHC relies on dye-labeled antibodies to detect their corresponding antigens. Although this method is simple and rapid, it suffers from lower sensitivity

arising from a lack of signal amplification. Indirect IHC uses a primary antibody which is specific to the target antigen, followed by the use of the dye-labeled secondary antibody which is reactive towards the species-specific constant region of the primary antibody. This method achieves higher sensitivity and signal amplification. However, it is also limited in its multiplexing ability by the limited species of primary antibody available.

Cell Imaging and Confocal Laser Scanning Microscopy

The advantage of confocal microscopy over normal microscopy is the improved image sharpness, particularly for images taken from thick section of specimens. This is because of the collection of light only from the thin plane of focus and the exclusion of out-of- focus light. It is especially important for collecting fluorescence and trying to resolve fine structures deep within a specimen, since the fluorescence from all planes would otherwise be collected and would obscure the resolution and contrast of the structure. Confocal laser scanning microscopy (CLSM), for example, is one of the most frequently used techniques for the spatial localization and quantification of a distribution of different markers. Through the use of multiple laser lines and filters, it is possible to investigate the distribution of multiple biomarkers through the use of dye-labeled antibody probes, which is extremely useful in histopathology and IHC.

Flow Cytometry/Fluorescence Activated Cell Sorting

Flow cytometry and its more specialized successor, Fluorescent Activated Cell Sorting (FACS), are powerful technologies employed in molecular toxicology. They are able to identify, characterize and separate heterogeneous cell populations based on morphology, by using light scattering and on molecular phenotype, by using fluorophore dye-conjugated targeted probes. Flow cytometry can be used to distinguish between cell characteristics such as cell size and shape, granularity of cytoplasm, membrane potential, intracellular pH and can identify cellular components, including nucleic acid, proteins, enzyme activity and surface receptors.[27] The high-throughput (~5,000 cells/s) and multiparameter (up to 19-17 fluorescent signals, 2 scattering signals) capabilities permit the investigation of cells individually with high sensitivity and precision (Fig. 1).[28] One of the most common uses is the identification and separation of immune cells based on cell surface antigens such as CD4 and CD8.[29] Cells can also be permeabilized, to enable the detection of intracellular biomarkers indicative of cellular changes (such as DNA breakdown) caused by exposure to drugs or toxicants.[30] The ability to study the mechanisms associated with cell death through the correlation of multiple parameters in the same cell, such as the degree of apoptosis and the position in the cell cycle, the state of an organelle, or the analysis of proteins, such as bcl-2, c-myc and p53, demonstrates the powerful use of flow cytometry in molecular toxicology.[31]

DYE-BASED FLUORESCENT PROBES

In the techniques described above, the common factor is the use of fluorophores as reporter molecules. Although the use of fluorescent dye-conjugated probes is standard practice for labeling cellular targets, traditional dyes suffer drawbacks including broad emission profiles and rapid photobleaching. They also often require expensive and

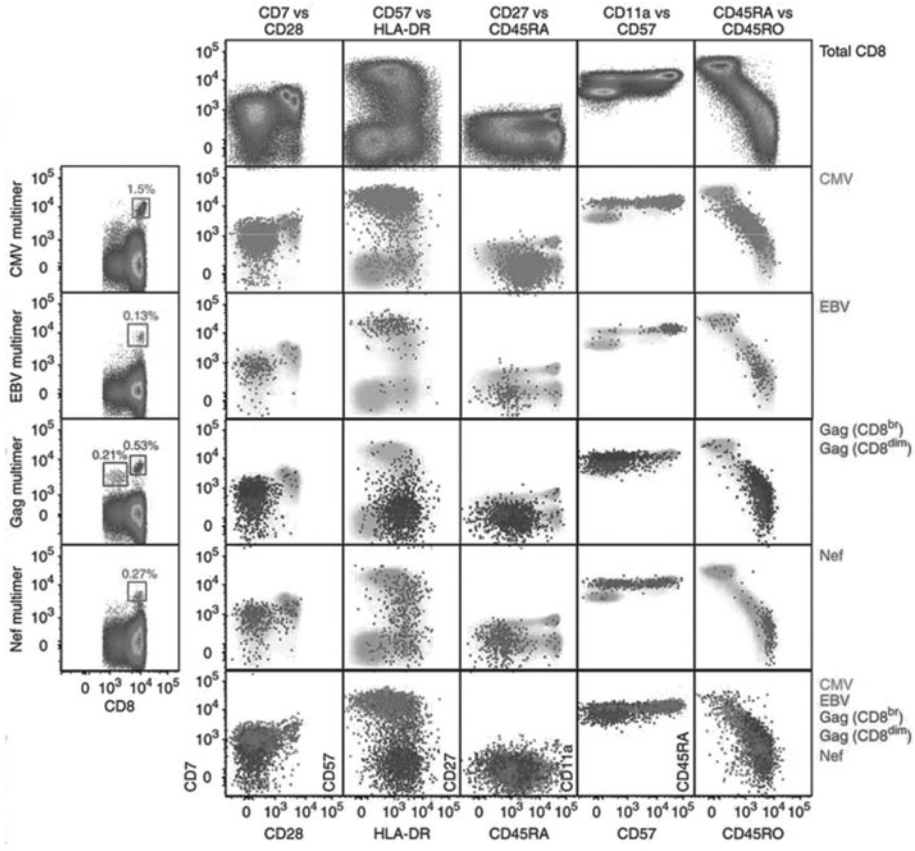

Figure 1. An examples of a 17-color FACS analysis of antigens with QDs. In the top panel, total CD8+ T-cells are distinguished by fluorescence intensity (bright, dim and zero) corresponding to the expression of phenotypic biomarkers (CD7, CD11a, CD27, CD28, CD45RA, CD45RO, CD57, HLA-DR). CD8+ T-cell populations are stained and categorized with QD-pMHCI conjugates specific to peptide epitopes derived from Human Immunodeficiency Virus (Nef and Gag), Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV); total T-cells are shown and percentages indicate sub-populations restricted within CD8+ T-cells (left column). In the remaining rows, for each antigen-specific population (color), phenotypic biomarker expression is compared to expression within the overall CD8+ T-cell population (light grey). The bottom row is an overlay of the staining patterns observed in all the antigen-specific cell populations. The fluorescence intensity for each labeled biomarker is indicated on the axes. This study demonstrates the power of multiparameter technology for studying the heterogeneous protein expression within CD8+ T-cells. Reprinted with permission from Nat Med 2006; 12(8):972-977.[28] ©2006 Macmillan Publishers Ltd. A color version of this image is available at www.landesbioscience.com/curie.

complex experimental set-ups to achieve true multiplexing and fluorescence-based IHC and FISH often suffer from low fluorescence efficiency and the high autofluorescence of FFPE tissue, which results in a low signal-to-noise outcome.[32-33]

In order to further advance the field of molecular toxicology, the detection of multiple signals is necessary to permit better differentiation between necrosis and apoptosis. For instance, simple colorimetric or fluorescent live/dead cell assays often cannot make this distinction, which is of fundamental importance in determining the cause of cell death.

Also, the spectral overlap of dyes prohibits the true multiplexed detection of multiple biomarkers. As a result, multiple assays, each indicating a specific characteristic, need to be employed in sequence, to enable proper conclusions to be drawn. For instance, propidium iodide, an indicator of membrane integrity, that is often compromised in necrosis, and Annexin V-based apoptosis assays, are commonly employed simultaneously to determine the type of cell death. The ability to concurrently probe and read-out multiple biomarkers promises to improve the ability of toxicologists to determine the mechanisms by which toxicants cause cell death.

QUANTUM DOT-BASED FLUORESCENT PROBES

The recent development of semiconductor nanocrystals, also know as quantum dots (QD), has made available a new class of fluorescent labels with unique advantages and applications. In contrast to organic dyes, the fluorescence emission spectra of QDs can be continuously tuned by changing the particle size and a single wavelength can be used for the simultaneous excitation of all the different-sized QDs. Surface-passivated QDs are highly stable against photobleaching and have narrow, symmetric emission peaks. It has been estimated that CdSe QDs are about 20-50 times brighter and thousands of times more stable than single dye molecules.[34] These properties offer new possibilities for the study of molecular targets relevant to molecular toxicology. QD photophysics and fictionalization will be discussed below, followed by a consideration of their applications for biomarker detection in homogeneous solution-based assays and cellular imaging assays.

Unique Photophysical Properties of Quantum Dots

As a consequence of their inorganic semiconductor composition, QDs contain electronic charge carriers—i.e., negatively charged electrons and positively charged holes—which constitute each exciton. Whereas bulk semiconductors exhibit composition-dependent bandgaps (the characteristic minimum energy required to excite an electron from its ground state in the valence band to the conduction band), the band gap in nano-sized semiconductors is determined by both composition and size. The nanometer sizes of QDs confine the excited charge carriers to spaces smaller than their Bohr radii in bulk semiconductors. This "quantum confinement" of the exciton is the principle responsible for the size-dependent optoelectronic properties apparent in QDs.[35-37] Smaller QDs experience a higher degree of confinement, which is associated with an increase in the bandgap energy. The size-dependent tuning of the bandgap results in a tunable QD emission wavelength, such that smaller particles emit at shorter wavelengths than larger particles (Fig. 2). By adjusting their sizes and compositions, QDs can now be prepared to emit fluorescence spanning the ultraviolet, visible and infrared wavelengths (400-4,000 nm).[38-42] The absorption of a photon with energy greater than the bandgap energy of a QD results in the excitation of an electron from valence band to the conduction band. The recombination of the constituent electron and the hole is accompanied by the conversion of the bandgap energy into an emitted photon of fluorescence.

QDs can absorb and emit light very efficiently, which permits highly sensitive detection relative to the use of conventional organic dyes and fluorescent proteins as biological probes. QDs have very large molar extinction coefficients, of the order of 0.5–5 $\times 10^6$ $M^{-1}cm^{-1}$,[43] which is approximately 10-50-times larger than those of organic dyes

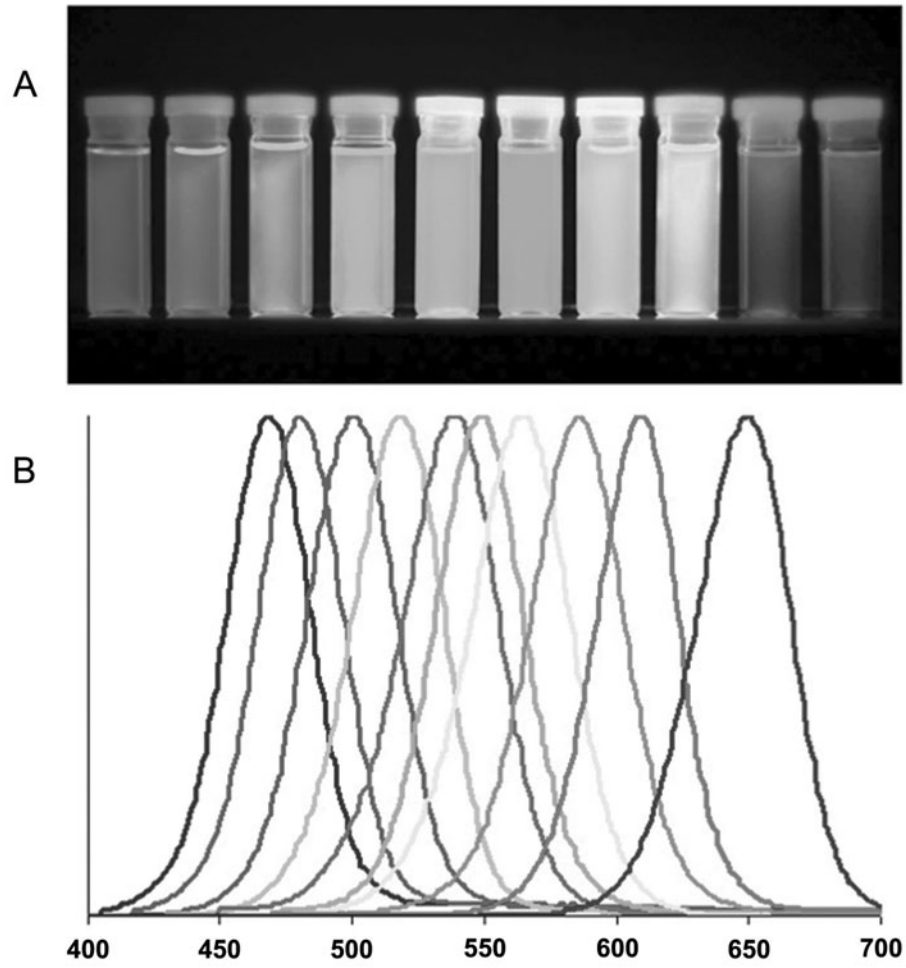

Figure 2. Multicolor CdSe/ZnS QDs. A) Size tunable emission of a series of QDs ranging in size from 2-6 nm (left to right), excited with a UV lamp. B) The corresponding size-tuned fluorescence emission spectra. Reprinted with permission from Nat Biotechnol 2001; 19(7):631-635.[62] ©2001 Macmillan Publishers Ltd.

(5–10 × 10^4 $M^{-1}cm^{-1}$). Combined with the fact that QDs can have quantum efficiencies similar to those of organic dyes (up to 85%)[41], individual QDs have been found to be 10-50 times brighter than organic dyes,[44-45] thus permitting the highly sensitive detection of analytes present at low concentrations. This is particularly important for the potentially low copy-number biomarkers present in the early stages of cell death. For the monitoring and tracking of biomarkers in live cells over extended periods of time, QDs exhibit greater photostability in comparison to conventional fluorophores (Fig. 3A), which is an important feature when new cytotoxicological assays are being developed for tracking the progression of the cell death process.

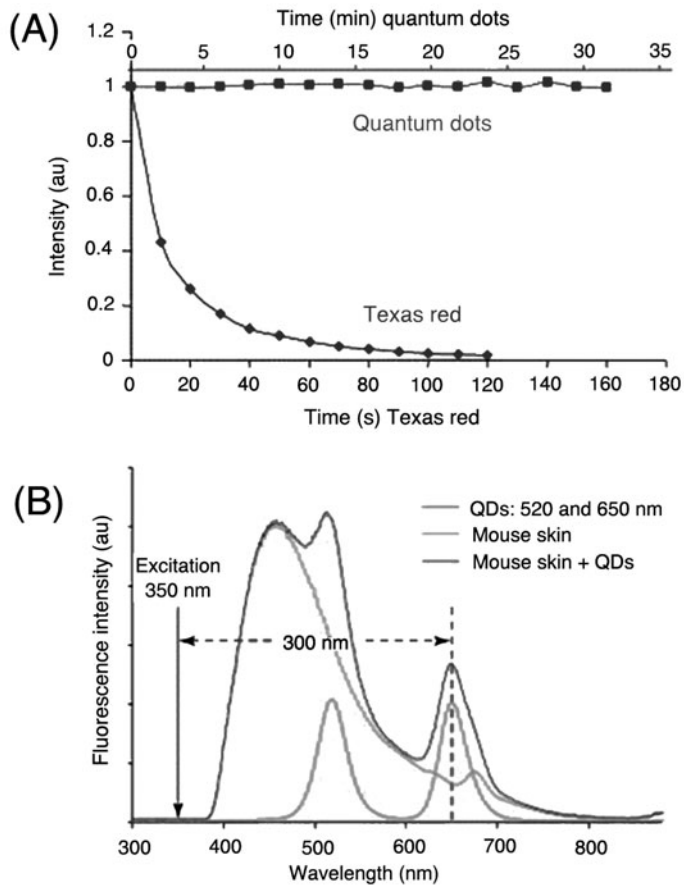

Figure 3. Advantages of the optical properties of QDs. A) Time-dependent fluorescence intensity of QDs *versus* an organic dye (Texas Red), demonstrating the resistance to photobleaching of QDs. B) Comparison of the Stokes shifts of quantum dots versus organic dyes, demonstrating that the QD fluorescent signal can be shifted away from the excitation and autofluorescence wavelengths.[34] Reprinted from Curr Opin Biotechnol 2005; 16:63-72;[34] © 2005 Elsevier Ltd. A color version of this image is available at www.landesbioscience.com/curie.

The multiplexing of QD signals is feasible due to the combination of broad absorption bands with narrow emission bands (Fig. 2). Broad absorption bands allow multiple QDs to be excited with a single source of short wavelength light, which simplifies instrumental design, increases detection speed and lowers cost. QD emission bands can be as narrow as 14 nm (full width at half maximum) in the visible range, thus enabling distinct signals to be detected simultaneously, with very little cross-talk.[39,46] By comparison, organic dyes and fluorescent proteins have narrow absorption bands and relatively wide emission bands, which considerably increase the difficulty of separating signals from distinct fluorophores. Biological tissue contains a variety of intrinsic fluorophores, particularly proteins and cofactors, which yield a background signal close to the emission wavelength of such

traditional fluorophores, resulting in decreased probe detection sensitivity. However, QDs can be tuned to emit in spectral regions in which autofluorescence is minimized, such as longer wavelengths in the red or infrared regions. Due to their broad absorption bands and large Stokes shifts, QDs can still be efficiently excited by wavelengths of light hundreds of nanometers shorter than the emission wavelength, whereas organic dyes require excitation close to the emission peak, which buries the signal in autofluorescence (Fig. 3B). These properties of QDs have been utilized to allow for the quantitative sensitive multiplexed detection of biomarkers labeled with QDs over background autofluorescence in tissue and cancer biopsies.[47] Sensitivity can also be increased by using time-gated light detection, because the excited state lifetimes of QDs (20-50 ns) are typically 1 order of magnitude longer than those of organic dyes and tissues (autofluorescence). Thus, QD fluorescence detection can be significantly enhanced by time-gating techniques that delay signal acquisition until background autofluorescence has decreased because of its shorter fluorescent lifetime.[48] In addition to the increased sensitivity provided by the unique photophysics of QDs, the ability to perform multiplexed detection by using multicolor QD probes simultaneously to image multiple biomarkers, represents a significant advancement for molecular toxicology, because of the large number of genes and proteins involved in cell death.

Bioconjugation of Quantum Dots for Biological Applications

Since the size-dependent properties of QDs are most pronounced for a monodisperse population of QDs, great strides have been made in the synthesis of highly homogeneous and crystalline QDs. The highest quality QDs are typically prepared at elevated temperatures in organic solvents, such as tri-n-octylphosphine oxide (TOPO) and hexadecylamine, both of which are high boiling-point solvents and contain long alkyl chains. These hydrophobic organic molecules serve as the reaction media and also coordinate with unsaturated metal atoms on the nanoparticle surface to prevent aggregation and the formation of bulk semiconductor. Since QDs are capped with a monolayer of the organic ligands, they are only soluble in hydrophobic solvents, such as chloroform and hexane. The most commonly used and best understood QD system is a core of CdSe, coated with a shell of ZnS to chemically and optically stabilize the core.

For biological applications, the hydrophobic dots are made water soluble and dispersed in aqueous buffer by one of two general strategies, as shown in (Fig. 4). The first approach (ligand exchange) replaces the hydrophobic monolayer of ligands on the QD surface with hydrophilic ligands. However, this method tends to cause particle aggregation and to decrease the fluorescence efficiency.[44] In the second approach (polymer coating) the native hydrophobic ligands are retained on the QD surface and amphiphilic polymers are absorbed to cover the hydrophobic QD surface and render it water soluble. Several polymers have been reported, including octylamine-modified polyacrylic acid,[49] PEG-derivatized phospholipids,[50] block copolymers[51] and amphiphilic polyanhydrides.[52] The hydrophobic domains strongly interact with the alkyl chains of the ligands on the QD surface, whereas the hydrophilic groups face outwards and render the QDs water soluble. Since the coordinating organic ligands (TOPO) are retained on the inner surface of the QDs, the optical properties of the QDs and the toxic elements of the core are shielded from the outside environment by a hydrocarbon bilayer.

The targeting ability and binding specificity of polymer coated QDs rely on their conjugation to bioaffinity ligands, such as monoclonal antibodies, peptides,

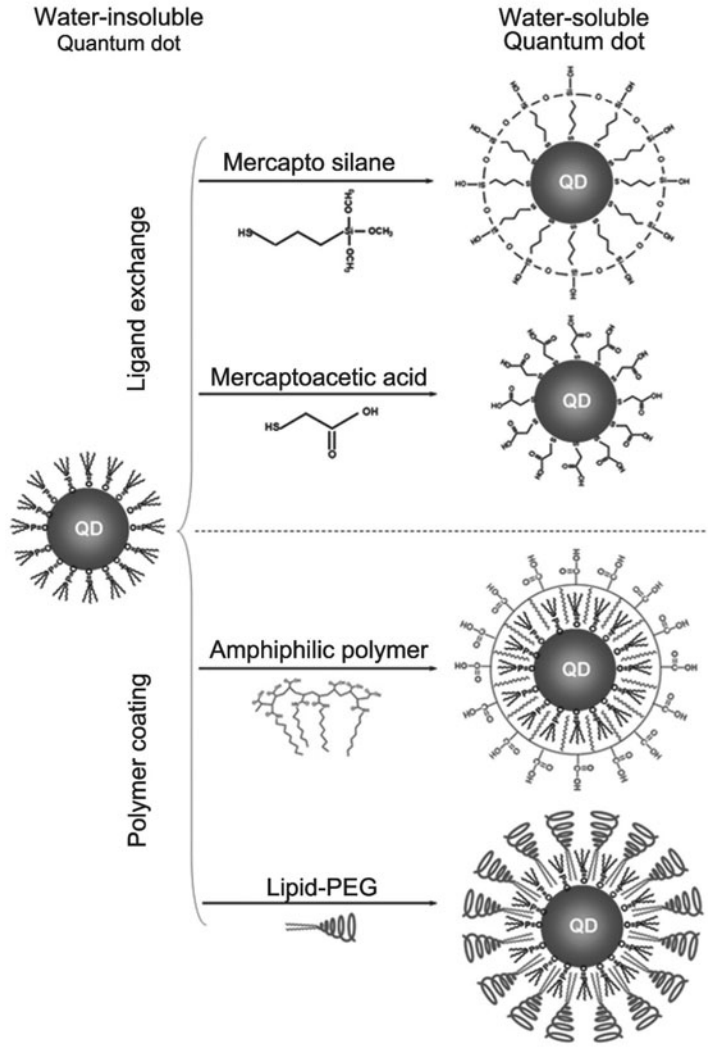

Figure 4. Water-solubilization schematic of hydrophobic tri-n-octylphosphine oxide (TOPO)-coated QDs. The surface coating thickness typically ranges from 1-2 nm. Ligand-exchange involves the replacement of TOPO ligands by heterobifunctional ligands such as mercapto silanes or mercaptoacetic acid (top panel). Polymer-coating is performed by coating the TOPO-coated QDs with an amphiphilic polymer, which is stabilized onto the QD surface through hydrophobic interactions of the alkyl chains (bottom panel). Figures not drawn to scale. Adapted with permission from Expert Rev Mol Diagn 2006; 6(2):231-244,[87] ©2006 Future Drugs Ltd.

oligonucleotides or small-molecule inhibitors. Additional fictionalization with PEG can improve biocompatibility and reduce nonspecific binding. The relatively large surface areas of QDs relative to their small molecule counterparts enable single QDs to be conjugated to multiple ligands for an overall multivalent effect and for multifunctionality. Several approaches exist for QD bioconjugation, including electrostatic adsorption,[53] covalent-bond

formation,[44] and streptavidin—biotin linking.[54] Ideally, the molecular stoichiometry and orientation of the attached biomolecules should be manipulated to permit access to the active sites of all conjugated enzymes and antibodies. However, this is very difficult in practice. Goldman and coworkers first explored the use of a fusion protein as an adaptor for immunoglobulin G antibody coupling.[55] The adaptor protein has a protein G domain that binds to the antibody Fc region and a positively charged leucine-zipper domain for electrostatic interaction with anionic QDs. As a result, the Fc end of the antibody is connected to the QD surface, with the target-specific $F(ab')_2$ domain facing outwards.

HOMOGENIZED SPECIMEN-BASED ASSAYS

Assays involving cell lysate or tissue extracts are powerful tools for the sensitive detection of low copy-number molecular targets. The higher sensitivity and ability for significant multiplexing are attributes which, in some applications, can outweigh such drawbacks as a loss of cell structure information and an inability to perform studies on living cells. The application of single QDs for detecting proteins and nucleic acids is discussed below, followed by a consideration of biomarker detection with QD-barcodes.

Protein Biomarker Detection by Quantum Dots

Screening for the molecular basis of cytotoxicity in its earliest stages, necessitates highly sensitive assays for detecting the biomarkers of apoptosis. The current gold standard for detecting low copy-number protein is the enzyme-linked immunosorbent assay (ELISA), which has a limit of detection in the pM range. Although such assays are used clinically, they are labor intensive, time consuming, difficult to multiplex and expensive. QDs could enhance the detection of low copy-number protein biomarkers. QDs have been successfully used as substitutes for organic fluorophores and colorimetric reagents in a variety of immunoassays for the detection of specific proteins. However, they have not demonstrated an increase in sensitivity (target concentration less than 100 pM).[55-56] Increasing the sensitivity of these probes may only be a matter of optimizing the bioconjugation parameters and assay conditions. Nonetheless, the multiplexing capabilities of these probes have already been demonstrated. Goldman and coworkers simultaneously detected four toxins by using four different QDs, which emitted at between 510 and 610 nm, in a sandwich immunoassay configuration, by using a single excitation source.[57] Although there was spectral overlapping of the emission peaks, deconvolution of the spectra revealed fluorescence contributions from all four toxins. Similarly, Makrides and coworkers demonstrated the ease of simultaneously detecting two proteins with two spectrally different QDs in a Western blot assay.[58]

Nucleic Acid Biomarker Detection by Quantum Dots

The early detection and identification of various genes associated with apoptosis offers the potential to significantly advance molecular toxicology, particularly in relation to the detection of mRNA transcripts associated with cell stress, injury or death by apoptosis. The current gold standard for the sensitive detection of nucleic acids is the PCR, combined with a variety of molecular fluorophore assays, commonly resulting in a detection limit in the fM range. However, like ELISAs, the clinical utility of nucleic

acid analysis for use in molecular toxicology is precluded by the time involved and its labor intensiveness, as well as its poor multiplexing capabilities. Recently, many types of new technologies have been developed for the rapid and sensitive detection of nucleic acids, most notably reverse transcriptase PCR and nanoparticle-based biobarcodes,[59] each of which have a limit of detection in the tens of molecules. However, QDs could also offer an advantage in this field, due to their multiplexing potential. Gerion and coworkers reported the detection of specific single nucleotide polymorphisms of the human p53 tumor suppressor gene, by using QDs in a microarray assay format with a detection sensitivity of 2 nM.[60] Recently, Zhang and coworkers developed a QD biosensor for DNA, analogous to the aforementioned protein biosensor (Fig. 5A).[61] However, in this case, fluorescence emission was monitored from the quenched QD donor, as well as from an acceptor reporter dye bound to the target DNA. Since QDs have broadband absorption compared with organic dyes, excitation of the QD at a short wavelength did not excite the dye, thereby resulting in extremely low background signals. This enabled the highly sensitive and quantitative detection of as few as 50 DNA copies and was sufficiently specific to differentiate single nucleotide differences. In conjunction with microfluidics, this technology could be improved for high-throughput biomarker screening and detection.

Multiplexed and High-Throughput Biomarker Detection with QD-Encoded Microbeads

Rather than using single QDs for identifying single biomarkers, it has been proposed that different colors of QDs could be combined into a larger structure, such as a microbead, in order to provide an optical barcode. With the combination of six QD emission colors and ten QD intensity levels for each color, 1 million different codes are theoretically possible. A vast assortment of biomarkers can be optically encoded by conjugation to these beads, thereby opening the door to the multiplexed identification of many biomolecules in the high-throughput screening of homogeneous biological samples. Pioneering work was reported by Han and coworkers in 2001, in which 1.2-μm polystyrene beads were encoded with three colors of QDs (red, green and blue) at different ratios and therefore different fluorescence intensity levels (Fig. 5B).[62] The beads were then conjugated to DNA, with the result that different nucleic acids could be distinguished by their spectrally distinct optical codes. These encoded probes were incubated with their complementary DNA sequences, which were also labeled with a fluorescent dye as a target signal. The hybridized DNA was detected by the simultaneous detection of the target signal and the probe optical code, via single-bead spectroscopy, by using only one excitation source. The bead code identified the sequence, while the intensity of the target signal corresponded to the presence and abundance of the target DNA sequence. Recently, the uniformity and brightness of the QD-encoded beads were substantially improved by Gao and Nie, by using mesoporous materials.[62-63] The high-throughput potential of this technology was realized by combining it with flow cytometry. For example, DNA sequences from specific alleles of human cytochrome P450 genes and N-acetyltransferases were correctly identified by hybridization to encoded probes.[64] It is worth mentioning that the long excited state of QDs and the blinking effect (isolated QDs show intermittent fluorescence emission, thus appearing to blink), do not interfere with bead decoding.[65] If three or more colors are used for microbead encoding, this identification would be considerably more difficult with organic dyes, because their emission peaks overlap, thus obscuring the distinct codes; also, multiple excitation sources are required. Once encoded libraries have been developed for

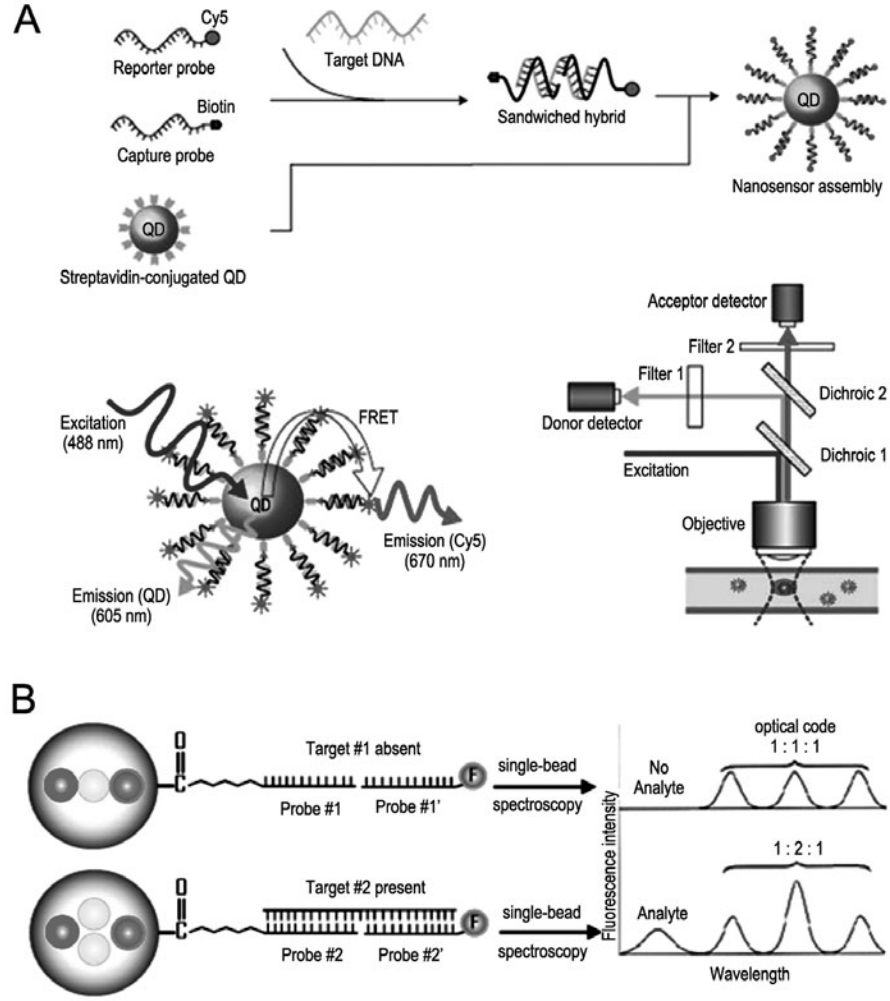

Figure 5. QD-based biosensors for homogeneous biological specimen assays. A) Single QD DNA sensors (top). Conceptual scheme showing the formation of a nanosensor assembly in the presence of targets. FRET-based emission from Cy5 (FRET acceptor) due to excitation of QD (FRET donor) and experimental setup (bottom panel). Reprinted with permission from Nat Mater 2005; 4(11):826-831;[61] © 2005, Macmillan Publishers Ltd B) Schematic illustration of DNA hybridization assays using QD-barcode beads. In the absence of a target molecule, only a QD barcode fluorescent signal is detected. In the presence of a target molecule, the barcode probe sequence and the reporter probe sequence are colocalized, resulting in the detection of the target sequence and its abundance. The blue dot can be either an organic fluorophore or single quantum dot. Reprinted with permission from Nat Biotechnol 2001; 19(7):631-635;[62] © 2011 Macmillan Publishers Ltd. A color version of this image is available at www.landesbioscience.com/curie.

the identification of nucleic acid sequences and proteins, the solution-based multiplexing of QD-encoded beads could quickly produce a vast amount of gene and protein expression data and lead to the discovery of new biomarkers for cell injury and cell death.

CELLULAR LABELING WITH QUANTUM DOTS

The evaluation of cell morphology through standard cell imaging techniques is an important and commonly practised technique, which facilitates the qualitative staging (classification) of the progression of disease or dysfunction (e.g., the histological staging of cancer or apoptosis). As such assessments are dependent upon the observer, they are somewhat qualitative and subjective which increases the difficulty in comparing assessments made by different observers. Specific quantitative information concerning changes in the concentrations of cellular biomarkers would help to refine qualitative morphological assessments and would provide greater insight into mechanisms of toxicity. Previously, successes have been achieved in cancer biomarker detection with colloidal gold and dye-doped silica nanoparticles. However, immunogold staining is essentially a single-color assay, whereas dye-doped silica nanoparticles are limited by the disadvantages associated with organic fluorophores. QDs would be better candidates for the quantitative staining of tissues for cytotoxicity biomarkers, due to their ability to detect multiple analytes simultaneously and because they have already been proven to be outstanding probes for the fluorescence detection of proteins and nucleic acids in cells.

Labeling of Fixed Cells and Tissues with Quantum Dots

The feasibility of using QDs for biomarker detection in fixed cellular monolayers was first demonstrated by Bruchez and coworkers in 1998.[45] By labeling nuclear antigens with green silica-coated QD and F-actin filaments with red QD in fixed mouse fibroblasts, these two spatially distinct intracellular antigens were simultaneously detected. This work and that of others have demonstrated that QDs are brighter and dramatically more photostable than organic fluorophores, when used for cellular labeling.[44,66] Many different cellular antigens in fixed cells and tissues have been labeled by using QDs, including specific genomic sequences,[67-68] mRNA,[69] plasma membrane proteins,[66,70-71] cytoplasmic proteins,[45,66] and nuclear proteins.[44,49] Also, it is apparent that QDs can be used in both primary and secondary antibody staining techniques. In addition, high-resolution actin filament imaging has been demonstrated by using QDs (Fig. 6A),[66] and the fluorescence can be correlated directly to electron micrograph contrast, due to the high electron density of QDs.[72-73] It has now become clear that QDs are superior to organic dyes for fixed cell labeling. The transition from fixed cell labeling to the staining of FFPE tissue sections is not straightforward, due to the high autofluorescence and the loss of antigen presentation associated with the embedding and fixation processes. Nonetheless, tissue-section labeling with QDs has been successful for the biomarker-specific staining of rat and mouse neural tissues,[74-75] human skin basal cell carcinomas,[70] and human tonsilar tissue.[76] High-throughput QD-based IHC and FISH has been achieved in FFPE tissues in a semi-automated process which permitted the simultaneous detection of gene expression and the cell lineage determination of multiple molecular targets.[77] Furthermore, the mRNA sequences of the apoptosis inhibitors, surviving and the X-linked inhibitor of apoptosis protein (XIAP) were simultaneously detected, in addition to the transcript for bcl-2, by QD-based quantitative and multiplexed FISH in FFPE cancer tissue biopsies (Fig. 7).[32] The recent advances in IHC for protein detection and FISH for nucleic acid detection using QD probes could revolutionize molecular toxicology, due to the large number of biomarkers that could be simultaneously monitored.

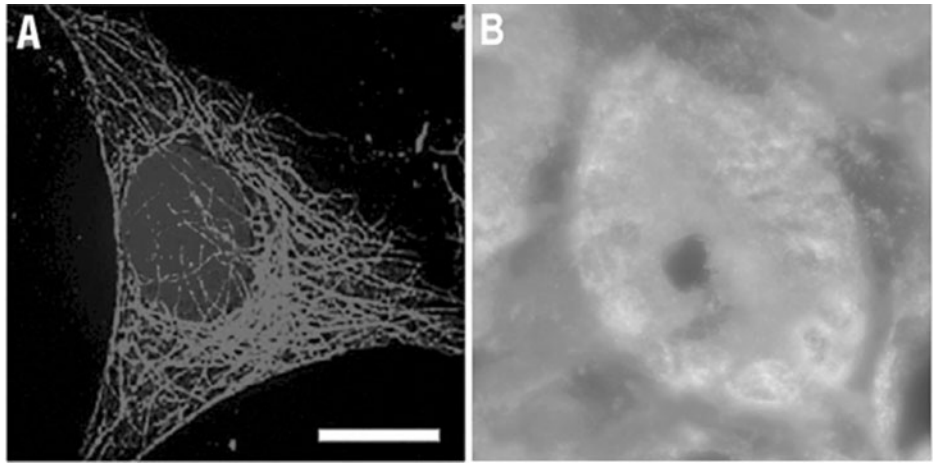

Figure 6. Cell and tissue-based molecular imaging. A) Red color QDs used to label microtubules in NIH-3T3 cells. Scale bar is 10 μm. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology, copyright 2003.[66] B) Immunostaining with QDs of formalin-fixed, paraffin-embedded human prostate tumor specimens. Red and green color QDs are used to label over-expressed p53 and EGF-1 biomarkers, respectively. QD fluorescence is clearly distinguishable from the background autofluorescence (blue) of the tissue, due to its large Stokes shift. Reprinted with permission from Nat Protoc 2007; 2(5):1152-1165;47 © 2007 Macmillan Publishers Ltd. A color version of this image is available at www. www.landesbioscience.com/curie.

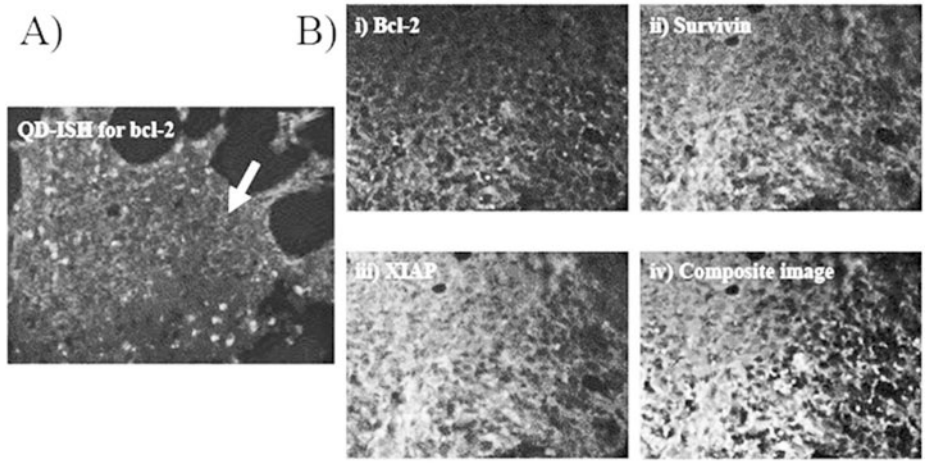

Figure 7. QD-based in situ hybridization. A) Red QD (655 nm) bcl-2 probes demonstrating bcl-2 upregulation (arrow) in formalin-fixed paraffin-embedded lymphoma tissue against autofluorescence (green). B) Triplex QD-based in situ hybridization. Intensity maps after spectral analysis of multiplexed detection of bcl-2, survivin and x-linked inhibitor of apoptosis protein (XIAP) mRNA transcripts with appropriate QD-oligonucleotide probes in bone-marrow infiltrated with acute myeloid leukemia. False color composite image of mRNA expression of bcl-2 (red), surviving (green) and XIAP (blue). This figure was adapted from Biochem Biophys Res Commun 2006; 348(2):628-636;[32] © 2006 Elsevier Ltd. A color version of this image is available at www.landesbioscience.com/curie.

Live Cell Imaging with Quantum Dots

In 1998, Chan and coworkers demonstrated that QDs conjugated to a membrane-translocating protein, transferrin, can cause the endocytosis of QDs by living cancer cells in culture.[44] The QDs retained their bright fluorescence in living cells and were not noticeably toxic, thus revealing that QDs could be used as intracellular labels in living cell studies. Subsequent living cell studies with QDs have focused on the labeling of plasma membrane proteins,[78-79] and on evaluating techniques for traversing the plasma membrane barrier.[80] It is becoming evident that QDs will become powerful tools for unveiling many aspects of cellular biology and for optically tagging cells to determine their lineages and distribution in multicellular organisms.[50] In this fast moving and exciting field, QDs have already been used to calculate plasma membrane protein diffusion coefficients,[79] and to observe a single ErbB/Her receptor (a cancer biomarker) and its internalization after binding to epidermal growth factor.[78] Furthermore, QD probes of living cells have prompted the discovery of a new filopodial transport mechanism.[78,81] While most of these studies have centered on biological discovery, a new clinically relevant cell motility assay for cancer diagnosis has already been developed from these living cell studies.[82-83] These advances demonstrate the great potential of QD-based research in molecular toxicology.

The benefits of QD photostability for live cell imaging are apparent when visualizing both rapid events (taking place within seconds), such as changes in cell membrane morphology typified by membrane blebbing and phosphatidylserine externalization and slower events which are to be tracked over extended periods of time. Indeed, QDs conjugated to Annexin V were demonstrated to be superior to organic dyes in this regard.[3,20,84] This work demonstrated that the high frequency visualization of membrane blebbing could be achieved with bright and photostable QDs (Fig. 8). This also opened up the possibility to produce images of time-dependent cell responses after stimulation with an apoptosis inducer, thereby permitting the study of the delays in the onset of apoptosis which depend on the cell type and inducer and to quantify the phosphatidylserine levels present on the outer membrane.

CONCLUSION

Nanotechnology and QDs in particular, have recently made available a host of new approaches for the improved quantitative detection of biomarkers, which demonstrates their great potential as research tools for use with both living cells and FFPE tissues. The incorporation of multiplexed QD-based assays into cell imaging, flow cytometry and other homogenized sample-based assays for detecting multiple biomarkers associated with cell injury and apoptosis, are predicted to have a significant impact on research in molecular toxicology. Although the use of QDs is competing with the uses of a large number of other highly promising and already established in vitro probes, such as small fluorophores, biobarcodes and metallic nanoparticles, the superiority of QD probes for cellular labeling has been demonstrated. Nevertheless, despite the encouraging results obtained so far, further improvements in fictionalization are needed, in order to control the number and orientation of biomolecules on the QD surface, before this technology can achieve widespread use in molecular toxicology. QDs will enable the sensitive and

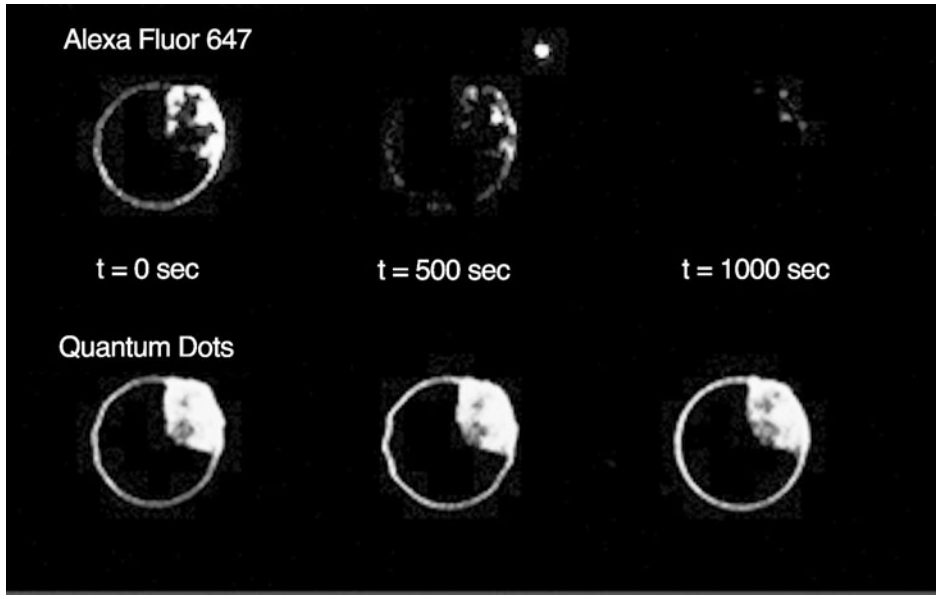

Figure 8. Live cell staining. Cell membrane phosphatidylserine staining efficiency comparison of relative photostability of red QD and Alexa Fluor 647-annexin V conjugates in living cells. Images taken every 10 seconds with laser intensity set at 10% for Alexa Fluor 647 and 20% for QDs. Reprinted with permission from Nano Lett 2006; 6(9):1863-1869;[3] © 2006 American Chemical Society. A color version of this image is available at www.landesbioscience.com/curie.

quantitative in situ detection of key regulatory factors associated with cellular injury and apoptosis, as well as of low copy-number transcription products and proteins. Future prospects for the use of QDs in research in molecular toxicology include QD-encoded beads for gene and protein profiling, which is only hindered by the technical challenge of developing libraries for screening a large number of targets. Also, work employing FRET dye/quenchers attached to QDs through peptide linkages containing proteolytic cleavage sequences,[85-86] might result in sensors for monitoring the activities of caspases and other enzymes activated during apoptosis. In summary, QDs are bright functionalizable nanoparticles, which not only demonstrate superior optical properties over traditional fluorophores, but also exhibit unsurpassed versatility as enabling components for new assays and for new potential discoveries in molecular toxicology.

REFERENCES

1. Vermes I, Haanen C. Apoptosis and programmed cell-death in health and disease. In: Sobotka H, Stewart CP, eds. Advances in Clinical Chemistry, Vol 31. New York: Academic Press, 1994:177-246.
2. Maines MD, Lawrence DA. Assessment of cell toxicity. In: Morgan KS, Maines MD, Sassa S et al., eds. Current Protocols in Toxicology. Hoboken: John Wiley and Sons, 2006:1:2.0.1-2.13.10.
3. LeGac S, Vermes I, van den Berg A. Quantum dots based probes conjugated to annexin V for photostable apoptosis detection and imaging. Nano Lett 2006; 6(9):1863-1869.
4. Ryan KM, Birnie GD. Myc oncogenes: the enigmatic family. J Biochem 1996; 3(14):713-721.
5. Evan GI, Wyllie AH, Gilber CS et al. Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992; 69:119-128.

6. Yonish-Rouach E. The p53 tumour suppressor gene: a mediator of a G1 growth arrest and apoptosis. Experientia 1996; 52:933-1017.
7. Yonish-Rouach E, Resnitzky D, Lotem J et al. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interlukin-6. Nature 1991; 3(53):345-347.
8. Adams JM, Cory S. The bcl-2 protein family: arbiters of cell survival. Science 1998; 281(5381):1322-1326.
9. Chao DT, Korsmeyer SJ. Bcl-2 family: regulators of cell death. Annu Rev Immunol 1998; 16(1):395-419.
10. Cory S, Adams JM. The bcl-2 family: regulators of cellular life-or-death switch. Nat Rev Cancer 2002; 2:657-656.
11. Hickenbery DM, Nunez G, Milliman C et al. Bcl-2 in inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990; 348:334-336.
12. Lowy DR, Willumsen BM. Function and regulation of ras. Annu Rev Biochem 1993; 62(1):851-891.
13. Marshall MS. Ras target proteins in eukaryotic cells. FASEB J 1995; 9(13):1311-1318.
14. Campbell SL, Khosravi-Far R, Rossman KL. Increasing complexity of ras signaling. Oncogene 1998:1395-1413.
15. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 1989; 49(17):4682-4689.
16. McClean S, Hill BT. An overview of membrane, cytosolic and nuclear proteins associated with the expression of resistance to multiple drugs in vitro. Biochem Biophys Acta 1992; 1114:107-127.
17. Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997; 326:1-16.
18. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002; 9:459-470.
19. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; 281(5381):1312-1316.
20. Fadok VA, Voelker DR, Campbell PA et al. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992; 148(7):2207-2216.
21. Martin SJ, Reutelingsperger CP, McGahon AJ et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of bcl-2 and abl. J Exp Med 1995; 182(5):1545-1556.
22. Andree HA, Reutelingsperger CP, Hauptmann R et al. Binding of vascular anticoagulant alpha (vac alpha) to planar phospholipid bilayers. J Biol Chem 1990; 265(9):4923-4928.
23. Jin L, Lloyd RV. In situ hybridization: methods and applications. J Clin Lab Anal 1997; 11(1):2-9.
24. Drake TJ, Tan W. Molecular beacon DNA probes and their bioanalytical applications. Appl Spectrosc 2004; 58(9):269A-280A.
25. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 1995; 14:303-308.
26. Nitin N, Santangelo PJ, Kim G et al. Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. Nucleic Acids Res 2004; 32(6):e58.
27. Rieseberg M, Kasper C, Reardon KF et al. Flow cytometry in biotechnology. Appl Microbiol Biotechnol 2001; 56:350-360.
28. Chattopadhyay PK, Price DA, Harper TF et al. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. Nat Med 2006; 12(8):972-977.
29. Orfao A, Ruiz-Arguelles A, Lacombe F et al. Flow cytometry: its applications in hematology. Haematologica 1995; 80:69-81.
30. Dallas CE, Evans DL. Flow cytometry in toxicity analysis. Nature 1990; 345:557-558.
31. Darzynkiewicz Z, Juan G, Li X et al. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 1996; 27:1-20.
32. Tholouli E, Hoyland JA, Di Vizio F et al. Imaging of multiple mRNA targets using quantum dot based in situ hybridization and spectral deconvolution in clinical biopsies. Biochem Biophys Res Commun 2006; 348(2):628-636.
33. Del Castillo P, Llorente AR, Stockert JC. Influence of fixation, exciting light and section thickness on the primary fluorescence of samples for microfluorometric analysis. Basic Appl Histochem 1989; 33:251-257.
34. Gao XH, Yang L, Petros JA et al. In vivo molecular and cellular imaging with quantum dots. Curr Opin Biotechnol 2005; 16:63-72.
35. Alivisatos AP. Semiconductor clusters, nanocrystals and quantum dots. Science 1996; 271(5251):933-937.
36. Murphy CJ, Coffer JL. Quantum dots: a primer. Appl Spectrosc 2002; 56(1):16A-27A.
37. Sapra S, Sarma DD. Evolution of the electronic structure with size in II-VI semiconductor nanocrystals. Phys Rev B 2004; 69(12):125304-125307.
38. Pietryga JM, Schaller RD, Werder D et al. Pushing the band gap envelope: mid-infrared emitting colloidal PbSe quantum dots. J Am Chem Soc 2004; 126(38):11752-11753.
39. Zhong X, Feng Y, Knoll W et al. Alloyed Zn(x)Cd(1-x)S nanocrystals with highly narrow luminescence spectral width. J Am Chem Soc 2003; 125(44):13559-13563.
40. Zhong X, Han M, Dong Z et al. Composition-tunable Zn(x)Cd(1-x)Se nanocrystals with high luminescence and stability. J Am Chem Soc 2003; 125(28):8589-8594.

41. Qu L, Peng X. Control of photoluminescence properties of CdSe nanocrystals in growth. J Am Chem Soc 2002; 124(9):2049-2055.
42. Kim S, Fisher B, Eisler HJ et al. Type-II quantum dots: CdTe/CdSe(core/shell) and CdSe/ZnTe(core/shell) heterostructures. J Am Chem Soc 2003; 125(38):11466-11467.
43. Leatherdale CA, Woo WK, Mikulec FV et al. On the absorption cross section of CdSe nanocrystal quantum dots. J Phys Chem B 2002; 106(31):7619-7622.
44. Chan WCW, Nie S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 1998; 281(5385):2016-2018.
45. Bruchez Jr M, Moronne M, Gin P et al. Semiconductor nanocrystals as fluorescent biological labels. Science 1998; 281(5385):2013-2016.
46. Li LS, Pradhan N, Wang Y et al. High quality ZnSe and ZnS nanocrystals formed by activating zinc carboxylate precursors. Nano Lett 2004; 4(11):2261-2264.
47. Xing Y, Chaudry Q, Shen C et al. Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. Nat Protoc 2007; 2(5):1152-1165.
48. Dahan M, Laurence T, Pinaud F et al. Time-gated biological imaging by use of colloidal quantum dots. Opt Lett 2001; 26:825-827.
49. Yu WW, Wang YA, Peng XG. Formation and stability of size-, shape- and structure-controlled CdTe nanocrystals. Chem Mater 2003; 15(22):4300-4308.
50. Dubertret B, Skourides P, Norris DJ et al. In vivo imaging of quantum dots encapsulated in phospholipid micelles. Science 2002; 298(5599):1759-1762.
51. Gao X, Cui Y, Levenson RM et al. In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 2004; 22(8):969-976.
52. Kirchner C, Liedl T, Kudera S et al. Cytotoxicity of colloidal CdSe and CdSe/ZnS nanoparticles. Nano Lett 2005; 5(2):331-338.
53. Mattoussi H, Mauro JM, Goldman ER et al. Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein. J Am Chem Soc 2000; 122(49):12142-12150.
54. Goldman ER, Balighian ED, Mattoussi H et al. Avidin: a natural bridge for quantum dot-antibody conjugates. J Am Chem Soc 2002; 124(22):6378-6382.
55. Goldman ER, Anderson GP, Tran PT et al. Conjugation of luminescent quantum dots with antibodies using an engineered adaptor protein to provide new reagents for fluoroimmunoassays. Anal Chem 2002; 74(4):841-847.
56. Goessl C. Noninvasive molecular detection of cancer—the bench and the bedside. Curr Med Chem 2003; 10(8):691-706.
57. Goldman ER, Clapp AR, Anderson GP et al. Multiplexed toxin analysis using four colors of quantum dot fluororeagents. Anal Chem 2004; 76(3):684-688.
58. Makrides S, Gasbarro C, Bello J. Bioconjugation of quantum dot luminescent probes for western blot analysis. Biotechniques 2005; 39(4):501-506.
59. Penn SG, He L, Natan MJ. Nanoparticles for bioanalysis. Curr Opin Chem Biol 2003; 7(5):609-615.
60. Gerion D, Chen F, Kannan B et al. Room-temperature single-nucleotide polymorphism and multiallele DNA detection using fluorescent nanocrystals and microarrays. Anal Chem 2003; 75(18):4766-4772.
61. Zhang CY, Yeh HC, Kuroki MT et al. Single-quantum-dot-based DNA nanosensor. Nat Mater 2005; 4(11):826-831.
62. Han M, Gao X, Su JZ et al. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat Biotechnol 2001; 19(7):631-635.
63. Gao X, Nie S. Doping mesoporous materials with multicolor quantum dots. J Phys Chem B 2003; 107(42):11575-11578.
64. Xu H, Sha MY, Wong EY et al. Multiplexed snp genotyping using the Qbead™ system: a quantum dot-encoded microsphere-based assay. Nucl Acids Res 2003; 31(8):e43.
65. Gao X, Nie S. Quantum dot-encoded mesoporous beads with high brightness and uniformity: rapid readout using flow cytometry. Anal Chem 2004; 76(8):2406-2410.
66. Wu X, Liu H, Haley KN et al. Immunofluorescent labeling of cancer marker her2 and other cellular targets with semiconductor quantum dots. Nat Biotechnol 2003; 21(1):41-46.
67. Pathak S, Choi SK, Arnheim N et al. Hydroxylated quantum dots as luminescent probes for in situ hybridization. J Am Chem Soc 2001; 123(17):4103-4104.
68. Xiao Y, Barker PE. Semiconductor nanocrystal probes for human metaphase chromosomes. Nucl Acids Res 2004; 32(3):e28.
69. Matsuno A, Itoh J, Takekoshi S et al. Three-dimensional imaging of the intracellular localization of growth hormone and prolactin and their mRNA using nanocrystal (quantum dot) and confocal laser scanning microscopy techniques. J Histochem Cytochem 2005; 53(7):833-838.
70. Sukhanova A, Devy J, Venteo L et al. Biocompatible fluorescent nanocrystals for immunolabeling of membrane proteins and cells. Anal Biochem 2004; 324(1):60-67.

71. Dressler C, Minet O, Beuthan J et al. Microscopical heat stress investigations under application of quantum dots. J Biomed Opt 2005; 10(4):041209.
72. Giepmans BNG, Deerinck TJ, Smarr BL et al. Correlated light and electron microscopic imaging of multiple endogenous proteins using quantum dots. Nat Meth 2005; 2(10):743-749.
73. Nisman R, Dellaire G, Ren Y et al. Application of quantum dots as probes for correlative fluorescence, conventional and energy-filtered transmission electron microscopy. J Histochem Cytochem 2004; 52(1):13-18.
74. Ness JM, Akhtar RS, Latham CB et al. Combined tyramide signal amplification and quantum dots for sensitive and photostable immunofluorescence detection. J Histochem Cytochem 2003; 51(8):981-987.
75. Chan P, Yuen T, Ruf F et al. Method for multiplex cellular detection of mRNAs using quantum dot fluorescent in situ hybridization. Nucl Acids Res 2005; 33(18):e161.
76. Sukhanova AV, Devy L, Artemyev J et al. Highly stable fluorescent nanocrystals as a novel class of labels for immunohistochemical analysis of paraffin-embedded tissue sections. Lab Invest 2002; 82(9):1259-1261.
77. Byers RJ, Vizio DD, O'Connell F et al. Semiautomated multiplexed quantum dot-based in situ hybridization and spectral deconvolution. J Mol Diagn 2007; 9(1):20-29.
78. Lidke DS et al. Quantum dot ligands provide new insights into erbb/her receptor-mediated signal transduction. Nat Biotechnol 2004; 22(2):198-203.
79. Dahan M, Levi S, Luccardini C et al. Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. Science 2003; 302(5644):442-445.
80. Derfus AM, Chan WCW, Bhatia SN. Intracellular delivery of quantum dots for live cell labeling and organelle tracking. Adv Mater 2004; 16(2):961-966.
81. Lidke DS, Lidke KA, Rieger B et al. Reaching out for signals: filopodia sense egf and respond by directed retrograde transport of activated receptors. J Cell Biol 2005; 170(4):619-626.
82. Parak WJ, Boudreau R, Le Gros M et al. Cell motility and metastatic potential studies based on quantum dot imaging of phagokinetic tracks. Adv Mater 2002; 14(12):882-885.
83. Pellegrino T, Parak WJ, Boudreau R et al. Quantum dot-based cell motility assay. Differentiation 2003; 71(9-10):542-548.
84. van Engeland M, Nieland LJW, Ramaekers FCS et al. Annexin V-Affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998; 31(1):1-9.
85. Medintz IL, Clapp AR, Brunel FM et al. Proteolytic activity monitored by fluorescence resonance energy transfer through quantum-dot-peptide conjugates. Nat Mater 2006; 5(7):581-589.
86. Chang E, Miller JS, Sun J et al. Protease-activated quantum dot probes. Biochem Biophys Res Commun 2005; 334(4):1317-1321.
87. Smith AM, Dave S, Nie S et al. Multicolor quantum dots for molecular diagnostics of cancer. Expert Rev Mol Diagn 2006; 6(2):231-244.

CHAPTER 9

ENGINEERING QUASI-VIVO® IN VITRO ORGAN MODELS

Tommaso Sbrana[1] and Arti Ahluwalia*,[1,2]

[1]Centro Interdipartimentale di Ricerca "E. Piaggio," University of Pisa, Pisa, Italy; [2]Kirkstall Ltd., The Sheffield BioIncubator, Sheffield, UK

**Corresponding Author: Arti Ahluwalia—Email: arti.ahluwalia@centropiaggio.unipi.it*

Abstract: Cell culture is the workhorse of biologists, toxicologists, tissue engineers and a whole host of research fields in both academia and industry. Having explored individual molecular mechanisms inside cells for decades using traditional cell culture techniques, 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. This chapter describes how tissue and organ models can be developed using the Quasi-Vivo® system and discusses how they may be used in drug toxicity studies.

INTRODUCTION

The fact that current in vitro cell culture systems are poorly representative of human or animal physiology is now widely accepted.[1] This has been generally attributed to the fact that the complexity of the physiological environment is not replicated in Petri dishes or microplates.[2] In fact, all cells are exquisitely sensitive to their microenvironment, which is rich in 3D cues from the extracellular matrix, other cells and from mechanical stimuli due to flow, concentration gradients and movement. Traditional methods for

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

investigating cellular responses in vitro are inadequate in this sense, since the complex interplay of mechanical and biochemical factors is largely absent.

A variety of methods have been proposed to refine the simplistic in vitro representations which are currently used in cell culture laboratories worldwide. For example, since the cross-talk between different tissues is important in modulating and enhancing cell function, the use of conditioned media or the addition of growth factors generated by stromal or other cells is increasingly common, as are cocultures.[3,4] Some recent approaches use microscale devices, combining cell culture and microfluidics to fabricate cells, organs or bodies on chips.[5] The organ-on-a-chip concept has met with limited success in the field of cell biology, because it is difficult to translate experimental methods from the milliscale to the microscale. As a result, in most research and industrial cell culture laboratories the gold standard is still very much the multiwell plate or Petri dish with monolayer monocultures.

For biologists and technicians to embrace new physiologically more-relevant culture methods, the transition from wells and dishes to other tools has to be as smooth as possible. For this reason, we have developed a 'system on a plate' modular MultiCompartmental Bioreactor (MCmB) array, known as Quasi-Vivo®, which enables microwell protocols to be transferred directly to the bioreactor modules without the need to redesign cell culture experiments (Quasi-Vivo® is a registered trademark of Kirkstall Ltd, Sheffield, UK).[6] The new system offers mechanical stimuli from flow and biochemical stimuli from cells placed in connected modules, thus offering a Quasi-Vivo® microenvironment to cells in vitro. Its design principles are based on the allometric scaling of cell numbers and the mean residence times of molecules in metabolic tissues, as well as consideration of oxygen tension and shear stress, which together can be combined to establish organ and system models.

Starting from a general description of the cell microenvironment, we describe the design and engineering of the Quasi-Vivo® MCmB modules and discuss their applications in toxicity testing and in setting up more-accurate organ models of biotransformation, adsorption and gaseous exchange. Other models, such as glucose metabolism and excretion, can also be designed by using the same principles.

DEFINING THE CELL ARENA

Cells in vivo are constantly subject to three types of cues or signals: biochemical cues, including ligands, signalling molecules and other cells; physicochemical cues, which comprises gradient-dependent factors such as surface properties, oxygen tension, pH and temperature; and finally, mechano-structural cues. The mechano-structural signals include the architecture in 2 and 3 dimensions, as well as mechanical forces such as stress and strain, all of which act in a nonlinear, but fairly constant, manner. The tissue microenvironment is surrounded by these cues in the form of the prestressed extracellular matrix, prestressed neighbouring cells, endocrine and paracrine signals, blood flow, body movement and forces and nutrient diffusion. Furthermore, cells themselves will remodel and modulate their own habitat, an aspect which is often ignored in in vitro experiments, as are most of the mechano-structural stimuli. Thus, cells co-ordinate their behaviour and function in response to signals (Fig. 1) from the macroscale to the microscale, within and without their tissues. Figure 1 highlights the cues which can be controlled in vitro by using engineering and design.[7] Many of these parameters can be modulated in the Quasi-Vivo® system, as described in this chapter.

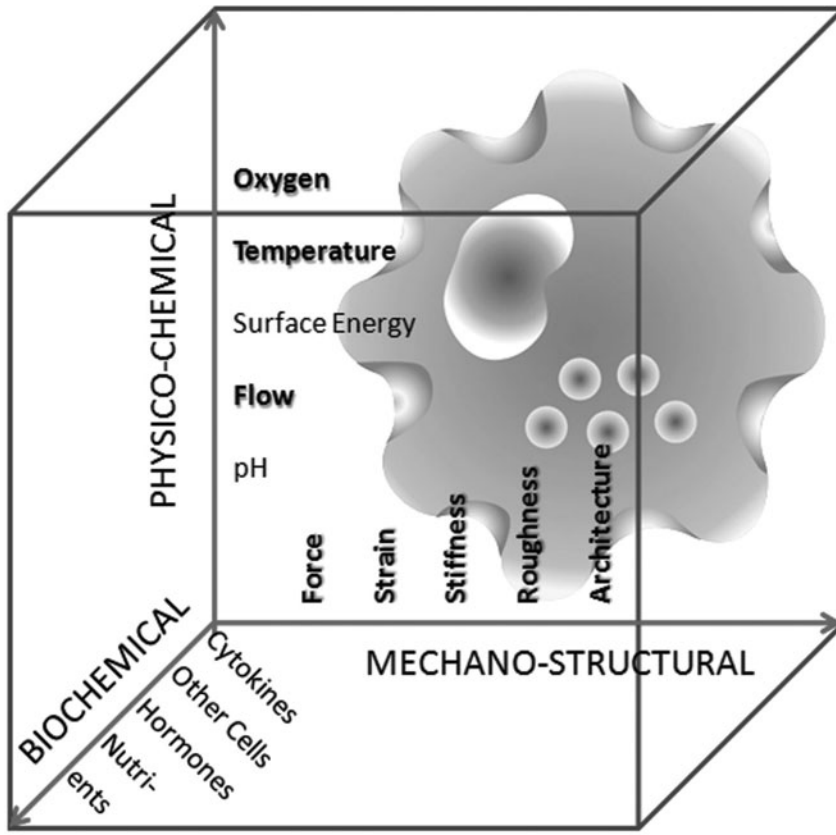

Figure 1. Some of the more important biochemical, physio-chemical and mechano-structural cues present in the cellular microenvironment. The cues in bold are those which can be controlled by using appropriate design and engineering.

Oxygen Concentration

Probably one of the best known physico-chemical cues is oxygen concentration. In incubators, the acid-base balance is strictly regulated through the use of buffers, which ensure that the pH and CO_2 tension remain similar to that seen in vivo. However, the limiting nutrient for cell culture is oxygen and all in vitro systems must maximize mass transport of this gas between the culture atmosphere and the cells. Typical oxygen concentrations in nonvascular tissue in vivo vary from 0.05 mM to 0.125 mM, corresponding to an atmospheric tension of 12.5% to 5%. In cell culture incubators the atmospheric oxygen concentration is the same as that in air, namely, around 20%. Hence the concentration of oxygen in fresh medium in the absence of cells is 0.2 mM. When oxygen consuming cells are added to the medium (Fig. 2), the concentration falls rapidly with depth as described in Figure 3. It is important to realize that, while the atmospheric oxygen tension is relatively

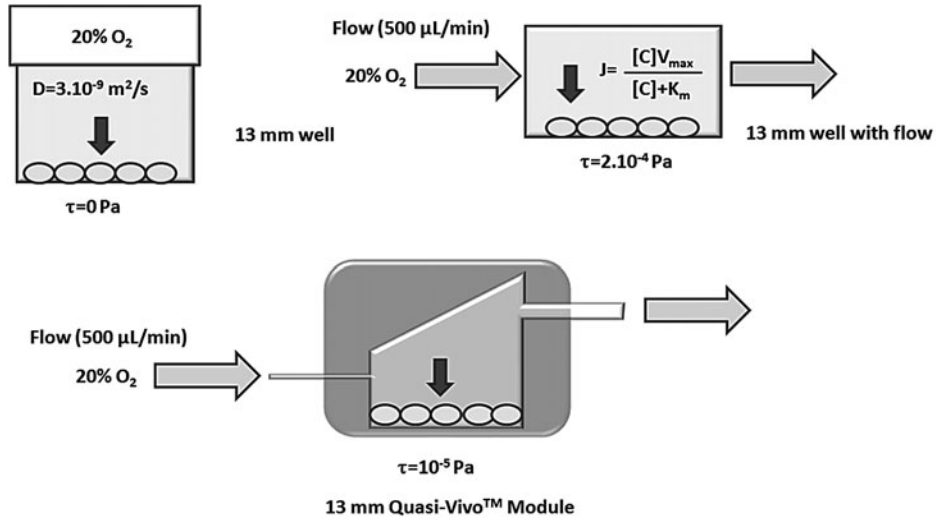

Figure 2. Three configurations used for modelling oxygen concentration profiles and shear stress. The calculated wall shear stress at the surface of the cell monolayer is reported for a flow rate of 500 µL/min.

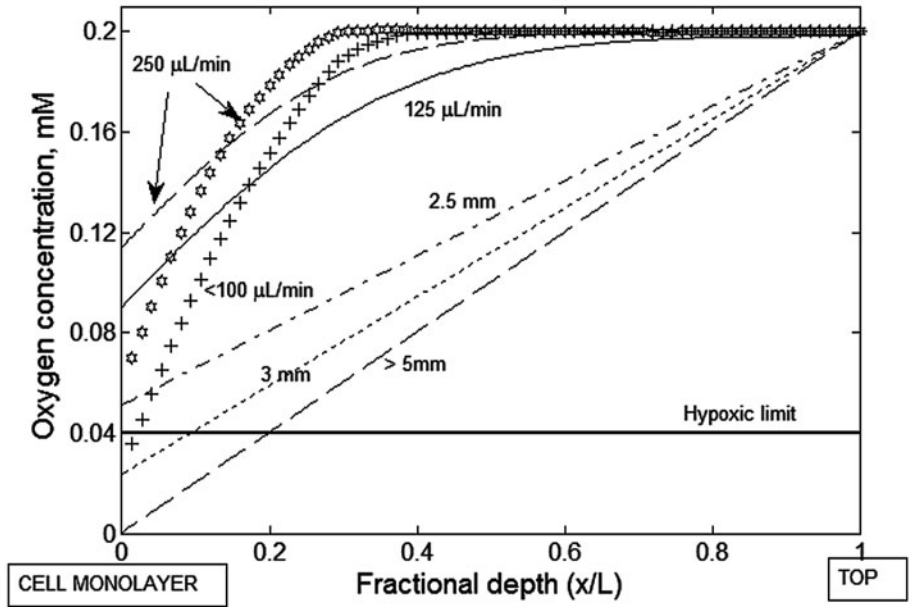

Figure 3. Oxygen concentration as a functional of normalized depth in wells with different medium heights (straight lines), in wells with flow tangential to the cell culture (····250 µL/min, —125 µL/min) and in the MCmB modules (∘∘∘∘250 µL/min, ++++ ,100 µL/min).

straightforward to control in hypoxic or hyperoxic incubators, the actual oxygen tension at the cell surface will vary greatly from one experiment to the other. It will depend mainly on the height of the medium, the intrinsic oxygen consumption rate of the cell culture and the thickness of any construct used. Hepatocyte monolayer cultures are particularly sensitive to in vitro culture conditions, due to their high oxygen consumption rate. In vivo hepatic oxygen concentrations are between 0.04-0.15 mM and hepatocyte function is compromised at 0.02 mM.[8] Figure 3 shows that a typical hepatocyte monolayer culture with a media height of over 3 mm will suffer from hypoxia.

Shear Stress and Kinetics

Kinetics is an often-overlooked problem in current in vitro systems for toxicity or metabolite testing. Usually, a drug or metabolite is added to culture medium and the steady state dose-response behaviour is assessed by using analytical tools. The assumption of stationary conditions is, however, quite inappropriate for most physiological processes, in which the extracellular milieu is in constant motion, due to pressure and concentration gradients established by blood flow in capillaries, a phenomenon known as interstitial flow.[9] Besides endothelial cells and non-adherent cells, most cells cannot support direct tangential fluid flow, but are instead constantly stimulated by a low velocity convective motion, which is modulated by the random fibrous architecture of the extracellular matrix; hence, recapitulating the dynamic interstitial transport environment in vivo is important in evoking more-realistic tissue responses in vitro. Interstitial flow rates are estimated to be of the order of 0.1-2 μm/s, while the shear stress on cell membranes due to this flow has been estimated by continuum models to range from 10^{-4} to 0.1 Pa,[10,11] although there is still some debate on precise values for different tissues. One of the main engineering issues in dynamic culture devices is thus the balance between high oxygen mass transfer and low wall shear stress to cells. For a given flow rate (Q) and fluid viscosity (μ), wall shear stress τ is inversely proportional to channel width (w) and height (h) (Eq. 1):

$$\tau = \frac{6Q\mu}{wh^2} \tag{1}$$

Hepatocytes are particularly sensitive to shear stresses and several reports describe the effects of flow and shear stress on in vitro cultures. Probably one of the most cited papers on hepatocytes and shear stress concludes that hepatocyte function is compromised at wall shear stresses greater than 0.03 Pa.[12] Little attention is paid to the fact that the authors used a top coculture of collagen-producing fibroblasts, which attenuated the shear force sensed by underlying heptocytes. In Vinci et al,[13] we show that the optimal shear stress for maintaining cytochrome P450 expression in human hepatocytes is of the order of 10^{-5} Pa.

To demonstrate the advantages of the MCmB modules in terms of oxygen transport and fluid dynamics, three different configurations can be modelled by using finite element methods: a cell culture well, a well with imposed flow and a MCmB module (Fig. 2). Figure 3 illustrates how oxygen concentrations at the bottom of a well decrease as the height of culture medium is increased from 2.5 mm to 5 mm in a high density rat hepatocyte culture. Convective motion of the medium will result in higher oxygen concentrations at the cell monolayer, but at the expense of nonzero wall stress (values reported in Fig. 2).

THE EDGE OF MICROFLUIDICS

Most cell- or organ-on-a-chip systems are based on microfluidics. Several reports demonstrate that these devices are capable of mimicking physiological interactions between cells.[5,14] The usual configuration comprises microchannnels and micro chambers for cell culture, generally fabricated from PDMS (polydimethylsiloxane) by using soft-lithography. Microscale devices require extreme downscaling of current cell-handling protocols and this is one of the principal reasons why they remain a "niche" research tool. The MCmB modules are also fabricated from PDMS and this is the only similarity between microfluidic devices and Quasi-Vivo®. PDMS is a biocompatible elastomer with high oxygen permeability, which is simple to mould into different shapes. It is also self adhesive and hydrophobic and will nonspecifically bind to small hydrophobic molecules, such as drugs.[15]

There are a number of differences between the Quasi-Vivo® system and microfluidic devices, perhaps the most obvious being size. While microfluidic systems use extremely small volumes, of the order of tens of microliters, each MCmB module has a volume of 2 mL, typical of cell cultures in dishes or wells. The height of the modules is 11 mm, whereas microfluidic circuits typically have heights of several tens of microns. Therefore, most microscaled devices are only suitable for 2D cultures composed of a few thousand cells, which is not representative of an organ, since they contain but little more than a few functional units. A functional unit is defined as a tissue entity which communicates through paracrinal signaling and corresponds to a cube with sides of 100 to 200 microns and from 1000 to 5000 cells. In engineering terms it is a volume of tissue which does not require vascularization.

By virtue of their small scale, microfluidic systems have extremely high surface area to volume ratios. As a consequence, they have high wall shear stresses (Eq. 1) and are particularly affected by high nonspecific surface adsorption. In addition, an often overlooked problem in microsystems for cell culture is the amplified edge effect, where, as a result of the low volume and high surface area, a large percentage of cells lie at the edges, rather than being surrounded by other cells. This is known to induce increased cytoskeletal tension, as well as increased cytokine production rates.[16,17] More recently edge effects have also been shown to direct changes in cell phenotype.[18]

One of the least reported practical problems with microfluidics systems is the presence of bubbles. Once again, the high surface area to volume ratio implies that surface forces play a major role in these systems. Therefore, differences in surface energies between dissolved gases and the bulk liquid lead to the formation of bubbles, which stick to the PDMS walls, no matter what flow rates or external forces (such as tilting and shaking) are used. The MCmB modules do not suffer from this problem due to their intelligent design features and size.

QUASI-VIVO® DESIGN

The modular bioreactor stems from the Multi Compartmental Bioreactor (MCB), in which the metabolic circuit has a fixed topology.[19,20] It was designed with the aim of reproducing a down scaled in vitro Quasi-Vivo® "human body", so as to better understand

interactions between different cell types in the presence of physical and biochemical stimuli, with particular reference to the metabolic system. Specifically, the Quasi-Vivo® system was designed by using allometric laws that mathematically link nonlinear quantities (e.g., organ mass, blood flow, blood retention time, metabolic rate). By using these laws, a bioreactor with chamber dimensions similar to those of a 24 well plate was realized, in which not only kinetic relationships, but also metabolic, volumetric and exchange rate relationships between cells are conserved.

Our initial studies on the MCB showed that for a given set of cell types, the overall system response is a function of three variables: Cell numbers and cell ratios; passage times or flow rates; and total volume. In order to elicit physiologically meaningful responses, a connected culture system requires physiologically scaled cell numbers and ratios and flow rates that do not cause shear stress (and therefore, damage to cells), but that allow adequate residence times in each compartment to enable the cells to process metabolic signals.

In the bioreactor, cell ratios and medium passage times in the metabolic chamber are scaled by using allometry. In this way, cell ratios are conserved to obtain physiologically-meaningful proportions between cell types in the system or organ under consideration. Having established cell ratios, we then use cell numbers and densities which are compatible with standard 12- or 24-multiwell plates, to facilitate media handling and assays, and to allow direct comparisons between the different experimental groups. The flow rate and volume will determine the residence and passage times of molecules, as well as the shear stress, and the level of cross-talk is likely to depend strongly on these variables. For a given volume, the allometric scaling model allows us to establish a sufficiently high flow rate to provide a dynamic stimulus in the form of flow and concentration gradients, but low enough to ensure that cross-talk signals can be transduced by the cells. The main difference between the MCmB modules and the original MCB system is that the former allows any tissue or organ model to be simulated simply by connecting the modular chambers in a desired configuration.

The bioreactor design process started with an analysis of the oxygen concentration and with an assessment of the minimum concentration of oxygen that is needed near the cell surface. Subsequently, a fluid dynamic model of the MCmB chamber was developed, in order to investigate the shear stress and to estimate the optimal chamber size for obtaining both adequate oxygen diffusion and low shear stress near the cell surface. The starting point for the module design was the 24-multiwell plate, the wells of which have an internal diameter of 13 mm. We focused on monolayer cultures, as they are commonly used in cell biology. Since hepatocytes are one of the most important cell types in drug metabolism and toxicity studies, the design process began by simulating a high density (400,000 cells/cm^2) monolayer culture of primary rat hepatocytes in a well. A high density value was used to estimate a "worst case" monolayer consumption. The results are shown in Figure 3. In a static culture, unless the height of medium is less than 3 mm, the cells will suffer from hypoxia. Hepatocytes are considered "difficult" cells to culture; they have high oxygen demands and they rapidly lose their phenotypic functions, particularly xenobiotic metabolism.[21] Several reports suggest that this is due to the absence of an adequately equipped micro-environment.[22] By applying flow to a well-shaped chamber, we can increase oxygen supply to reasonable levels, but the shear stress at the cell surface increases. To reduce the tangential forces acting at the bottom of the well-shaped chamber, while keeping the flow rate at levels which enable an adequate oxygen turnover, we designed specially-shaped inlets, which allow circulating media to

fill the chamber without directly impacting on the bottom of the well. The shear stress is reduced to acceptable levels as shown in Figure 2, while the oxygen concentration is above 4% for flow rates greater than 100 μL/min (Fig. 3).

Final adjustments to the design were implemented to overcome air bubbles, one of the biggest problems encountered in fluidic systems, particularly in microscale ones, as discussed previously. The patented sloping roof collects air bubbles and conveys them to the outlet, so there is no need to purge or de-gas the circuit. In addition, the base of the bioreactor is ridged, to permit the easy removal of 2D and 3D samples and to enable fluid motion and an oxygen supply underneath scaffolds (Fig. 4).[6]

Figure 5 shows how a simple 3-chamber Quasi-Vivo® experiment is set up. Sampling and infusion ports can be added to the outlet and inlet of each chamber by including a 3-way stopcock with a septum cap. The recommended sampling and infusion site is the mixing chamber, which serves as both a fluid reservoir and for gas exchange. A sterile

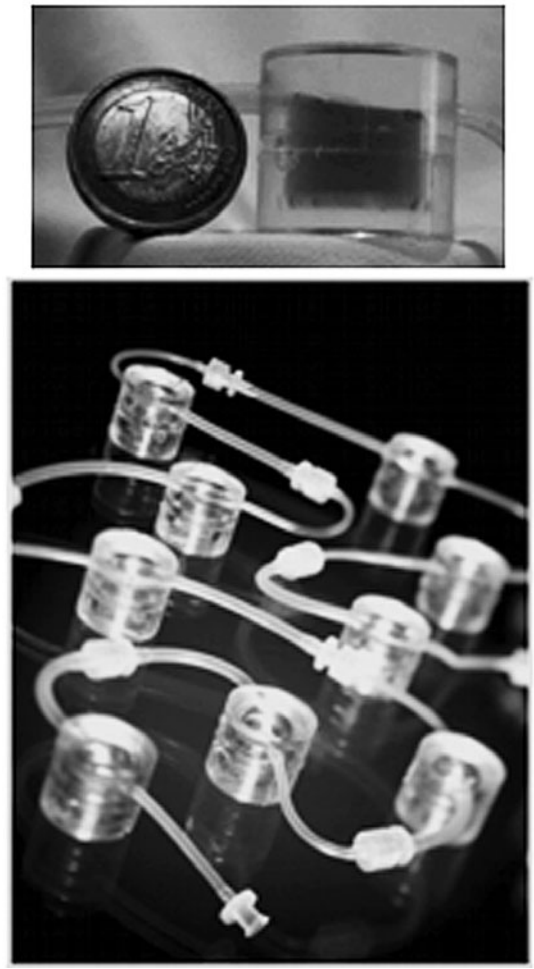

Figure 4. An MCmB module and Quasi-Vivo® circuit.

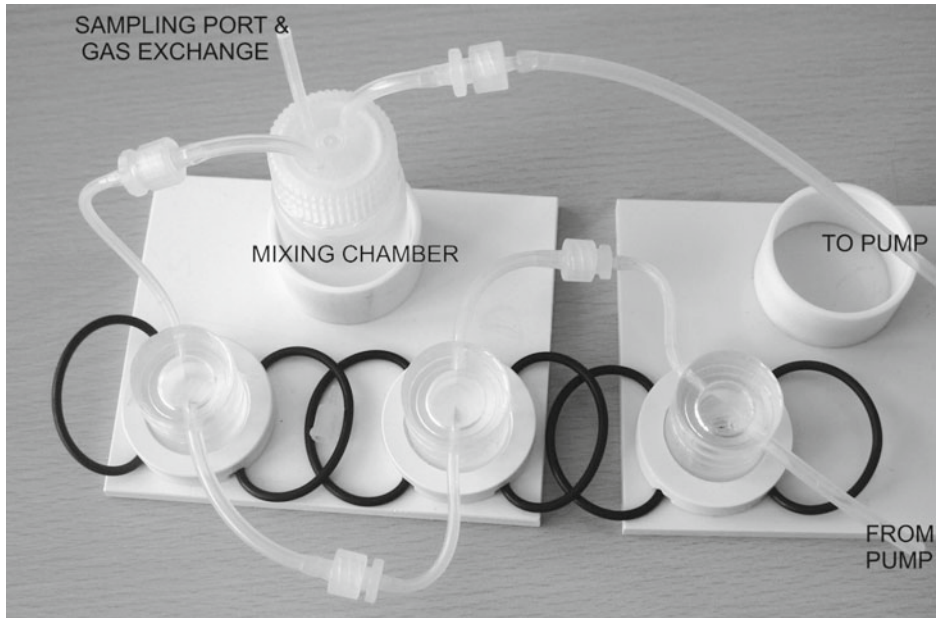

Figure 5. A simple 3-chamber Quasi-Vivo® circuit a showing the mixing chamber with sampling and gas exchange ports.

filter is usually placed on the gas exchange and sampling port. Typical circuit volumes are 2 mL per chamber plus at least 1 mL for the mxing chamber and 1 mL per meter of tubing, giving a total of 3.2 mL for 1 chamber.

In the following section, we describe how allometric scaling can be used to assemble Quasi-Vivo® organ models to represent biotransformation and absorption and exchange processes in vitro.

ALLOMETRIC SCALING

Allometry is the science of scaling and deals with changes in body size and relationships among different parameters and processes in all organisms, as a function of body mass, M.[23] The basic allometric equation can be used to correlate physiological variables between organisms of different sizes (Eq. 2):

$$Y = a \cdot M^b \tag{2}$$

where Y is the physiological parameter (for example time, length, metabolism), M is the mass of the organism, a is a proportionality factor and b is the allometric exponent. b varies according to the manner in which the parameter scales. Typically $b = 1$ for volumes and cell numbers and lies between 0 and 1 for rates (metabolic rates, flow rates, etc.), while it is negative for frequencies (cardiac frequencies, respiratory frequencies, etc.).

Traditionally, to estimate the value of *b*, data from different species are collected and plotted in a log-log graph in relation to animal body mass. *b* is the slope of the straight line which follows the data. Figure 6 is an example of the basal metabolic rates (BMRs) of different mammals in relation to their body masses. The slope of the straight line is 0.75, so *b* for BMR is 3/4. Note that these data refer to a young adult and one should keep in mind the variability between individuals. Indeed, allometric data generally refer to median values for a population or species.

The steps involved in estimating the value of *a* are more complicated, as *a* varies from parameter to parameter. A couple of well-known features and the allometric relation that links these parameters to the body mass, are necessary to evaluate *a.* If the aim is to estimate a particular feature, such as the BMR, the body masses and BMRs of different mammalian species can be found in the literature. The equation that links the BMR to the body mass is well known and it is valid for all mammals (Eq. 3):

$$BMR = a_{BMR} M^{\frac{3}{4}} \tag{3}$$

Then a_{BMR} can be estimated by substituting BMR and *M* for a given species.

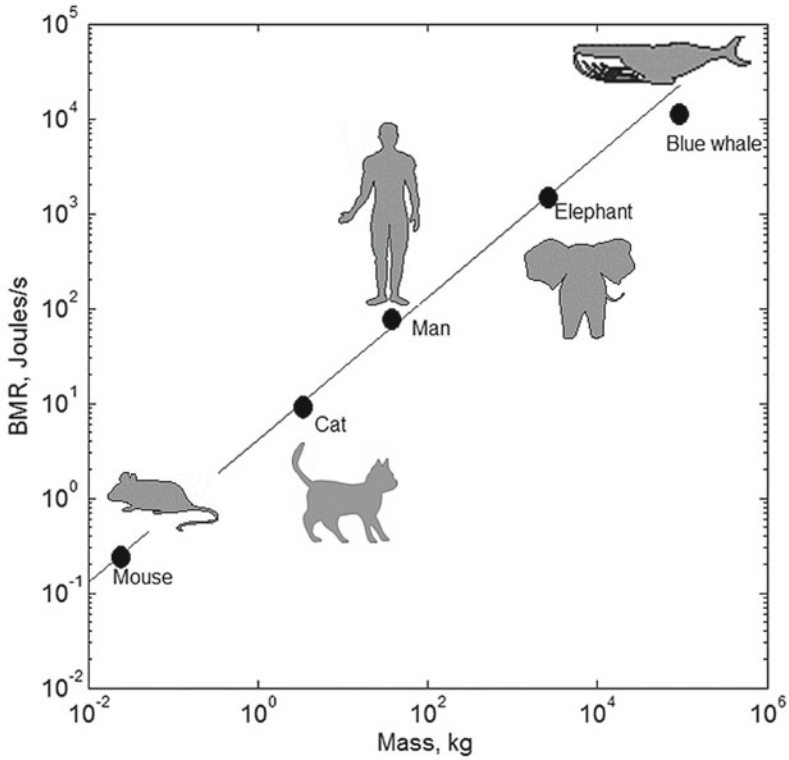

Figure 6. Basal metabolic rate of mammals in relation to body mass. The value of *b* is 3/4.

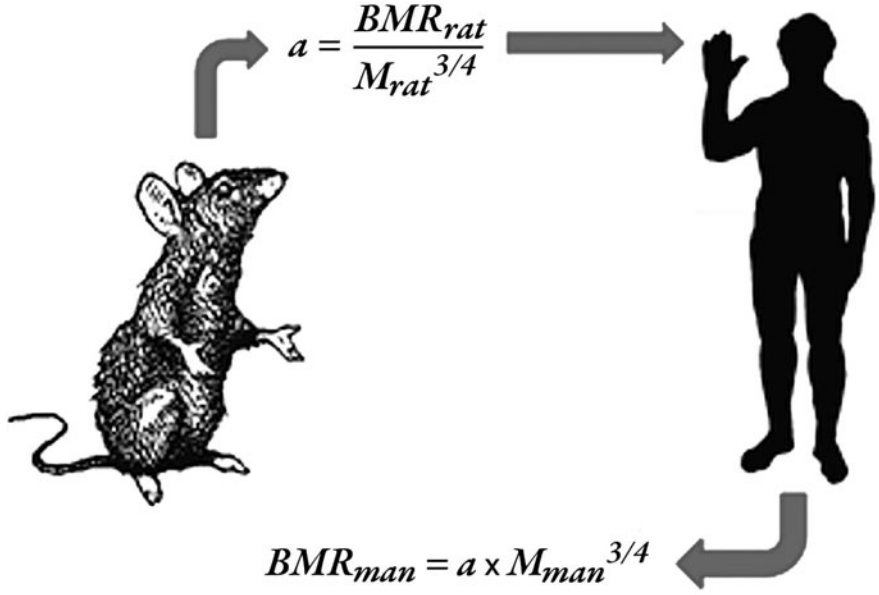

Figure 7. An algorithm for estimating the human BMR from rat data.

Subsequently, a_{BMR} can be used to evaluate the human BMR for a given body weight, by using Equation 3. Figure 7 summarizes the passages to evaluate the human BMR. The starting points are the body mass and the BMR of a rat.

The allometric approach can be used to evaluate the relations between different physiological tissues, in order to ensure that the same relationships are conserved in the bioreactor environment. Here, we show how allometry can be used to set up models of biotransformation, adsorption and gas exchange in the Quasi-Vivo® system.

Experimental Set Up: Quasi-Vivo® Biotransformation

Hepatocytes are the main orchestrators of metabolism and represent the starting point for most organ models. As cells are usually plated in monolayers, we begin the allometric design process by considering the metabolism of a 2D culture of human hepatocytes in a single MCmB module. For reference, we use a 70 kg, 20-year-old male (M_{man}), with a the tabulated BMR_H of 88 J/s or 1795 kcal/day and a liver mass of 1.4 kg, with about 2×10^{11} hepatocytes (see Table 1 for data sources).

The surface area of the hepatocyte module is known (1.33 cm^2), so we can estimate the number of hepatocytes which can be seeded in a monolayer. A confluent layer of hepatocytes has a density of about 2×10^5 cells/cm^2.[24] In the module, this corresponds to 2.6×10^5 cells. The liver generates 27% of the total BMR of a human, corresponding to 23.76 J/s. Then, assuming that the total metabolic contribution of the liver is due only to hepatocytes, the BMR per human hepatocyte is 119 pJ/s, while the equivalent BMR of the Quasi-Vivo® liver is 30 μJ/s.

Despite being a metabolic organ, liver mass scales with an exponent of $\beta \sim 1$. The explanation usually given for this is that small organisms have a larger number of metabolic organelles (mitochondria) per cell (β for the number of mitochondria per cell is 4/3). Then the total BMR of the Quasi-Vivo® system is 111 μJ/s (30/0.27).

We can now use the allometric equation (Eqs. 2 and 3) to estimate the equivalent mass of the Quasi-Vivo® liver ($M_{\downarrow(liver/QV)}$), as well as the total body mass that the Quasi-Vivo® system mimics (M_{QV}):

$$\begin{aligned} 88\frac{J}{S} &= a_{BMR}\,70^{\frac{3}{4}} \\ 111\mu\frac{J}{S} &= a_{BMR}M_{QV}^{\frac{3}{4}} \end{aligned} \tag{4}$$

giving $M_{QV} \sim 1$ mg, which represents the equivalent mass of the organism represented by the Quasi-Vivo® system.

Having established the metabolic parameters of the most metabolically-important organ, we now show an example of how to use the allometric approach to add a module to mimic the human lung.

Lung: Quasi-Vivo® Gas Exchange Model

The allometric relation that links the surface area (S) to mass (M) is (Eq. 5):[25]

$$S = aM^{\frac{2}{3}} \tag{5}$$

This equation holds true for organs responsible for exchange, such as the skin, lungs and intestines. Given that human lung mass is 800 g and its surface area is 80 m^2, the constant a relating lung area to body mass can be estimated. Having established the total mass of our Quasi-Vivo® system, we can calculate the equivalent Quasi-Vivo® lung area by using the same constant. The surface area of the chamber which simulates the lung is 4.7×10^{-4} m^2.

This value can be used to design a new chamber or to set up an allometric experiment by using the current modules. In particular, the ratio between the surface areas of the liver chamber and the lung chamber provides the relationship between the number of modules to mimic hepatic tissue and to simulate the lung:

$$\frac{\text{Area liver module}}{\text{Area lung module}} = \frac{4.7\ \text{cm}^2}{1.33\ \text{cm}^2} = 3.5 \tag{6}$$

Thus, the most sensible approach is to use one liver chamber and four lung chambers in series.

Similar considerations can also be used to design Quasi-Vivo® skin and intestinal adsorption, glucose metabolism, excretion and nutrient transport. Some examples are shown in Table 1.

Flow Rates

The flow velocity should allow medium to remain in contact with cells for sufficient time to allow them to transduce external signals from cells in adjoining modules. However, if the time is too long, then the cells may initiate down-regulation or other

Table 1. Allometric-based Quasi-Vivo® configurations for some physiological models

Quasi-Vivo® Physiological Model	Cells	Modules	Relationships
Biotransformation	Hepatocytes	1 liver	
Gas Exchange and Biotransformation	Hepatocytes, Lung epithelial cells	4 lung epithelia (confluent layer) 1 liver	Lung mass, b = 1 (800 g) Lung area, b = 2/3 (80 m^2)
Absorption and Biotransformation	Hepatocytes, Skin epithelia	12 liver, 1 skin epithelia (confluent layer)	Skin mass, b = 0.87, (4-8 kg) Skin area, b = 2/3 (1.8 m^2)
Nutrient absorption and Biotransformation	Hepatocytes, Intestinal epithelial cells	1 liver, 9 intestinal epithelia (confluent layer)	Intestinal bed, b = 2/3 (200 m^2)
Biotransformation and Nutrient transport	Hepatocytes, Endothelial cells	4 liver, 1 endothelial cell (confluent layer)	Capillary bed, b = 11/12 Total capillary bed area = 500 m^2 *
Biotransformation, Nutrient transport and Nutrient absorption	Hepatocytes, Endothelial cells, Intestinal epithelial cells	4 liver, 1 endothelial, 32 intestinal epithelia	

Data sources: (1) The Physics Factbook, www.hypertextbook.com. (2) Lindstedt SL & Schaeffer PJ. Use of allometry in predicting anatomical and physiological parameters of mammals. Laboratory Animals 2002; 36: 1-1. (3) Sohlenius-Sternbeck AK. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. Toxicol In Vitro 2006; 20(8):1582-1586.

* This values varies greatly in the literature; we use the value of 500 m^2 based on measurements by Kamiya A, Takeda S, Shibata M. Mat Biol 1987; 49(3):351-361, but it should be noted that (a) there is not enough blood to fill all capillaries, (b) additional capillaries are opened due to exercise, stress or action, so 500 m^2 probably corresponds to a resting state and could be doubled if necessary.

processes which limit cross-talk with cells in downstream modules. Given their high sensitivity to shear stress, hepatocytes are the flow rate limiting cells in a Quasi-Vivo® system. The flow rate of the adult human liver is 1000-1500 mL/min. The allometric exponent (b) for flow rates is 3/4, giving a maximum flow rate of ~2 μL/min in our Quasi-Vivo® system (a total passage time of about 1000 minutes per module). This flow rate is rather low and, according to the oxygen transport model shown in Figure 3, is not sufficient for ensuring an adequate oxygen concentration at the bottom of the module. Alternatively, we can choose to reproduce the mean organ perfusion time in the liver, which is about 2-5 minutes, giving a flow rate of about 400 μL/min. This is close to the experimentally-determined optimal flow rates of 300 and 250 μL/min for hepatocytes (see refs. 6 and 13, respectively).

Three Dimensionality

One of the most important structural cues, which has not been sufficiently emphasized, is the 3D architecture of tissues. Cells in vivo are surrounded by an extracellular matrix through which both biochemical and mechanical signals are transmitted and transferred to neighbouring cells. The models presented here are based on monolayer cultures, simply because this is the standard method used for in vitro preparations. For this reason, we have considered organ models based mainly on absorption and permeation. The Quasi-Vivo® modules are designed to house tissue slices and scaffolds of up to 5 mm in height,[6] both of which can be used to increase cell density and reduce the number of chambers required to represent exchange organs.

TRANSLATING QUASI-VIVO® DATA TO PREDICTIVE TESTING

Long Term Studies

A number of studies on cell sustainability have been conducted using the Quasi-Vivo® chambers. In Vinci et al,[26] HepG2 cells were seeded on 3D polylactide-coglycolide scaffolds and cultivated in three chambers placed series for up to 7 days. Both cell viability and albumin production rates were increased with respect to static monolayer controls over the entire culture period. In a different set of experiments carried out in collaboration with INSERM, Montpellier, human hepatocytes were cultured in collagen sandwich in 8 chambers in series over a 3 week period. Gene expression studies revealed a significant upregulation of P450 activity compared with static controls, with little decline of mRNA expression and xenobiotic activity over time.[13] Longer term and repeated exposure studies are currently being carried out.

Predictive Testing

Current in vitro cytotoxicity models are limited by their incomplete modeling of the cellular microenvironment and of the other cell types and interactions within a tissue, organ, system and whole body.[27] The Quasi-Vivo® system was designed to minimize these discrepancies by building organ models of increasing the complexity in a stepwise manner. We have shown that just by using hepatocytes in the system, it is possible to approach a response which is more predictive of in vivo behavior. A hepatotoxic drug, diclofenac, was used to demonstrate how the Quasi-Vivo® system can modulate cell response and sensitivity. Diclofenac is an arylacetic, nonsteroidal anti-inflammatory drug (NSAID), which is frequently prescribed in treating rheumatic diseases and as an analgesic.[28] The use of diclofenac has been associated with a mild to severe hepatotoxicity in a small, but significant, number of patients. Indeed, diclofenac-associated hepatitis is more common than had previously been realised and the drug now carries a regulatory warning. Diclofenac toxicity was detected in the Quasi-Vivo® system at concentrations two orders of magnitude lower than in conventional hepatocyte cultures. Table 2 lists comparative IC50 values

Table 2. Diclofenac IC50 values

Method	IC50
Quasi-Vivo®	50 ± 20 μM
Multiwell	590 ± 63 μM
Reference 29, comparison	392 ± 34 μM

for the drug in standard 24-multiwells and in the Quasi-Vivo® system (Vozzi et al, in preparation) as well as from reference 29. In humans, toxicity has been known to occur in cases with plasma concentrations of 4.2 μM diclofenac (the typical value of maximal plasmal concentration associated with efficacy). This concentration would cause detectable toxicity in the Quasi-Vivo® system, but not in a multiwell system.

CONCLUSION

Thus, the Quasi-Vivo® system is poised at the interface between drug discovery and animals, representing an enabling technology which shifts the assessment of drug effects in humans to a very early stage, resulting in a reduction in animal testing and progress in the Three Rs. While a number of issues, such as subject variability, the allometrics of dosage and the establishment of standard 3D cultures, still have to be addressed, new tools such as Quasi-Vivo® are set to become future standards in toxicology testing.

REFERENCES

1. Kirkpatrick J, Fuchs S, Hermanns I et al. Cell culture models of higher complexity in tissue engineering and regenerative medicine, Biomaterials 2007; 28(34):5193-5198.
2. Zhang S. Beyond the petri dish. Nat Biotechnol 2004; 22(2):151-152.
3. Cantòn I, Cole DM, Kemp EH et al. Development of a 3D human in vitro skin co-culture model for detecting irritants in real-time. Biotechnol Bioeng 2010; 106(5):794-803.
4. Van de Bovenkamp M, Groothuis GM, Meijer DK et al. Liver fibrosis in vitro: cell culture models and precision-cut liver slices. Toxicol In Vitro 2007; 21(4):545-557.
5. Sung, JH, Esch MB, Shuler ML. Integration of in silico and in vitro platforms for pharmacokinetic-pharmacodynamic modeling. Expert Opin Drug Metab Toxicol 2010; 6(9):1063-1081.
6. Mazzei D, Guzzardi MA, Giusti S et al. A low shear stress modular bioreactor for connected cell culture under high flow rates. Biotechnol Bioeng 2010; 106(1):127-137.
7. Di Nardo P, Minieri M, Ahluwalia A. Engineering the stem cell niche and the differentiative micro and macroenvironment: technologies and tools for applying biochemical, physical and structural stimuli and their effects on stem cells. In: Artmann GM, Minger S, Hescheler J, eds. Stem Cell Engineering: Principles and Applications. Heidelberg: Springer-Verlag, 2010:41-48.
8. Smith MK, Mooney DJ. Hypoxia leads to necrotic hepatocyte death. J Biomed Mater Res A 2007; 80(3):520-529.
9. Swartz MA, Fleury ME. Interstitial flow and its effects in soft tissues. Annu Rev Biomed Eng 2007; 9:229-256.
10. Pedersen JA, Boschetti F, Swartz MA. Effects of extracellular fiber architecture on cell membrane shear stress in a 3D fibrous matrix. J Biomech 2007; 10:1484-1492.
11. Wang DM, Tabell JM. Modeling interstitial flow in an artery wall allows estimation of wall shear stress on smooth muscle cells. Trans ASME 1995; 117:358-363.
12. Tilles AW, Baskaran H, Roy P et al. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. Biotechnol Bioeng 2001; 73(5):379-389.
13. Vinci B, Duret C, Klieber S et al. Medium flow stimulates expression and activity of detoxication genes in primary human hepatocytes in a multicompartment modular bioreactor. Biotech J 2011;6(5):554-564.
14. Wei CW, Cheng JY, Young TH. Elucidating in vitro cell-cell interaction using a microfluidic coculture system. Biomed Microdevices 2006; 8(1):65-71.

15. Tirella A, Marano M, Vozzi F et al. A microfluidic gradient maker for toxicity testing of bupivacaine and lidocaine. Toxicol in Vitro 2008; 22:1957-1964.
16. McBeath R, Pirone DM, Nelson CM et al. Cell shape, cytoskeletal tension and RhoA regulate stem cell lineage commitment. Dev Cell 2004; 6(4):483-495.
17. Francis K, Palsson BO. Effective intercellular communication distances are determined by the relative time constants for cyto/chemokine secretion and diffusion. Proc Natl Acad Sci USA 1997; 94:12258-12262.
18. Kilian KA, Bugarija B, Lahn BT et al. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci USA 2010; 107(11):4872-4877.
19. Vozzi F, Heinrich JM, Bader A et al. Connected culture of murine hepatocytes and HUVEC in a multicompartmental bioreactor. Tissue Eng Part A 2009; 15(6):1291-1299.
20. Guzzardi MA, Vozzi F, Ahluwalia A. Study of the cross-talk between hepatocytes and HUVEC using a novel multi compartmental bioreactor: a comparison between connected cultures and co-cultures. Tissue Eng Part A 2009; 15(11):3635-3644.
21. Guillouzo A. Liver cell models in vitro toxicology. Environ Health Perspect 1998; 106:551-532.
22. Nahmias Y, Berthiaume F, Yarmush ML. Integration of technologies for hepatic tissue engineering. Adv Biochem Eng Biotechnol 2007; 103:309-329.
23. West BG, Brown JH, eds. Scaling in Biology. New York: Oxford University Press, 2000.
24. Pichard L, Raulet E, Fabre G et al. Human hepatocyte culture. Methods Mol Biol 2006; 320:283-293.
25. Lindstedt SL, Calder WA. Body size, physiological time and longevity of homeothermic animals. The Q Rev Biol 1981; 56(1):1-16.
26. Vinci B, Cavallone D, Mazzei D et al. In vitro liver model using microfabricated scaffolds in a modular bioreactor. Biotechnol J 2010; 5(2):232-241.
27. Guillouzo A, Guguen-Guillouzo C. Evolving concepts in liver tissue modeling and implications for in vitro toxicology. Expert Opin Drug Metab Toxicol 2008; 1279-1294.
28. Boelsterli UA. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. Toxicol Appl Pharmacol 2003; 192(3):307-322.
29. Roque B, Ponsoda X, Jover R et al. Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. J Pharmacol Exp Ther 1999; 288(1):65-72.

CHAPTER 10

ECVAM AND NEW TECHNOLOGIES FOR TOXICITY TESTING

Michel Bouvier d'Yvoire,[1] Susanne Bremer,[2] Silvia Casati,[2] Mara Ceridono,[2] Sandra Coecke,[2] Raffaella Corvi,[2] Chantra Eskes,[3] Laura Gribaldo,[4] Claudius Griesinger,*[,2] Holger Knaut,[5] Jens P. Linge,[6] Annett Roi[1] and Valérie Zuang[1]

[1]REACH Unit, DG Enterprise and Industry, European Commission, Brussels, Belgium; [2]European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection (IHCP), DG Joint Research Centre (JRC), European Commission, Ispra, Italy; [3]SeCAM, Agno, Switzerland; [4]Molecular Biology and Genomics Unit, Institute for Health and Consumer Protection (IHCP), DG Joint Research Centre (JRC), European Commission, Ispra, Italy; [5]Skirball Institute, Langone Medical Center, New York University, New York, USA; [6]Global Security and Crisis Management Unit, Institute for the Protection and Security of the Citizen (IPSC), DG Joint Research Centre (JRC), European Commission, Ispra, Italy

**Corresponding Author: Claudius Griesinger—Email: claudius.griesinger@ec.europa.eu*

Abstract: The development of alternative empirical (testing) and non-empirical (nontesting) methods to traditional toxicological tests for complex human health effects is a tremendous task. Toxicants may potentially interfere with a vast number of physiological mechanisms thereby causing disturbances on various levels of complexity of human physiology. Only a limited number of mechanisms relevant for toxicity ('pathways' of toxicity) have been identified with certainty so far and, presumably, many more mechanisms by which toxicants cause adverse effects remain to be identified. Recapitulating in empirical model systems (i.e., in vitro test systems) all those relevant physiological mechanisms prone to be disturbed by toxicants and relevant for causing the toxicity effect in question poses an enormous challenge. First, the mechanism(s) of action of toxicants in relation to the most relevant adverse effects of a specific human health endpoint need to be identified. Subsequently, these mechanisms need to be modeled in reductionist test systems that allow assessing whether an unknown substance may operate via a specific (array of) mechanism(s). Ideally, such test systems should be relevant for the species of interest, i.e., based on human cells or modeling mechanisms present in humans. Since much of our understanding about toxicity mechanisms is based on studies using animal model systems (i.e., experimental animals or animal-derived cells),

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

designing test systems that model mechanisms relevant for the human situation may be limited by the lack of relevant information from basic research. New technologies from molecular biology and cell biology, as well as progress in tissue engineering, imaging techniques and automated testing platforms hold the promise to alleviate some of the traditional difficulties associated with improving toxicity testing for complex endpoints. Such new technologies are expected (1) to accelerate the identification of toxicity pathways with human relevance that need to be modeled in test methods for toxicity testing (2) to enable the reconstruction of reductionist test systems modeling at a reduced level of complexity the target system/organ of interest (e.g., through tissue engineering, use of human-derived cell lines and stem cells etc.), (3) to allow the measurement of specific mechanisms relevant for a given health endpoint in such test methods (e.g., through gene and protein expression, changes in metabolites, receptor activation, changes in neural activity etc.), (4) to allow to measure toxicity mechanisms at higher throughput rates through the use of automated testing. In this chapter, we discuss the potential impact of new technologies on the development, optimization and use of empirical testing methods, grouped according to important toxicological endpoints. We highlight, from an ECVAM perspective, the areas of topical toxicity, skin absorption, reproductive and developmental toxicity, carcinogenicity/genotoxicity, sensitization, hematopoeisis and toxicokinetics and discuss strategic developments including ECVAM's database service on alternative methods. Neither the areas of toxicity discussed nor the highlighted new technologies represent comprehensive listings which would be an impossible endeavor in the context of a book chapter. However, we feel that these areas are of utmost importance and we predict that new technologies are likely to contribute significantly to test development in these fields. We summarize which new technologies are expected to contribute to the development of new alternative testing methods over the next few years and point out current and planned ECVAM projects for each of these areas.

INTRODUCTION

Traditionally, toxicological testing has been relying on a rather limited suite of test methods, typically requiring the use of experimental animals and characterised by often observational readouts of adverse effects that focused on the downstream end of the causative chain leading to toxicity. This approach is increasingly being criticized[1] for essentially four scientific reasons intrinsically linked to the traditional model systems used:

Criticism 1. *"Wrong biology"*: Traditional testing paradigms are focusing on animal instead human biology with the consequence that the findings in animal models or models based on animals cells or cell lines may be of debatable relevance for the species of interest. Consequently, predictions on possible hazards and risk have to be extrapolated from animals to humans, adding considerable uncertainty.

Criticism 2. *"Extrapolation and uncertainty"*: Such across-species extrapolation has been criticized for being based on arbitrary judgment and not the careful evaluation of scientific evidence. This extrapolation is a considerable source of uncertainty and the focus of a good toxicity testing paradigm should be to reduce uncertainty wherever possible or at least to describe the level of (un)certainty in a transparent manner.

Criticism 3. *"Lack of mechanistic information"*: Traditional toxicity testing relies to a great extent on the observation of effects on a phenomenological level and does neither provide information on the mechanism of action nor on the dynamics of the biological reaction to the exposure. Knowledge of the mechanism by which xenobiotics trigger toxic

effects however is critical to improve the design of new pharmaceuticals and chemical products that are less toxic—at least with respect to those toxicity mechanisms that are known, understood and somehow embodied in standardized toxicity testing methods.

Criticism 4. *"High dose paradigm"*: Traditional toxicological testing has, for various reasons, been focusing on high dosing which led to an additional uncertainty regarding the extrapolation of adverse effects to be anticipated at lower exposure levels.

In this chapter we explore, from an ECVAM perspective aiming at the validation of standardized methods, how new technologies from biology and bioengineering may help to improve toxicity testing and how these technologies may address the weaknesses of the traditional testing paradigm as outlined above (criticism 1 to 4). We have subsumed all those approaches under the term 'new technologies' which have emerged over the last decades and, more importantly, have not yet been systematically integrated into the standardized toxicity testing regime, or, for some endpoints, have only very recently lead to standardized and internationally accepted guidelines for toxicity testing (e.g., OECD test guidelines). Such technologies may thus be regarded as 'new' to toxicity testing, independent of the fact that they may have been utilized for quite some time already within their original discipline of basic research (e.g., molecular biology, bio-medicine, pharmacology) as tools for basic and, to some extent, applied research and development.

This approach led us to describe the anticipated positive impact of tissue engineering (e.g., reconstructed human epidermis or corneal models), human-derived cells and cell lines, stem cells, 'omics' techniques (genomics, proteomics, metabonomics), imaging techniques, high-throughput testing, and, notably, conceptual approaches such as integrated testing strategies for toxicity testing. The overwhelming majority of new technologies rely on the use of alternative approaches to animal testing, i.e., the use of in vitro models as test systems which model the biological target organ or organism and/or the relevant physiological pathways known to be targeted by toxicants. A notable exception to this observation is the use of transgenic animals (e.g., in carcinogenicity testing). Although a potentially powerful tool, transgenic animals do not allow addressing some of the criticism of traditional toxicology (i.e., "wrong biology" and "extrapolation and uncertainty"). We refrained from including the use of transgenic animals in this chapter.

Finally, we would like to alert the reader to a recent comprehensive overview about the status of test methods for cosmetics testing, in particular with regard to the 2013 deadline of the Cosmetics Directive. This publication, prepared by an expert group co-ordinated by ECVAM, may be of interest to the reader wishing to gain insight into the current status of available test methods for the purpose of cosmetics testing and for the endpoints (1) toxicokinetics, (2) skin sensitization, (3) repeated-dose toxicity, (4) carcinogenicity and (5) reproductive toxicity.[2]

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

The relationship between chemical exposure and reproductive/developmental toxicity is highly complex, involving interactions between multiple organs and organ systems in both parents and their offspring at a variety of different time points and life stages. The inherent complexity of the vertebrate reproductive system represents a significant challenge to the development of in vitro and in silico models aimed at reducing, and ultimately replacing, animal use in this area of toxicology. As described for other complex toxicological endpoints, it will be nearly impossible to cover the whole reproductive cycle with all possible target

cells and mechanisms in vitro. Therefore, the possible applicability of testing strategies should be discussed to establish which toxicological information is necessary to identify potential adverse effects to mammalian reproduction and how various pieces of the necessary total information can be generated by a well-characterized subset of testing and nontesting methods. Conceptual testing strategies can be set up and are currently under development. Such testing strategies could be based either on existing information or on prevalence studies.

Within the 6th Framework Programme of the European Union (EU) a transnational Integrated Project (ReProTect, www.reprotect.eu), involving 35 partners from industry, academia and governmental institutions, had been set up aiming to develop and optimize in vitro tests that are able to detect key effects and mechanisms associated with reproductive toxicity. It was one of the objectives of this project to provide a toolbox of in vitro tests covering various aspects of reproductive toxicity, so that selected tests can be used as building blocks to compose testing batteries and strategies according to the toxicological questions at hand. In a series of strategic workshops, the adverse effects of reproductive toxicants have been discussed and various in vitro models have been evaluated. Major toxicological targets which could lead to adverse effects on mammalian fertility have been identified, such as effects on Leydig or Sertoli cells, folliculogenesis, germ cell maturation, the motility of sperm cells, steroidogenesis and fertilization, and on the pre-implantation embryo. In particular, for these chemical target cells, ReProTect has been optimizing in vitro tests based on primary cells/tissues, cell lines and genetically engineered cells.[3] Test protocols have been developed and the most predictive toxicological endpoints have been selected, to provide relevant information on the functionality and viability of these target cells. As a result of the ReProTect project ECVAM is already receiving (as of 2010/11) submissions for test method prevalidation and, potentially, also validation.

Other toxicologically sensitive aspects during mammalian reproduction include the processes of embryo implantation and embryo-maternal interactions. Human endometrial cell culture, human endometrial explant culture, endometrial-endothelial primary cells, microvascular fragment models and in vitro human trophoblast cell systems, as well as the human placental perfusion method, are currently being evaluated for their predictivity to assess chemical effects on implantation. Furthermore, transcriptional tests that detect the (anti)-estrogenic and (anti)-androgenic activities of compounds have been optimized and are now being statistically analyzed for their predictive power. A protocol for evaluating the ability of chemicals to bind to the estrogen receptor will be validated under the umbrella of the respective OECD (Organisation for Economic Co-operation and Development) validation management group.[4]

In addition to the complexity of mammalian reproduction, pronounced interspecies variations have been described, often showing less than a 60% correlation between the different laboratory mammalian species with regard to the assessment of developmental toxicity. Human embryonic stem cells for embryotoxicity testing promise to enhance the predictive value of such tests with regard to the estimation of human risk. After intensive public discussion, the legal and ethical frameworks for using human embryonic stem cell lines have been developed or are currently being further developed in the majority of the EU Member States. Due to the fact that an embryotoxicity test based on murine embryonic stem cells has already been validated,[5-7] the humanization of the tests will focus on the detection of chemicals, in order to circumvent the problems caused by interspecies variations. For a more comprehensive discussion on the potential use of stem cells, see the chapter by Stummann and Bremer.

CARCINOGENICITY AND GENOTOXICITY

According to the 7th Amendment to the EU Cosmetics Directive 76/768/EEC, in vivo genotoxicity testing in Europe has been banned for cosmetics since 2009. This is problematic, since in vivo genotoxicity assays are routinely used to address the biological relevance of positive results obtained in the standard battery of in vitro genotoxicity assays which yield a high percentage of false positive results for noncarcinogens.[8] In a report prepared by ECVAM and DG Enterprise, timetables for phasing out animal testing required an added step in the genotoxcity testing strategy.[9] Since skin cells are the site of first contact, it was proposed to establish genotoxicity testing in reconstituted 3D skin models. To address this, the EU Cosmetics Association (COLIPA), in collaboration with ECVAM and the UK National Centre for the 3Rs (NC3Rs), is co-ordinating a global, multi-laboratory study to assess the micronucleus and Comet endpoints in different 3D reconstituted human skin models.[10]

The high rate of false positives in the current in vitro testing battery is in part due to the choice of cell lines commonly used for genotoxicity testing.[11] Such cell lines typically lack normal metabolism, leading to reliance on exogenous activation systems, impaired p53 function (e.g., Aroclor-induced rat S9) and altered DNA repair capability.[11] Therefore, there is a need for new test systems which improve specificity without compromising sensitivity. At present, there are some systems under development and consideration which meet some of the requirements identified, but none of them meet all of the criteria. For example, reporter assays such as the Green Screen HS assay, are being developed. This assay uses human lymphoblastoid TK6 cells transfected with the GADD45a genotoxic stress response gene that, in turn, is linked to a gene encoding a green fluorescent reporter protein.[12] Multicentre trials are under way, to determine the reliability and relevance of the assay[12] and the assay has been submitted to ECVAM for eventual validation.

The impact of metabolism on the false positive rate in in vitro genotoxicity tests is not known. However, variation of the metabolizing system can have dramatic effects on the results of in vitro tests. Much effort will still be required to introduce a broader spectrum of metabolic capabilities into the test system. A comprehensive review on the possibility to incorporate metabolic capacity into existing test systems has been published as report of an ECVAM workshop.[13]

Higher throughput variants of the most commonly used genotoxicity/mutagenicity assays (e.g., the Ames and micronucleus tests) have been developed by industry. In the pharmaceutical sector these are used for the screening of new molecules at an early stage of drug development. Within the 6th Framework Programme, an EU sponsored project (COMICS) has developed a comet assay that has an increased throughput, by using multi-well format and cell arrays.[14]

The use of automated scoring of micronuclei in the in vivo micronucleus test has been recognized as acceptable for regulatory use.[15] In particular, flow cytometric analysis allows the scoring of micronuclei in peripheral blood, instead of bone marrow sampling. Consequently, chromosome damage can be monitored in rodents and in other species as part of routine toxicology studies (e.g., the 28-day repeated dose study) by sampling at different time points in the same animal. Therefore, the application of this method has the potential to lead to a significant reduction of the number of animals used for in vivo genotoxicity testing.[16]

In vitro cell transformation assays (CTAs) may contribute to the assessment of the genotoxic and nongenotoxic carcinogenic potential of compounds.[17] An ECVAM study

has demonstrated for 2 standardised CTA protocols that these assays are transferable and reproducible between-laboratories.[18-21] To facilitate the scoring of the transformed colonies or foci, which is still done manually under the microscope, several improvements are being investigated, including the detection of the transformation phenotype by using ATR-FTIR spectroscopy,[22] image analysis[23] or by the inclusion of molecular biomarkers.[24] Transcriptomics analysis is also used for mechanistic investigation of cellular transformation,[25,26] while activities to increase the throughput of the assay are ongoing such as one using soft agar colony screening[27] and the Bhas 42 96-well plate method.[28] Ideally, human-cell based systems would be clearly preferable to the rodent-cell based systems for cell transformation assays. Although this recommendation has already been made in 1999, no progress has been made in this respect.[29]

The developing field of toxicogenomics is expected to have an impact on assessing genetic toxicity as well as carcinogenicity. Furthermore, in the area of genetic toxicology, the use of transcriptomics technology has the potential to distinguish mechanisms of genotoxicity, e.g., those that have a threshold from those that act without a threshold.[30]

Regulatory testing for carcinogenicity currently requires a two-year rodent bioassay. Therefore, a significant refinement would be achieved by shortening the duration of in vivo studies while still ensuring the identification of the carcinogenic potential of tested chemicals through using early genetic effects as reliable indicators. Currently, toxicogenomics-based studies applied to in vivo models are carried out mainly by the pharmaceutical industries.[31] With the generation of high quality data and the subsequent population of databases with expression profiles, the use of toxicogenomics is expected to become more powerful.[32]

An initiative in this area is the carcinoGENOMICS project (www.carcinogenomics.eu), which is sponsored by the EU Commission within Framework Programme 6. The aim of the project is to develop a battery of predictive in vitro tests accounting for various modes of carcinogenic activity. The tests are designed to cover carcinogenicity in major target organs, including the liver, lungs and kidneys. Furthermore, pathway-associated gene expression will be combined with metabolic profiles generated in vitro, representing a highly innovative approach which might lead to bioinformatic models to predict the carcinogenic potential of a compound.

SENSITIZATION

Given the complexity of this toxicological endpoint, it is generally believed that no single alternative non-animal test will be able to substitute on its own for the currently used animal assays, in particular with regard to potency assessment. However, the mechanisms underlying sensitization are fairly well understood which offers to opportunity to develop and validate a suite of mechanism-based assays that address different biochemical and immunological mechanisms of the causal cascade leading to sensitization. In vitro and in chemico methods used in combination with in silico models may, however, provide sufficient information to allow the identification of potential skin allergens and the characterization of their relative potency. Progress has been made in the development of alternative methods that cover some of the key mechanisms involved in sensitization, e.g., skin bioavailability, protein binding, epidermal inflammation, dendritic cell (DC) activation, dendritic cell migration and T-cell proliferation.[33,34] Among these, the Direct Peptide Reactivity Assay (DPRA) which is addressing protein reactivity, the human Cell

Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitisation Test (MUSST) which monitor the expression of cell surface markers associated with DC activation are currently being evaluated in an ECVAM co-ordinated study. Another test method, the KeratinoSens™, has undergone an external prevalidation study[35] and is under evaluation at ECVAM. The KeratinoSens™ test method is based on a keratinocyte adherent cell line transfected with a selectable plasmid which contains the luciferase gene under the transcription control of SV40 promoter fused with the ARE (antioxidant response element) from the AKR1C2 gene. The activity of ARE-binding transcription factors in the cells in response to exposure to chemicals is measured using light-producing luciferase substrates.

Despite the progress made, efforts are still needed to identify the most relevant/sensitive endpoints to increase the predictive performance of existing tests that address these endpoints, to design new in vitro/in chemico/in silico assays for these key endpoints, which should ideally be of high throughput, and to develop strategies to combine these assays for allowing a complete assessment of the allergic potential of chemicals that may be absorbed by the human skin to an extent sufficient to trigger the relevant mechanisms (see also section on skin absorption below). Besides the conventional approaches, omics technologies are being applied already at the research level, but are however not yet ready for more standardized use within the context of predictive assays for hazard or risk assessment.

The Sens-it-iv integrated project, cosponsored by the EU Commission within Framework Programme 6, is aimed at the development of in vitro testing strategies for the replacement of the animal tests used for the identification of potential skin and respiratory allergens. Within Sens-it-iv, genomics and proteomics techniques have been extensively employed, to:

- identify new biomarkers able to discriminate sensitisers from nonsensitisers and from irritants;
- improve the understanding of relevant biological pathways activated by sensitizing chemicals;
- identify changes in the proteomic and genomic profiles of cells induced by treatment with sensitizing substances or derived from cross talking between the relevant cells; and
- detect the interactions of chemicals and haptens with cellular and noncellular proteins.

To achieve these objectives, high-density DNA microarray technologies for the gene analysis and a combination of high-resolution two-dimensional electrophoresis (2-DE) have been used in Sens-it-iv, together with mass spectrometry (MS) for the proteomic analysis.

The project also applied techniques such as MS and Nuclear Magnetic Resonance (NMR) in conjunction with other methods, to explore the role of metabolism in allergenicity, including investigations on the fate of chemicals susceptible of spontaneous or metabolic activations and the characterization of the metabolites generated, the disposition of these compounds in the target cells and the interaction of compounds with proteins. Information derived from this analysis has been used to define the metabolic capability of the in vitro cell methods evaluated by the project (ref: http://www.sens-it-iv.eu/). ECVAM was actively participating in the project and it is expected that Sens-it-iv will yield a number of in vitro methods that can potentially be used for hazard prediction and/or as building blocks for testing strategies aiming to assess the relative potency of sensitizers.

TOPICAL TOXICITY

Eye Irritation

In the area of eye irritation, four organotypic assays have already been accepted by regulators for detecting severe ocular irritants.[36] Other advanced in vitro methods are currently undergoing validation by ECVAM.[37] Notably, ECVAM and COLIPA are jointly sponsoring an ECVAM-co-ordinated full prospective validation study on two reconstructed tissue models. Although these assays appear to be promising for specific purposes and applicability domains, they may not fully address the relevant mechanisms of ocular toxicity. Some examples include the reversibility of effects due to tissue remodelling following inflammation. To achieve full replacement of the animal test, the use of tiered test strategies has been recommended, which exploit the strengths of particular in vitro assays, in order to address the required ranges of irritation potential, chemical classes and/or mechanisms of action. In 2010 ECVAM has, together with external experts, published a simple approach of aligning test methods in a strategic manner. This so-called bottom-up/top-down approach describes how to use specific eye irritation test methods for either identifying serious eye irritants from the rest of the chemicals or for identifying non-irritants (i.e., chemicals that do not require classification) from the rest of the population.[38] ECVAM is promoting this approach as means of a testing strategy using various in vitro methods which may in their totality and if used in this strategic manner allow replacing the Draize animal test. It was moreover recommended to advance the development of mechanistically-based models, in order to address the currently existing mechanistic gaps.[38]

The ICCVAM/ECVAM symposium on Mechanisms of Chemically-Induced Ocular Injury and Recovery, held in May 2005, identified the following aspects of ocular irritation where further investigation and development would be helpful:

- the assessment of depth of injury as potential indicator of irreversibility/ reversibility of effects;[39]
- the use of complex human models (e.g., 3D human corneal tissue reconstructs); and
- the use of inflammatory mediators (e.g., cytokines, adhesion proteins) as potential markers and predictors of ocular irritation.

In addition, investigation of the potential effects on the ocular nervous system could be of value. Such an endpoint is not always identifiable in the animal test, although it is important to avoid pain and discomfort during clinical trials and to the end consumer. In addition, it might be possible to gain an insight into the neurogenic components of inflammation, which could contribute to the general chemically-induced inflammatory response of the cornea.[40]

Currently, the COLIPA eye irritation research programme addresses some of the above mentioned mechanistic features, by supporting the development of mechanistically based assays, such as 3D human reconstructs and the assessment of inflammatory mediators and toxicogenomics as biomarkers to predict eye irritation. In addition, various isolated efforts exist within the scientific community addressing the same endpoints. ECVAM is following such efforts, to ensure that the most mechanistically relevant assays proceed toward validation according to internationally agreed principles, and eventually lead, most likely in combination with other assays, to the full replacement of specific animal tests, in particular the Draize-test.

Skin Irritation and Corrosion

Local hazardous effects of chemicals on skin will, depending on the severity of the inflicted damage, either lead to an irreversible local destruction of the skin (corrosion) or will trigger an active tissue reaction in the form of a reversible inflammatory response (skin irritation). The reasons why dermal toxicity model systems are among the most well developed and used in vitro methods include: (a) the number of validated models available: (b) the scientific sophistication of how the target organ is modelled in vitro; and (c) the ability of several methods in this area to dispense completely with the animal test (i.e., as full replacement methods).

Therefore, is there any further need to address dermal toxicity by developing alternative methods? Before discussing the application of new technologies for investigating skin irritation and corrosion, the current status of models and parameters ("endpoints") will be briefly reviewed.

Six methods have been validated for the assessment of skin corrosion. Four of them (the EpiDerm™, EPISKIN™, SkinEthic™ and EST-1000™ models) are reconstructed human epidermis (RhE) models.[41-46] In their overall design (use of human derived keratinocytes, representative tissue architecture), they closely mimic the biochemical and physiological properties of the outer layers of human skin, i.e., the epidermis (the EpiDerm™ model) and the epidermis and underlying dermis (the EPISKIN™ and SkinEthic™ models). In these model systems, the impairment of cell viability, as measured by vital dyes (e.g., MTT), is used as a predictor for corrosivity. These three methods and the TER assay (based on the change of transcutaneous electrical resistance of explanted rat skin due to chemical challenge) were validated as full replacements, although the provisions in the respective OECD guideline may demand the generation of additional data to support negative predictions.

With regard to skin irritation, ECVAM has during the last four years validated three commercially available test methods based on the principle of "Reconstructed human Epidermis" (RhE).[47,48] The three RhE-based test methods validated in 2007 and 2008 are the Episkin, the updated Epiderm and the SkinEthic assays—which had previously been validated by ECVAM for skin corrosion testing using different protocols. Inflammatory processes are responses to local tissue damage induced by mechanical stimuli (e.g., noxious stimuli), or by exposure to xenobiotic chemicals that have infiltrated the tissue. The latter may be glycoproteins or glycolipids on the surface of infectious agents (virus, bacteria, fungi etc.) or chemicals following topical exposure. The primary event in the signalling cascade triggering an inflammatory response during human contact dermatitis (i.e., skin irritation) appears to be damage to keratinocytes[49] which respond with the expression of immune-associated antigens[50] and the release of inflammatory mediators.[51] This demonstrates that keratinocyte damage is a key trigger of localised inflammatory reaction during skin irritation following topical exposure to chemicals. Importantly, this primary triggering mechanism is measured in RhE test methods: The protocols and prediction models of the RhE test methods are based on the measurement of cell viability (through reduction of the vital dye MTT) to predict whether a substance should be considered irritant or non-irritant (nonclassified). While it is agreed that there may be hitherto unknown mediators that, in addition to those known[52] may contribute to the inflammatory reaction in skin, there is very good evidence that the primary event seems to be tissue and cell damage. Since RhE lacks vascularisation, macroscopic physiological symptoms typical of inflammation (e.g., erythema, oedema, heat, pain) cannot be measured

in RhE model systems. The monitoring of causal factors other than cell damage acting upstream within the inflammatory cascade may improve both the predictive capacity of these tests as well as their biological/mechanistic relevance. This is supported by data from the ECVAM validation study: The sensitivity of one of the assays could be increased by measuring release of the inflammatory mediator interleukin (IL) 1α as a secondary endpoint in case of negative MTT assay results. Further work is required to progress such endpoints to potential routine use since the variability associated with the measurement of inflammatory mediators is currently high. In the ECVAM validation studies, all three methods were found applicable for the determination of the presence and absence of hazardous (skin irritant) properties of substances and, although originally validated taking the EU classification system into account, showed also satisfactory performance under the UN GHS-compliant rules for classification and labeling[53] and hence can also be used under the CLP Regulation EC 1272/2008.[54] In addition to substantial engagement towards the validation of full replacement methods for skin irritation, ECVAM has moreover critically contributed to the international acceptance of the RhE technology for regulatory purposes by providing key input for the drafting of the EU test method on in vitro skin irritation (method B.46), accepted in 2009 in the EU, as well as the expert consultations concerning the OECD draft test guideline 439 for in vitro skin irritation testing.[54]

Due to their experimental accessibility, in vitro methods hold the promise of allowing the direct measurement of causal factors involved in physiological responses to chemical challenges, rather than purely assessing the macroscopic or cellular symptoms of chemically-induced tissue trauma. Models for assessing hazardous skin effects are ideal candidates for advancing the parameters measured to predict toxicity: Firstly, the causal chain between the release of inflammatory mediators in response to a chemically-induced trauma and the primary or acute inflammatory response is well understood. Secondly, many methods for assessing topical toxicity are reconstructed tissue models which are based on cells of human origin and thus are biologically more relevant. However, the importance of choosing those parameters (or causal factors) with great care is perhaps best illustrated by a consideration of what exactly an increased IL-1α might mean when irritating or corrosive chemicals are tested in a skin model. In contrast to most extracellular proteins which are released by the classical ER/Golgi pathway, finally crossing the membrane barrier via vesicular release, IL-1α is secreted into the extracellular space via the nonclassical pathway that is independent of vesicles. Export probably happens via multi-protein complexes across the plasma membrane.[55,56] Thus, it is conceivable that, in the case of chemical destruction of the plasma membrane—a mechanism of toxicity associated with many irritant chemicals—IL-1α that is freely available in the plasma might leak through plasma membranes whose barrier function has been impaired by the chemical challenge. Therefore, increased levels of IL-1α might not reflect the active inflammatory response of the traumatised cell but rather provide readouts for plasma membrane impairment and cell damage with a higher sensitivity than the MTT assay. This example might be taken as supporting the notion that the identification of as many mechanistically relevant endpoints as possible is crucial to arriving at a situation where the appropriate and most relevant causal factor can be chosen from a multitude of available readouts, so that meaningful and reliable parameters are used as toxic predictors for each given test setting and/or toxic event.

To further the development of mechanistic parameters, ECVAM also assessed two promising new predictive technologies: toxicogenomics and toxicometabonomics (c.f. section on metabonomics below). In the toxicogenomic approach, eight chemicals

(four irritants and four non-irritants) were used at sub-cytotoxic doses to examine the differential expression of mRNA by quantitative real-time PCR in the EPISKIN™ model. The data were compared with those from preparations under control conditions. The mRNA extracted from the tissue models was transcribed into cDNA and hybridized on the DualChip human aging gene chip. The chip contains 240 genes involved in keratinocyte biology, as well as in senescence and stress responses. A subset of 30 genes appeared to be selectively up-regulated or down-regulated by either irritants or non-irritants and thus might serve as a predictor for these two classes of toxic effect. The genes identified are involved in cellular stress responses, cellular signalling, cell growth and cell cycle regulation, or protein metabolism, or are genes coding for cytokine or structural proteins. Two interesting examples are the small inducible cytokine A5 (CCL5), a mediator of the acute inflammatory response, and tissue activator inhibitor 1 (PAI-1), which controls the extracelluar proteolytic plasminogen-plasmin cascade involved in matrix integrity and tissue remodelling. Remarkably, none of the differently regulated genes was involved in apoptosis but the genes are involved in processes highly relevant for chemically-induced trauma, i.e., inflammation or tissue remodelling, including cell division.[57] Such toxicogenomic mapping, together with a toxicoproteomic approach, can be used to confirm whether up-regulated genes are really translated into augmented protein levels and might increase our understanding of the pathways of toxicity involved in corrosion and irritation and lead to the development of better tests.

Within an ECVAM in-house research project using the metabonomics approach, the EPISKIN™ model has also been used to study the ability of test chemicals to alter metabolic profiles. Here, Mass Spectrometry (MS) was used to identify the changes in metabolite levels after chemical challenge, following the metabolic "foot-printing" approach, i.e., by analyzing differential changes in extracellular metabolites released into the culture medium.[58,59] Changes in the metabolic state restricted to the cell interior (the "finger-printing" technique) are not yet being assessed. It has been shown that differential, but yet unidentified, metabolite-peaks can be identified in response to irritant chemicals, which are absent in controls and in non-irritant profiles (unpublished results). Such specific patterning of peaks, together with their identification, might lead to the development of specific biomarkers useful for predicting toxicity. Alternatively this approach may improve our understanding of toxicity pathways, especially when combined with a toxicogenomic and toxicoproteomic strategy. While it has been demonstrated that the metabonomic approach can distinguish irritants from non-irritants it now needs to be assessed whether this approach is sufficiently reliable, robust and transferable and more powerful than classical endpoints with regard to the accuracy of its predictions.

Skin Absorption

New technologies such as tissue engineering allowing the reconstruction of human epidermis or full-thickness skin models (i.e., modeling epidermal and dermal layer) hold the promise to improve standardized skin absorption testing for toxicological risk assessment. Skin absorption is one of the exposure routes by which toxicants may enter the human body (along with inhalation, ingestion and, in the context of pharmaceuticals, injection). Understanding to what extent and how readily substances are taken up through the skin and may become available on a systemic level in the human body is thus of tremendous importance for toxicology. The skin is most readily exposed in many scenarios of occupational and consumer exposure. In particular, skin absorption is of pivotal importance for assessing

possible adverse effects of cosmetics on human health since the majority of cosmetics are applied directly, voluntarily and often repeatedly on skin. Additional momentum in this field stems from the emerging field of nanotechnological applications which will require robust tools to estimate the potential absorption of such materials through the skin[60,61] to arrive at risk assessments that are based on a realistic estimation of the possible systemic availability of these materials. Moreover, the growing interest in transdermal delivery of drugs (also but not only, of nanosized dimensions)[62] adds a further dimension to the importance of a reliable methodology for estimating skin absorption.

Skin absorption can be differentiated into (a) dermal adsorption of substances at the stratum corneum layer, (b) dermal absorption of substances in the epidermis and dermis and (c) the percutaneous penetration into the subcutaneous layer and systemic availability. However, due to extensive vascularisation of the dermis and its vicinity to the epidermis, substances found in both epidermis and dermis may be already considered as potentially bioavailable and having passed into the systemic compartment.[63] Skin absorption has traditionally been tested through animal experimentation (OECD TG 427 on in vivo skin absorption[64]), but since 2004 also an in vitro OECD test guideline is available that describes the use of excised skin for measuring absorption/penetration of substances into a diffusion cell (OECD TG 428 on in vitro skin absorption[65]). Importantly, viable and nonviable skin (including skin which has been stored in the freezer) may be used since the process of skin absorption appears based not on active transport mechanisms but on the passive properties of the skin (in particular its barrier function) and the physicochemical properties of the molecules to which the skin is exposed. Guidance on the conduct of skin absorption studies has been published by OECD also in 2004 (OECD Guidance Document Nr. 28 for the conduct of skin absorption studies[66]). For the in vitro method, skin from various mammalian species (e.g., rat, pig, guinea pig) or human donors can be used. While the basic design of mammalian skin is the same across species, considerable differences exist with regard to the thickness of the skin, the number of cell types and, of particular relevance, the density of skin appendages such as hair follicles/shafts and sweat or sebum glands which potentially allow the absorption of substances via the "shunt pathway": Substances may circumvent the epidermal stratum corneum with its considerable barrier function and enter the dermis and thus the systemic compartment via the lateral (nonstratified) walls of such appendages. Consequently, penetration through animal skin (e.g., rat) skin is generally much greater than through human skin due to the much higher density of, in particular, hair follicles.[67] Pig skin appears to model more accurately human skin absorption properties.[68] While the current in vitro skin absorption method has a number of advantages over the in vivo method (e.g., replicate measurements possible, intended use exposure can be studied, different physical forms can be assessed), there are also several considerable drawbacks: First, when using human skin, supply of skin samples may be limited and there is considerable donor variability, the latter also being an issue in case of samples of animal skin. Second, there is considerable variability associated with the existing methods due to different (nonstandardized) study designs, donor and receptor media, experimental set-ups[69] and, in particular, due to the use of different skin types[70-73] which confounds the comparability of published data. [72,74] This has led to the conclusion that the in vitro methodology based on excised human or animal skin requires further development and standardization[73] despite the fact that it is already laid down in an OECD test guideline. Finally, the available in vitro technology, at least if based on excised animal skin, propagates one of the known difficulties of the in vivo skin absorption methods which is

the generally higher permeability of animal skin compared to human skin. Although this issue has been mentioned in the OECD's guidance document on skin absorption testing (2004), it is, with the current suite of available methods, far from solved.

Currently, the emphasis towards an improvement of skin absorption technology is clearly on reconstructed human epidermis or full thickness models as already used for skin corrosion and skin irritation testing. Recently a number of groups have made considerable progress in using ECVAM-validated reconstructed human epidermis (RhE) models (such as the Episkin™, Epiderm™, SkinEthic™ models) and other models (e.g., the Phenion™ full thickness model) for purposes of skin absorption measurements of chemicals and pharmaceuticals[75-78] and some protocols have already undergone (pre) validation.[79,80] Obvious advantages of this approach include better quality control of the test system (i.e., quality controlled reconstructed tissue batches vs acutely prepared animal skin) and improved species relevance (RhE models are based on normal human keratinocytes). Moreover, the availability of one validated test system (RhE) allowing the testing of three endpoints (skin corrosion, irritation and absorption) by using specific protocols constitutes an obvious logistical and economical benefit for end users of test methods. However, despite all progress, this approach still suffers from the fact that reconstructed human skin in vitro generally shows a considerably weaker barrier function than human skin, complicating the accurate estimation of skin absorption/penetration. Zghoul et al,[75] for instance, explored the use of reconstructed human skin equivalents for assessing drug absorption in comparison to human skin and, while concluding that such technologies have potential as a pharmaceutical test system to study dermal drug transport from topical formulations, observed a five times higher flux rate than in human epidermis. A recent review on the use of various reconstructed skin models as alternatives to human and pig skin for in vitro permeation testing of drugs further stressed the issue of differences of barrier function between human skin and human skin reconstructed in the dish.[81] This problem may either be addressed by altering (i.e., increasing) the barrier function of these RhE models or by adjusting the prediction models in order to take different barrier functions/diffusion rates into account. Importantly, recent progress in mathematical models for skin absorption[82] as well as QSAR models[83] may help in defining and refining the parameters of prediction models of empirical test systems based on tissue-engineering and may, in addition, support the interpretation of empirical data (whatever their origin) and thus support the risk assessment of substances taken up through the skin.

In summary, owing to the key role of exposure data via the dermal route in the context of cosmetics safety as well as the advent of nanotechnological applications and the growing interest in transdermal drug delivery, improved skin absorption test methods are urgently required. These should be relevant for humans, reproducible and robust enough in order to allow, in comparison with existing methods, a more accurate estimation and prediction of the expected human dermal absorption and percutaneous penetration of substances. New developments in tissue engineering such as reconstructed human epidermis (RhE) models and, in particular, full-thickness models, hold the promise to provide standardized test methods of human relevance and low batch-to-batch variability. However, before these methods can be used for routine testing, further optimization and rigorous independent assessment/validation are still required. ECVAM is following development, optimization and validation activities of human-based skin tissue models and has encouraged the submission of these test methods for ECVAM validation.

Phototoxicity

Phototoxicity is an acute toxic response that is elicited after the initial exposure of the skin to certain chemicals and subsequent exposure to light, or that is induced by skin irradiation after the systemic administration (oral, intravenous) of a chemical substance. The assessment of whether a chemical is likely to cause adverse phototoxic effects is required, if the chemical absorbs UV or visible light and is intended for human use.

Several animal tests used for predicting acute phototoxicity in humans exist, but none of them has been scientifically validated. It was proven during an international EU/ECVAM/COLIPA validation exercise that the phototoxic potential of chemicals can be predicted by using the 3T3-NRU in vitro phototoxicity test (3T3-NRU-PT).[84,85] This in vitro test, which involves the use of the immortalized mouse fibroblast cell line, Balb/c 3T3, gained regulatory acceptance in all the EU Member States in 2000[86] and in the OECD Member States in 2004 as Test Guideline (TG) 432 and is now widely used in the chemical and cosmetic industries.[87]

Determination of the phototoxic potential of a chemical in the 3T3-NRU-PT is often the first step in a sequential phototoxicity testing strategy. If a chemical provides a negative result in the 3T3-NRU-PT, no further testing is required in most instances. However, if the result is positive, the chemical may still be applied topically to the skin at safe concentrations, depending on the absorption and accumulation of the chemical in the skin. Thus, in addition to the information on phototoxic potential, as assessed using the 3T3-NRU-PT, additional testing may be required to obtain combined information about the phototoxicity and bioavailability of the chemical in the skin and about the relative phototoxic potential of the chemical, i.e., its phototoxic potency.

Ideally, a photopotency test should be performed in vivo on human volunteers, but this is often not acceptable for ethical reasons, especially if the chemical is a potential photoallergen. Reconstituted, 3D human skin models could offer an effective means of avoiding the need for confirmatory testing in vivo in animals, especially since such models are characterized by having both viable primary skin cells and skin barrier functions. In addition, high-quality, reconstituted human skin models are now available from a number of commercial sources, as illustrated above. These have been used successfully in the routine safety testing of skin products in various laboratories, since they are directly relevant to the organ of major concern. Such human 3D models could offer the following advantages when compared to the 3T3-NRU-PT:

1. A wide spectrum of chemicals or complex mixtures can be applied, simulating more closely the topical application to the skin.
2. Test concentrations more similar to real exposure conditions, including dermatological patch techniques, can be used.
3. Test materials can be applied in an undiluted form, so that aqueous solubility of the test material is not a limiting factor.
4. Materials with extreme pH values can be tested.
5. Histology can be performed on exposed and control samples.
6. Exposure to light can better be adapted to real-life situations, e.g., exposure time and the spectrum of simulated sunlight (a higher dose of short-wave light in the UVB range).
7. Depending on the barrier function of the stratum corneum, the absorption and penetration of the original chemicals or molecules created during exposure could provide more relevant results than tests performed on simpler systems (giving fewer false-positive results).

The first phototoxicity studies performed on a reconstituted human skin model were carried out by using the Skin^2 model, although the production of this model was discontinued in 1996.[88] The protocol was then successfully transferred to another reconstituted human skin model, EpiDerm™.[89] Preliminary studies had already shown that in vivo photoallergens which are not acute photoirritants (e.g., coumarin, 6-methyl-coumarin, musk ambrette), are correctly classified as negative by the skin model phototoxicity tests, while they are positive in the 3T3-NRU-PT.[90,91] Based on the promising outcome of an ECVAM-funded prevalidation study on the EpiDerm™ model[92] and the proposal by industry to apply the model in a tiered strategy to identify those chemicals that are predicted to be likely to be phototoxic in the 3T3-NRU-PT but are negative in vivo,[93] the European Medicines Agency (EMA) has suggested, in a Draft Guidance Document on Photosafety Testing, that confirmatory testing can be performed on such a skin model.[94] A feasibility study on whether the prevalidated human 3D model in vitro phototoxicity test, EpiDerm-PT, could successfully be used for phototoxic potency testing was performed, showing the usefulness of reconstructed human tissue models for prediction of phototoxicity of topically applied substances and formulations. This study showed that, in certain cases, the human situation may be underpredicted and that a precautionary factor of about 10 should be considered for extrapolation.[95]

Since the introduction of the EMA guidance document in 2002, the 3T3-NRU-PT was used extensively within the pharmaceutical industry. A number of companies found they were encountering a high percentage of positive results with the 3T3-NRU-PT (up to 50%). Importantly, the test, by evaluating photo-cytotoxicity, does only provide information on the intrinsic phototoxic *potential* of substances. While substances positive in the 3T3-NRU-PT assay are likely to be phototoxic, it is important to note that the test does not directly predict whether or not a substance acts as a phototoxic compound.

A survey of the European Federation of Pharmaceutical Industries and Associations (EFPIA) member companies was thus performed to capture industry experience.[96] The phototoxicity data was provided by 10 EFPIA member companies on 361 pharmaceutical candidates covering a broad range of different pharmacologies, including many nontopical ones. Of the 349 compounds tested in the 3T3-NRU-PT, 157 compounds (i.e., 44%) produced a PIF value of >5 and were classified as positive and 30 compounds (i.e., 8%) produced PIF values between 2 and 5 and were classed as equivocal. Of these 187, 26 were retested either on animals or humans and 85% of the retested substances were found to be negative in the animal model/human clinical tests. The survey concluded that the results of the 3T3-NRU-PT assay on the phototoxic potential of substances do not correlate very well with in vivo phototoxicity in animals and humans in the clinic. An independent survey was conducted in Japan by the Japan Pharmaceutical Manufacturers Association (JPMA), showing analogous results. A meeting took place between ECVAM and EFPIA in July 2010 at which the results of these surveys were discussed, and it was agreed that a focused expert workshop on the 3T3-NRU-PT would be held to present the 'in use' experience with the 3T3-NRU-PT applied to pharmaceuticals and to discuss why it differs from the result in the original validation exercise. This workshop, jointly organized by ECVAM and EFPIA, was held on the 25-27/10/2010 in Somma Lombardo, Italy.[35] experts from industry, academia and regulatory bodies were invited to contribute their experiences with the 3T3-NRU-PT. During the workshop, the assay methodology was reviewed, data from EFPIA and JPMA surveys were presented and reasons for different outcomes were discussed. These reasons include deviations in the protocol applied by industry as compared to the OECD Test Guideline 432, and differences between compound sets used

in the original validation study and compounds routinely encountered in nonclinical safety evaluation of candidate drug molecules. Experts from the cosmetics and pharmaceutical industries presented their experience with the 3T3-NRU-PT and evidence was presented for phototoxic clinical symptoms that could be linked to certain relevant molecules. To reduce the percentage of positive results, it was suggested to consider testing only for compounds showing a Molar Extinction Co-efficient (MEC) >1000 L/mol/cm, to limit the top concentration of test material under irradiation to 100 μg/mL, and to consider higher top concentrations without irradiation only to establish IC_{50} values for PIF calculation (if needed). In addition, data on the use of the assay collected from the pharmaceutical industry should be reviewed and, depending on data review, it was proposed to apply PIF < 5 or MPE < 0.15 thresholds for "negative" results (nonphototoxic) more generally (according to validation data), rather than PIF < 2 or MPE < 0.1. It was agreed that different PIF/MEP thresholds may have to be defined for topical (e.g., cosmetic) and nontopical (e.g., systemic pharmaceutical) compounds, since the current cut-off values defined in the OECD Test Guideline 432 appear to work well for chemicals relevant to the cosmetic industry. The state of play of alternatives to the 3T3-NRU-PT and the potential usefulness of 3D models as a second-tier test were described and discussed. The conclusions of the workshop were that test users should try to adhere as much as possible to protocol and OECD Test Guideline 432 standards and should be able to share data in the future in a common format including all relevant parameters. There should be a follow-up on outliers/anecdotal reports appearing not to support the proposed triggers in a tiered testing strategy (identify maximum IC_{50} values obtained under irradiation, identify minimum PIF/MPE values obtained, identify if retesting is required to confirm outliers). The newly formed ICH S10 EWG should follow-up on workshop proposals to better define how data based on OECD TG 432 can be used for risk assessment of pharmaceuticals. The workshop report is in preparation and will be submitted for publication later in 2011.

ALTERNATIVE APPROACHES TO IN VIVO TOXICOKINETICS: PREDICTION OF KINETICS INTEGRATING INFORMATION GENERATED IN SILICO AND IN VITRO

Understanding toxicokinetics, or more precisely the kinetics of a chemical that has entered the human body, is essential for assessing its potential toxic effects, which depend on the concentration to which target cells and organs are exposed, the so called internal exposure (see the extensive discussion on toxicokinetics in the recent review report on alternatives for cosmetics testing[2]).

Alternative techniques for predicting toxicokinetics of chemicals in animals or in man were developed mainly by the pharmaceutical industry for the purposes of weeding out pharmaceutical compounds likely to have undesirable kinetic, safety or efficacy profiles early on in the drug screening and development process. In vivo but also a variety of in vitro and in silico tools have been developed for predicting the absorption, distribution, metabolism and excretion (ADME) of drug candidates.[2] These predictions are usually done at various stages of drug development, by using all the available evidence and generating additional meaningful information from toxicokinetic experiments. The toxicokinetic information generated can be used, in particular, to select substances to be further developed, to direct further testing and to assist in the experimental design of the toxicodynamic evaluations to determine the toxicity profiles, thus providing

savings in terms of cost, time and animal use. Although some adjustments are probably necessary to increase the applicability of these methods for assessing the toxicity of chemicals used in other sectors, such as avoiding the use of radio-labeled materials, there is no reason not to use these techniques. Some sectors such as the cosmetics sector are being confronted with specific deadlines for phasing out not only topical toxicity testing but also systemic toxicity testing in animals including conventional toxicokinetic methods. It is evident that, for this sector, the basis of any future complete replacement will depend to a large extent on the methods derived from toxicokinetics[2] and from reliable methodologies assessing likely systemic availability of chemicals following topical exposure (see section on skin absorption above). Understanding the underlying toxicokinetic processes will provide the key to predicting all the other areas of toxicological concern and help rationalize the tests required—knowing the bioavailability of the relevant uptake routes will reveal whether systemic toxicity tests or just local toxicity tests are necessary. Moreover, the ability to relate toxicodynamic information from an in vitro nominal concentration-effect relationship to an in vivo dose-effect relationship—essential for risk assessment purposes—relies per se on toxicokinetics. Whilst the challenges related to developing accurate toxicokinetic models cannot be underestimated, many international collaborative research efforts are beginning to start up. Such efforts should undoubtedly be supported since the rewards are immeasurable and impact a wide area of fields, including the support of other EU policies, such as those related to the cosmetic and chemical sectors (e.g., REACH).

Breakthroughs in toxicokinetics not only promise to bring the panacea of complete replacement of animal tests but also to provide much more reliable data on the effects of chemicals in humans—information that can never be extrapolated in a straightforward way from animal tests.

In practice, the prediction of the toxicokinetic and toxicodynamic behavior of a chemical rests upon the use of physiologically-based compartmental pharmacokinetic (PBPK) models and data generated from a series of relevant in vivo, in silico or in vitro models. In silico models or in vitro techniques have been developed for estimating parameter values used to predict absorption, metabolic clearance, distribution and excretion. Blaauboer[97,98] reviewed the techniques involved in toxicokinetic prediction by using physiologically-based kinetic models. A general discussion on the in silico methods used to predict ADME is provided by Boobis et al.[99]

As for all predictions made by using models, including those using animal models, the potential variability in the target population and the uncertainty of the predictions made have to be balanced against the objectives of the prediction. Experimental in vivo evaluation of the predictions made and refinement of the PBPK-models used is usually necessary[100,101] and has to be carefully planned on a case-by-case basis. Strategies for integrating the predicted and experimental kinetic information generated routinely during drug development are to be found in the literature.[102-104] The principles described for pharmaceutical applications are relevant to kinetics simulation and prediction in the field of chemical safety since they permit the integration of the available kinetic or kinetically-relevant information from the very beginning of the risk assessment process. This strategy starts in the initial stages of compound development (e.g., pharmaceuticals, biocides, etc.) with simulations that can be generated by using only physicochemical characteristics, which themselves can be derived from in silico models (QSARs/QSPRs). The strategy proposed by Jones et al[104] for the set of (pharmaceutical) compounds under investigation, led to correct prediction of pharmacokinetics in man

for approximately 70% of the compounds. According to the authors, sufficiently accurate predictions were achieved mainly for compounds that were cleared by hepatic metabolism or renal excretion, the absorption and distribution of which were governed by passive processes. Significant mis-predictions were made when other elimination processes (e.g., renal and biliary excretion) or active processes were involved, or when the assumptions of flow-limited distribution and well-mixed compartments were not valid. In addition to the parent compound, metabolites may contribute significantly, or even predominantly, to the overall exposure-response relationship. In such cases a separate study program on the relevant metabolites may become necessary. To enable this high throughput, highly sensitive analytical methods will be essential to establish the ADME of the parent and its possible metabolites that may be present only in very low concentrations. Without such analytical tools a wide-spread use of toxicokinetic approaches in sectors different from the pharmaceutical sector will remain challenging.

HEMATOPOIESIS

Hematopoiesis is an elaborate process, in which pluripotent hematopoietic stem cells (PHSCs) differentiate into many types of highly-specialized circulating blood cells. At least 95% of hematopoietic cells fall into morphologically-recognizable cell lineages. Dormant PHSCs are recruited into the cell cycle by many cytokines such as IL1, IL3, IL4, IL6, IL11, IL12, SCF (stem cell factor), G-CSF (granulocyte-colony stimulating factor), M-CSF (macrophage-colony stimulating factor), Epo (erythropoietin), LIF (leukemia inhibitory factor), FLk2/FLT3 (tyrosine kinase receptor ligand) and TPO (thrombopoietin). Lineage-specific factors support the survival, proliferation and maturation of progenitors that are committed through the hypothetical stochastic expression of specific groups of differentiation genes.

Due to its rapid turnover, the hemopoietic tissue has the capacity to respond quickly to an increased demand for mature cells (for example, following blood loss or infection) and can maintain this response for prolonged periods of time. This rapid rate of renewal also makes the hemopoietic system a sensitive target for xenobiotic toxicity. Therefore, hematotoxicology is concerned with adverse effects of xenobiotics and pharmacological levels of endogenous substances on the number of cells and their functions. Mature and maturing erythrocytes, leukocytes and platelets circulate in the bloodstream, where they are usually exposed to higher concentrations of xenobiotics than are any other cell types. Xenobiotic exposure can lead to cytotoxic effects on cell function or to cytolysis, either directly or in concert with immune mechanisms. Xenobiotics may interfere with proliferative activity and with the complex regulation pathways that modulate differentiation.

The toxicological evaluation of the hematopoietic system is part of most preclinical and clinical safety studies and has become routine in monitoring a variety of novel and conventional therapies in humans and animals. The latter tendency makes this easily accessible tissue particularly useful in monitoring for systemic toxicity, while primary hematotoxicity ranks alongside liver and kidney side effects as an important source of attrition. In recent years, there has been increasing interest among industry and regulatory bodies in the development and use of in vitro tests for predicting in vivo hematotoxicity. Some in vitro tests have already been validated.[105] In vitro hematotoxicology provides the opportunity to study effects of toxicants directly on relevant human target tissues, offering a means of gaining the experience necessary for applying this kind of model to

other continuously-renewing tissues in the body. In particular, fetal cord blood stem cells are used for the clonogenic assays, because they are a more reliable and relevant target for toxicity testing than are immortalized cells. Given their plasticity, these fetal stem cells can generate a perpetual supply of healthy, normal human cells for use in disease modeling, drug discovery and toxicology, because they can potentially generate suitable models for cardiotoxicity, hepatotoxicity, genotoxicity/epigenetic and reproductive toxicology.[106]

One topic for the future would be the automation of scoring colonies in the clonogenic assays, which would provide the opportunity to refine the performance of the tests in terms of their accuracy and repeatability, as well as to reduce personnel costs. An algorithm that recognizes and scores CFU-GM has been developed.[107] This process, data fusion, relies on a classifier designed to process images of layers sampled from a three-dimensional (3D) domain and forming a stack.

Currently, the U.S. Food and Drug Administration (FDA) requires new drugs to undergo short-term toxicity testing in two species, as well as separate testing for long-term toxicology. It is likely that stem cell technologies will be the driving factor in convincing the FDA to accept in vitro preclinical studies as sufficient for the approval of drugs for human clinical testing.

A third area is the use of the omics techniques. A review has been published on gene expression profiling in peripheral blood cells for pharmacogenomics studies.[108] Some of the earliest biomarker discovery studies in whole blood and peripheral blood cells to monitor solid tumor cancers are described in this chapter. The same approach could be used to detect developmental disorders after xenobiotic exposure, by using gene profiling with human cord blood cells.

NEUROTOXICITY

Neurotoxicology is the study of the adverse effect of substances on developing or mature nervous systems. Classically, the neurotoxic potential of substances is assessed in animal models by histopathology, biochemistry and—more recently—behavioral studies after controlled exposure. This testing regime is lengthy, costly, uses large numbers of animals and is therefore not suitable for the screening of large numbers of substances. However, a series of new technical developments may overcome some of these shortcomings. These are (1) human embryonic stem cells, (2) alternative nonmammalian species; (3) high throughput screening; (4) metabonomics and (5) micro-electrode arrays. We believe that these technologies may facilitate the identification of potential neurotoxicants as briefly reviewed below.

Human embryonic stem (hES) cells are noncommitted or only partly-committed cells that have the potential to self renew and the ability to differentiate into a variety of specialized cells. Directing hES cells toward a neuronal fate offers the possibility to study in vitro the effect of substances on the neuronal differentiation process in a human-derived system. Although the use of hES cells still faces many technical and ethical problems, it may provide a useful model for neurotoxicity prediction that neither requires animal experimentation nor is complicated by the problem of interspecies differences. Umbilical cord blood stem cells and induced pluripotent stem cells may be alternative cell sources with lesser ethical problems than hES.

Alternative nonmammalian species encompass invertebrate and vertebrate model organisms. They offer various technical advantages for neurotoxicity studies. For example,

in transparent species such as the zebrafish, the expression of fluorescent reporter genes in subsets of the nervous system allows various aspects of neuronal development and function to be monitored in live animals or embryos. This offers the unique possibility to study the impact of a substance on the nervous system in its natural context and in real time (dynamics), both of which are very difficult to accomplish in vitro or in the current mammalian models. The key assumption underlying this approach is that what one learns from nonmammalian species is predictive for mammals and humans. This assumption makes sense because biological processes are often highly conserved across the animal phyla. On the other hand, there are many examples of exceptions attributed to interspecies differences and the extrapolation to the human situation must be handled with appropriate care.

High throughput screening (HTS) is based on automated assays where thousands of tests can be performed in a short period of time. Very simple neurotoxicological assays, involving the use of stable cell lines and biochemical endpoints, exist. They should be amenable to automated testing and—given the current speed of development—it may become feasible to also automate more complex neurotoxicological assays.

So far, screening systems based on nonmammalian species that involve the expression of fluorescent reporter genes, have been successfully used in various contexts. For example, substances affecting programmed cell death can be detected in living zebrafish embryos by using the vital dye, acridine orange.[109] Where this is possible, automated neurotoxicological assays will lower costs, time and the numbers of animals used, as well as generate many data points and—due to larger sample numbers—statistical robustness.

Metabonomics (also called metabolomics or metabolic profiling) is the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to physiological stimuli or genetic modification. The main parameters are, therefore, changes in the levels of small molecules within the cell (fingerprint) or in the extracellular lumen (footprint). Since most of these small molecules are cell metabolites, this technique provides a snap-shot of the metabolic state of a biological system. Frequently, neurotoxic substances directly or indirectly affect the composition of the nervous system's metabolites. This results in a specific metabonomic profile characteristic for certain types of substances. A comparison of the metabonomic profile of control and neurotoxicant-treated samples may reveal metabolites whose absence or presence is characteristic for a given toxicant, as well as casting some light on the mode of action of a toxicant as each toxicant is likely to yield a unique or distinct metabolic "finger- or footprint".[110] Currently, the slow speed of analysis and the rather high cost of metabonomics are making it less amenable to HTS but its comprehensive nature and the likely technical advances may render it one of the endpoints of choice in the near future.

Micro-electrode arrays (MEA) permit the simultaneous recording of spontaneous and evoked field potentials from multiple neurons within neuronal networks and therefore studies that are technically difficult or impossible to perform in vivo. Growing neuronal cells or tissue on such neuro-sensor MEA-chips offers the possibility to study the dynamics and strength of the effects of substances on the electrical activity of neurons and neuronal circuits.[111] Among the in vitro approaches to neurotoxicity, this endpoint is one of the most sensitive and most neuronal-specific. Although neuronal cells or tissue grown on neurosensor MEA-chips may only partly reflect the in vivo situation, this technique represents a novel and comprehensive method for identifying substances on the basis of their characteristic effects on network electrical activity. So far the bottleneck for this is the availability of robust and standardized human neuronal cells and tissue that grow on the MEA-chip.

ECVAM DATABASE SERVICE ON ALTERNATIVE METHODS (DB-ALM)

User-oriented documentation of, as well as access to, quality controlled information on new alternative techniques are becoming increasingly important. The Internet availability of the ECVAM DataBase service on ALternative Methods to animal experimentation (DB-ALM, http://ecvam-dbalm.jrc.ec.europa.eu) is responding to this demand. It represents a further step in advancing Europe's activities to promote animal alternatives. DB-ALM provides ready-to-use information, presented as evaluated data-sheets on various aspects of advanced and alternative methods, that is used by more than 2000 subscribers from the scientific community and regulatory authorities, and from other stakeholders with an interest in alternatives to animal testing. The database includes alternative methods at all stages of development and validation. For the time being its focus is on in vitro toxicity assessment methods for chemical compounds and/or formulations but the database is not restricted to this and can be used to include information on the mode of action that is covered by the various methods and on other experimental approaches. The DB-ALM provides method-summary descriptions and detailed protocols that allow every experienced laboratory to implement a described test method. The database also includes evaluation studies, details on formal validation studies and individual test results.[112] Moreover, the data sector INVITTOX has been adapted for use within the context of REACH to ensure the availability of relevant and adequately documented methods. A web-based INVITTOX template is under development that will allow test developers to present, from the very beginning on, their methods in this format which may become an international reporting standard for test methods.

A new data sector on persons and institutions active in the field of alternative methods has been launched in 2008 and can refer so far to over 200 entries. The DB-ALM is, moreover, being complemented with a comprehensive inventory of data retrieval systems with relevance for the animal alternatives area, providing user guidance and search procedures to facilitate the identification of the information required. This "ECVAM Search Guide" will be published in 2011 to be followed by an electronic version soon after. This will be highly relevant for ethical committees which, in line with the provisions for project authorisations of Directive 2010/63/EU, must make sure that scientists have carried out a comprehensive search for alternatives before applying for any animal experiment. All these information systems, together with those under development and related ones, will be made available through a new interactive portal to be finalised and launched during 2011.

CONCLUSION

The new technologies we have described in this chapter may prove to be pivotal in developing a new toxicity testing paradigm since they directly address the three major drawbacks of traditional toxicology:

- Human derived cells, either used in simple cell culture systems or, preferably, in reconstructed tissue models, hold the promise to generate predictions on adverse effects that are much more relevant to the human situation. It should be stressed that the use of these cells does not abolish uncertainty altogether, since issues of individual genetic background as well as metabolism are not addressed.

- Combined with high-throughput technologies and new approaches for recreating metabolism in the dish, test systems based on human cells will allow the efficient and cost effective testing of xenobiotics over a wide range of doses, resulting in a more accurate handle on dose-reponse and mechanism of action at the relevant exposure level.
- 'Omics' approaches are particularly amenable to high-throughput approaches and may be used to establish possible patterns of toxicity, i.e., correlations between the activation of specific biological pathways hitchhiked by xenobiotics and their chemical structure.

These new technologies will be especially important to tackle the more complex endpoints, i.e., those human health effects that have shown to be notoriously difficult with respect to the introduction of new alternative testing approaches. "Complex endpoints" (such as, for example, reproductive toxicity, carcinogenicity) are characterised by an elaborate network of physiological feedback loops (e.g., hormonal regulation) and/or involve a yet unknown number of possible mechanisms of action triggering downstream toxic effects.

It has been argued in the past that in vitro methods, by their very nature of being based on limited and reduced test systems in the dish, are not able to address complex physiological networks and their disturbance by toxicants and that they are consequently unable to cover all mechanisms of action due to—again—their intrinsic limitations (e.g., only one cell type, absence of metabolic activity, absence of tissue-specific cell-cell interactions etc.). It may therefore be tempting to suggest that the measurement of more downstream effects in entire experimental animals is irreplaceable. Considering the developments in the field discussed in this chapter, we would like to contest this assumption on the basis of a few considerations which we consider of key importance.

Traditionally, research in the area of alternatives to animal models has focused too much on the desire to reproduce "the animal in the dish", i.e., to reproduce the traditional model system (animal) in an in vitro system (cell culture) not employing the use of animals. Thus the intrinsic limitations of the original test method may be faithfully reproduced in the in vitro test system, even if the latter is based on human cells. ECVAM and the OECD have already addressed this by considering, for instance, for the selection of reference chemicals for in vitro test methods based on human cells, not only data from animal studies but also, where available and produced in an ethical manner, data from human exposure (e.g., in vitro skin irritation testing). While replacement of animal testing is feasible and already implemented in the area of topical toxicity (e.g., skin irritation, skin corrosion testing, skin absorption and phototoxicity), it seems clear that no single in vitro method will be able to reproduce the complex biology of systemic endpoints. However, progress in the design of testing and data integration strategies (so-called "integrated testing strategies") will allow recapitulating the key mechanisms of complex endpoints through the combination of a suite of well-characterised in vitro methods that can be used in a flexible manner and will—in their totality—address a wide range of mechanisms. Moreover, progress in coculturing technologies may allow the study of interactive mechanisms and physiological feedback loops that may be targeted by toxicants.

Finally, the new emerging paradigm of toxicity testing[1] will have important reverberations on the necessity to evaluate and validate new in vitro methods. Today it appears that toxicology will gradually phase out the traditional fixed suite of observational test methods and progress towards the combined use of data from various sources including both tools for generating empirical data as well as computational approaches. These

individual elements will be arranged in flexible integrated testing strategies. This new emerging "tool-box for toxicology" will require, however, that the individual building blocks of the strategies are well understood in terms of their reliability and their capacity to produce relevant measurements that, in combination with other information, allow assessing toxicological pathways and/or to predict human health effects. The rigorous evaluation and validation of these individual building blocks will become therefore an important aspect for ensuring that the integrated approaches which make use of such building blocks, are well characterized in terms of their applicability, limitations and specific contributions to an integrated risk assessment approach and are indeed reliable and robust enough to be utilized in standardized manner and that, each element on its own, does indeed provide an adequate subset of information with regard to the human situation.

REFERENCES

1. Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council. Toxicity Testing in the 21st Century: A Vision and a Strategy. Washington, DC: National Academies Press, 2007: Available at: http://www.nap.edu/catalog.php?record_id=11970#toc.
2. Adler S, Basketter D, Creton S et al. Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010. Arch Toxicol 2011; 85:367-485.
3. Bremer S, Balduzzi D, Cortvrindt R et al. The effects of chemicals on mammalian fertility. Altern Lab Anim—ATLA 2005; 33:391-416.
4. OECD. Information webpage on work on endocrine disruptors. Paris: Organisation for Economic Co-operation and Development, 2011: Available at: http://www.oecd.org/document/61/0,3746,en_2649_34377_2348733_1_1_1_1,00.html.
5. EC ECVAM. Statement on the Scientific Validity of the Embryonic Stem Cell Test (EST)—an In Vitro Test for Embryotoxicity. Ispra: European Centre for the Validation of Alternative Methods, 2001: Available at http://ecvam.jrc.it/publication/Embryotoxicity_statements.pdf.
6. Genschow E, Spielmann H, Scholz G et al. The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. Altern Lab Anim—ATLA 2002; 30(2):151-176.
7. Marx-Stoelting P, Adriaens E, Ahr HJ et al. A review of the implementation of the embryonic stem cell test (EST). The report and recommendations of an ECVAM/ReProTect Workshop. Altern Lab Anim—ATLA 2009; 37(3):313-328.
8. Kirkland D, Aardema M, Henderson L et al. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and noncarcinogens I. Sensitivity, specificity and relative predictivity. Mutat Res 2005; 584:1-256.
9. Maurici D, Aardema M, Corvi R et al. Genotoxicity and mutagenicity. Altern Lab Anim—ATLA 2005; 33(Suppl 1):117-130.
10. Aardema MJ, Barnett BC, Khambatta Z et al. International prevalidation studies of the EpiDerm 3D human reconstructed skin micronucleus (RSMN) assay: transferability and reproducibility. Mutat Res 2010; 701(2):123-131.
11. Kirkland D, Pfuhler S, Tweats D et al. How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop. Mutat Res 2007; 628:31-55.
12. Hastwell PW, Chai LL, Roberts KJ et al. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. Mutat Res 2006; 607:160-175.
13. Coecke S, Ahr H, Blaauboer BJ et al. Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. Altern Lab Anim—ATLA 2006; 34(1):49-84.
14. Azqueta A, Meier S, Priestley C et al. The influence of scoring method on variability in results obtained with the comet assay. Mutagenesis 2011; 26(3):393-399.
15. Hayashi M, MacGregor JT, Gatehouse DG et al. In vivo erythrocyte micronucleus assay. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion on nonhematopoietic target cells and a single dose-level limit test. Mutat Res 2007; 627:10-30.

16. Pfuhler S, Kirkland D, Kasper P et al. Reduction of use of animals in regulatory genotoxicity testing: identification and implementation opportunities—report from an ECVAM workshop. Mutat Res 2009; 680(1-2):31.
17. Yamasaki H, Mesnil M, Nakasawa H. Interaction and distinction of genotoxic and nongenotoxic events in carcinogenesis. Toxicol Lett 1992; 64-65:597-604.
18. Vanparys PH, Corvi R, Aardema M et al. ECVAM prevalidation of three cell transformation assays. ALTEX 2010; 27:267-270.
19. EC ECVAM. ESAC opinion based on the ESAC peer review of an ECVAM-coordinated prevalidation study concerning three protocols of the Cell Transformation Assay (CTA) for in vitro carcinogenicity testing. Ispra: European Centre for the Validation of Alternative Methods, 2011.
20. EC ECVAM. ESAC Working Group Peer Review Consensus Report on an ECVAM-coordinated study concerning three protocols of the Cell Transformation Assay (CTA) for in vitro carcinogenicity testing. Ispra: European Centre for the Validation of Alternative Methods, 2011.
21. EC ECVAM. ECVAM recommendation on the in vitro Cell Transformation Assay for carcinogenicity testing. Ispra: European Centre for the Validation of Alternative Methods, 2011.
22. Walsh MJ, Bruce SW, Pant K et al. Discrimination of a transformation phenotype in Syrian golden hamster embryo (SHE) cells using ATR-FTIR spectroscopy. Toxicol 2009; 258:33-38.
23. Urani C, Stefanini FM, Bussinelli L et al. Image analysis and automatic classification of transformed foci. J Microsc 2009; 234:269-279.
24. Poth A, Kunz S, Heppenheimer A. Bhas cell transformation assay as a predictor of carcinogenicity. ALTEX 2007; 14 (Special Issue):519-521.
25. Ao L, Liu JY, Liu WB et al. Comparison of gene expression profiles in BALB/c 3T3 transformed foci exposed to tumor promoting agents. Toxicol In Vitro 2010; 24:430-438.
26. Rohrbeck A, Salinas G, Maaser K et al. Toxicogenomics applied to in vitro carcinogenicity testing with Balb/c 3T3 cells revealed a gene signature predictive of chemical carcinogens. Toxicol Sci 2010; 118:31-41.
27. Thierbach R, Steinberg P. Automated soft agar assay for the high-throughput screening of anticancer compounds. Anal Biochem 2009; 87:318-320.
28. Ohmori K, Umeda M, Tanaka N et al. Non-Genotoxic Carcinogen Study Group in the Environmental Mutagen Society of Japan. An inter-laboratory collaborative study by the Non-Genotoxic Carcinogen Study Group in Japan, on a cell transformation assay for tumour promoters using Bhas 42 cells. Altern Lab Anim—ATLA 2005; 33(6):619-639.
29. Combes R, Balls M, Curren R et al. Cell transformation assays as predictors of human carcinogenicity. ECVAM Workshop Report 39. Altern Lab Anim—ATLA 1999; 27(5):745-767.
30. Aubrecht J, Caba E. Gene expression profile analysis: an emerging approach to investigate mechanisms of genotoxicity. Pharmacogenomics 2005; 6:419-428.
31. Ellinger-Ziegelbauer H, Stuart B, Wahle B et al. Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. Mutat Res 2005; 575:61-84.
32. Corvi R, Ahr HJ, Albertini S et al. Meeting report: Validation of toxicogenomics-based test systems: ECVAM-ICCVAM/NICEATM considerations for regulatory use. Environ Health Perspect 2006; 114:420-429.
33. Basketter D, Casati S, Gerberick F et al. Subchapter 3.4. Skin Sensitisation. In: Eskes C, Zuang V, eds. Alternative (non-animal) Methods for Cosmetics Testing: Current Status and Future Prospects. Altern Lab Anim—ATLA 2005; 33(Suppl 1):83-103.
34. Casati S, Aeby P, Basketter DA et al. Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. The report and recommendations of ECVAM workshop 51. Altern Lab Anim—ATLA 2005; 33(1):47-62.
35. Natsch A, Bauch C, Foertsch L et al. The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. Toxicol In Vitro 2011; 25(3):733-744.
36. EC. Manual of Decisions for Implementation of the 6th and 7th Amendments to Directive 67/548/EEC on Dangerous Substances. Updated version of July 2004 (EUR 20519). Ispra: European Chemicals Bureau, European Commission JRC, 2004.
37. Eskes C, Bessou S, Bruner L et al. Subchapter 3.3. Eye Irritation. In: Eskes C, Zuang V, eds. Alternative (non-animal) Methods for Cosmetics Testing: Current Status and Future Prospects. Altern Lab Anim—ATLA 2005; 33(Suppl 1):47-81.
38. Scott L, Eskes C, Hoffmann S et al. A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. Toxicol In Vitro 2010; 24(1):1-9.
39. Maurer JK, Parker RD, Jester JV. Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. Regul Toxicol Pharmacol 2002; 36:106-117.

40. Garle MJ, Fry JR. Sensory nerves, neurogenic inflammation and pain: missing components of alternative irritation strategies? A review and a potential strategy. Altern Lab Anim—ATLA 2003; 31(3):295-316.
41. EC ECVAM. ESAC Statement on the scientific validity of the Episkin test (an in vitro test for skin corrosivity). Ispra: European Centre for the Validation of Alternative Methods, 1998: Available at: http://ecvam.jrc.it/publication/EPISKIN_statement.pdf.
42. EC ECVAM. ESAC Statement on the scientific validity of the rat skin transcutaneous electrical resistance (TER) test (an in vitro test for skin corrosivity). Ispra: European Centre for the Validation of Alternative Methods, 1998: Available at: http://ecvam.jrc.it/publication/TER_statement.pdf.
43. EC ECVAM. ESAC Statement on the application of the EpiDerm human skin model for skin corrosivity testing. Ispra: European Centre for the Validation of Alternative Methods, 2000: Available at: http://ecvam.jrc.it/publication/EpiDerm_statement.pdf.
44. EC ECVAM. ESAC Statement on the application of the Corrositex assay for skin corrosivity testing. Ispra: European Centre for the Validation of Alternative Methods, 2000. Available at: http://ecvam.jrc.it/publication/CRTX_statement.pdf.
45. EC ECVAM. ESAC Statement on the Application of the Skinethic™ Human Skin Model. Ispra: European Centre for the Validation of Alternative Methods, 2006: Available at: http://ecvam.jrc.it/publication/ESAC25_statement_SKINETHIC_correction_on181206_C.pdf
46. EC ECVAM. ESAC Statement on the scientific validity of an in vitro test method for skin corrosivity testing. Ispra: European Centre for the Validation of Alternative Methods, 2009: Available at: http://ecvam.jrc.it/publication/ESAC30_skincorrosion_revised_20100921.pdf.
47. EC ECVAM. ESAC Statement on the validity of in vitro tests for skin irritation. Ispra: European Centre for the Validation of Alternative Methods, 2007: Available at: http://ecvam.jrc.it/publication/ESAC26_statement_SkinIrritation_20070525_C.pdf.
48. EC ECVAM. ESAC Statement on the scientific validity of in vitro tests for skin irritation testing. Ispra: European Centre for the Validation of Alternative Methods, 2008: Available at: http://ecvam.jrc.it/publication/ESAC_Statement_SkinEthic+EpiDerm%20FINAL%200812-01.pdf.
49. Willis CM, Stephens CJ, Wilkinson JD. Epidermal damage induced by irritants in man: a light and electron microscopic study. J Invest Dermatol 1989; 93:695-699.
50. Willis CM, Stephens CJ, Wilkinson JD. Selective expression of immune-associated surface antigens by keratinocytes in irritant contact dermatitis. J Invest Dermatol 1991; 96:505-511.
51. Spiekstra SW, Toebak MJ, Sampat-Sardjoepersad S et al. Induction of cytokine (interleukin-1alpha and tumor necrosis factor-alpha) and chemokine (CCL20, CCL27 and CXCL8) alarm signals after allergen and irritant exposure. Exp Dermatol 2005; 14:109-116.
52. Fluhr JW, Darlenski R, Angelova-Fischer I et al. Skin irritation and sensitization: mechanisms and new approaches for risk assessment. Skin Pharmacol Physiol 2008; 21:124-135.
53. EC ECVAM. ESAC Statement on the performance under UN GHS of three in vitro assays for skin irritation testing and the adaptation of the reference chemicals and defined accuracy values of the ECVAM skin irritation performances standards. Ispra: European Centre for the Validation of Alternative Methods, 2009: Available at: http://ecvam.jrc.it/publication/ESAC31_skin-irritation-statement_20090922.pdf.
54. Griesinger C, Barroso J, Zuang V et al. Explanatory background document to the OECD draft test guideline on in vitro skin irritation testing. Ispra: European Centre for the Validation of Alternative Methods, 2009.
55. Prudovsky I, Mandinova A, Soldi R et al. The nonclassical export routes: FGF1 and IL-1alpha point the way. J Cell Sci 2003; 116(24):4871-4881.
56. Nickel W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur J Biochem 2003; 270:2109-2119.
57. Borlon C, Godard P, Eskes C et al. The usefulness of toxicogenomics for predicting acute skin irritation on in vitro reconstructed human epidermis. Toxicology 2007; 241:157-166.
58. Zuang V, Eskes C, Griesinger C et al. ECVAM key area topical toxicity: update on activities. AATEX 2007; 14(Special Issue):523-528.
59. EC-ECVAM. ECVAM Technical Report 2006-2007. Ispra: European Centre for the Validation of Alternative Methods, 2008: Available at: http://ecvam.jrc.it/publication/ECVAM%20Technical%20Report%202006-2007%20final.pdf.
60. Smijs TG, Bouwstra JA. Focus on skin as a possible port of entry for solid nanoparticles and the toxicological impact. J Biomed Nanotechnol 2010; 6(5):469-484.
61. Baroli B. Penetration of nanoparticles and nanomaterials in the skin: fiction or reality? J Pharm Sci. 2010; 99(1):21-50.
62. Schroeter A, Engelbrecht T, Neubert RH et al. New nanosized technologies for dermal and transdermal drug delivery. A review. J Biomed Nanotechnol 2010; 6(5):511-528.
63. Steiling W, Kreutz J, Hofer H. Percutaneous penetration/dermal absorption of hair dyes in vitro. Toxicol In Vitro 2001; 15(4-5):565-570.

64. OECD. OECD guideline for the testing of chemicals Nr. 427. Skin absorption: in vivo method. Paris: Organisation of Economic Co-operation and Development, 2004.
65. OECD. OECD guideline for the testing of chemicals Nr. 428. Skin absorption: in vitro method. Paris: Organisation of Economic Co-operation and Development, 2004.
66. OECD. OECD series on testing and assessment Nr. 28. Guidance document on the conduct of skin absorption studies. Paris: Organisation of Economic Co-operation and Development, 2004.
67. Van der Sandt JJM, Meuling WJA, Elliott GR et al. Comparative in vitro-in vivo percutaneous absorption of pesticide propoxur. Toxicol Sci 2000; 58:15-22.
68. Barbero AM, Frasch HF. Pig and guinea pig skin as surrogates for human in vitro penetration studies: a quantitative review. Toxicol In Vitro 2009; 23(1):1-13.
69. Ng SF, Rouse JJ, Sanderson FD et al. Validation of a static Franz diffusion cell system for in vitro permeation studies. AAPS Pharm Sci Tech 2010; 11(3):1432-1441.
70. EDETOX Project. Evaluations and predictions of dermal absorption of toxic chemicals. Newcastle-Upon-Tyne: EDETOX Project, 2004: Available at: http://research.ncl.ac.uk/edetox/EDETOX%20PDF%20Amended.pdf.
71. Williams FM. EDETOX Evaluations and predictions of dermal absorption of toxic chemicals. Int Arch Occup Environ Health 2004; 77(2):150-151.
72. van de Sandt JJ, van Burgsteden JA, Cage S et al. In vitro predictions of skin absorption of caffeine, testosterone and benzoic acid: a multi-centre comparison study. Regul Toxicol Pharmacol 2004; 39(3):271-281.
73. Chilcott RP, Barai N, Beezer AE et al. Inter- and intralaboratory variation of in vitro diffusion cell measurements: an international multicenter study using quasi-standardized methods and materials. J Pharm Sci 2005; 94(3):632-638.
74. Schmook FP, Meingassner JG, Billich A. Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. Int J Pharm 2001; 215(1-2):51-56.
75. Zghoul N, Fuchs R, Lehr CM, Schaefer UF. Reconstructed skin equivalents for assessing percutaneous drug absorption from pharmaceutical formulations. ALTEX 2001; 18(2):103-6.
76. Netzlaff F, Lehr CM, Wertz PW et al. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity and substance transport. Eur J Pharm Biopharm 2005; 60(2):167-178.
77. Schäfer-Korting M, Mahmoud A, Lombardi Borgia S et al. Reconstructed epidermis and full-thickness skin for absorption testing: influence of the vehicles used on steroid permeation. Altern Lab Anim—ATLA 2008; 36(4):441-52.
78. Ackermann K, Borgia SL, Korting HC et al. The Phenion full-thickness skin model for percutaneous absorption testing. Skin Pharmacol Physiol 2010; 23(2):105-112.
79. Schäfer-Korting M, Bock U, Gamer A et al. Reconstructed human epidermis for skin absorption testing: results of the German prevalidation study. Altern Lab Anim—ATLA 2006; 34(3):283-294.
80. Schäfer-Korting M, Bock U, Diembeck W et al. The use of reconstructed human epidermis for skin absorption testing: Results of the validation study. Altern Lab Anim—ATLA 2008; 36(2):161-187.
81. Van Gele M, Geusens B, Brochez L et al. Three-dimensional skin models as tools for transdermal drug delivery: challenges and limitations. Expert Opin Drug Deliv 2011; 8(6):705-720.
82. Mitragotri S, Anissimov YG, Bunge AL et al. Mathematical models of skin permeability: An overview. Int J Pharm 2011; 418(1):115-29.
83. Ghafourian T, Samaras EG, Brooks JD et al. Validated models for predicting skin penetration from different vehicles. Eur J Pharm Sci 2010; 41(5):612-616.
84. Spielmann H, Balls M, Brand M et al. EC/COLIPA project on in vitro phototoxicity testing: first results obtained with the Balb/c 3T3 cell phototoxicity assay. Toxicol In Vitro 1994; 8:793-796.
85. Spielmann H, Balls M, Dupuis J et al. The international EU/COLIPA In vitro phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxicol In Vitro 1998; 12:305-327.
86. EC. Commission Directive 2000/33/EC of 25 April 2000 adapting to technical progress for the 27th time Council Directive 67/548/EC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Annex V B.41 Phototoxicity—in vitro 3T3 NRU phototoxicity test. Off J Eur Comm 2000; L136:98-107.
87. OECD. OECD guidelines for the testing of chemicals Nr. 432. In vitro 3T3 NRU phototoxicity test. Paris: Organisation for Economic Co-operation and Development, 2004.
88. Edwards SM, Donnelly TA, Sayre RM et al. Quantitative in vitro assessment of phototoxicity using a human skin model, Skin^2. Photodermatol Photoimmunol Photomed 1994; 10:111-117.
89. Liebsch M, Barrabas C, Traue T et al. Entwicklung eines in vitro Tests auf dermale Phototoxizitaet in einem Modell menschlicher Epidermis (EpiDerm^{TM}). ALTEX 1997; 14:165-174.
90. Liebsch M, Döring B, Donelly TA et al. Application of the human dermal model Skin^2 ZK 1350 to phototoxicity and skin corrosivity testing. Toxicol In Vitro 1995; 9:557-562.
91. Api AM. In vitro assessment of phototoxicity. In Vitro Toxicol 1997; 10:339-350.

92. Liebsch M, Traue D, Barrabas C et al. Prevalidation of the EpiDerm phototoxicity test. In: Clark D, Lisansky S, Macmillan R, eds. Alternatives to Animal Testing II: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry. Brussels/Newbury: CPL Press, 1999: 160-166.
93. Jones PA, King AV, Lovell W et al. Phototoxicity testing using 3-D reconstructed human skin models. In: Clark D, Lisansky S, Macmillan R, eds. Alternatives to Animal Testing II: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry. Brussels/Newbury: CPL Press, 1999:160-166.
94. EMEA. Note for guidance on photosafety testing (CPMP/SWP/398/01). London: European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products, 2002: Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003353.pdf.
95. Kejlová K, Jírová D, Bendová H et al. Phototoxicity of bergamot oil assessed by in vitro techniques in combination with human patch tests. Toxicol In Vitro 2007; 21(7):1298-303.
96. Lynch AM, Smith MD, Lane AS et al. An evaluation of chemical photoreactivity and the relationship to photogenotoxicity. Regul Toxicol Pharmacol 2010; 58(2):219-223.
97. Blaauboer BJ, Bayliss MK, Castell JV et al. The use of biokinetics and in vitro methods in toxicological risk evaluation. Altern Lab Anim—ATLA 1996; 24(4):473-497.
98. Blaauboer BJ. The necessity of biokinetic information in the interpretation of in vitro toxicity data. Altern Lab Anim—ATLA 2002; 30(Suppl 2):85-91.
99. Boobis A, Gundert-Remy U, Kremers P et al. In silico prediction of ADME and pharmacokinetics. Report of an expert meeting organised by COST B15. Eur J Pharm Sci 2002; 17:183-193.
100. Parrott N, Paquereau N, Coassolo P et al. An evaluation of the utility of physiologically based models of pharmacokinetics in early drug discovery. J Pharm Sci 2005; 94:2327-2343.
101. EPA. Approaches for the application of Physiologically Based Pharmacokinetic (PBPK) Models and supporting data in risk assessment. EPA/600/R-05/043F. Washington, DC: US Enivronmental Protection Agency, 2007.
102. Parrott N, Jones H, Paquereau N et al. Application of full physiological models for pharmaceutical drug candidate selection and extrapolation of pharmacokinetics to man. Basic Clin Pharmacol Toxicol 2005; 96:193-199.
103. Theil FP, Guentert TW, Haddad S et al. Utility of physiologically based pharmacokinetic models to drug development and rational drug discovery candidate selection. Toxicol Lett 2003; 138:29-49.
104. Jones HM, Parrott N, Jorga K et al. A novel strategy for physiologically based predictions of human pharmacokinetics. Clin Pharmacokinet 2006; 45:511-542.
105. Pessina A, Albella B, Bayo M et al. Application of the CFU-GM assay to predict acute drug-induced neutropenia: an international blind trial to validate a prediction model for the maximum tolerated dose (MTD) of myelosuppressive xenobiotics. Toxicol Sci 2003; 75:355-367.
106. Davila JC, Cezar GG, Thiede M et al. Use and application of stem cells in toxicology. Toxicol Sci 2004; 79:214-223.
107. Crosta G, Fumarola L, Malerba I et al. Scoring CFU-GM colonies in vitro by data fusion: A first account. Exp Hematol 2007; 35(1):1-12.
108. Burczynski ME, Dorner AJ. Transcriptional profiling of peripheral blood cells in clinical pharmacogenomic studies. Pharmacogenomics 2006; 7:187-202.
109. Parng C, Seng WL, Semino C et al. Zebrafish: a preclinical model for drug screening. Assay Drug Dev Technol 2002; 1:41-48.
110. van Vliet E, Morath S, Eskes C et al. A novel in vitro metabolomics approach for neurotoxicity testing, proof of principle for methyl mercury chloride and caffeine. Neurotoxicology 2008; 29(1):1-12.
111. van Vliet E, Stoppini L, Balestrino M et al. Electrophysiological recording of re-aggregating brain cell cultures on multi-electrode arrays to detect acute neurotoxic effects. Neurotoxicology 2007; 28(6):1136-1146.
112. Roi AJ, Flego M. ECVAM's Database Service on Alternative Methods (DB-ALM)—Online. ALTEX 2006; 23(Special Issue):177-180.

CHAPTER 11

MEDIUM TO HIGH THROUGHPUT SCREENING: Microfabrication and Chip-Based Technology

Yuan Wen, Xudong Zhang and Shang-Tian Yang*

William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio, USA

**Corresponding Author: Shang-Tian Yang—Email: yang.15@osu.edu*

Abstract: Medium to high throughput screening for toxicity testing can provide a wealth of information with significant time and cost savings. New technologies, such as microfabrication, microfluidics and chip-based technology, combined with advanced cell culture and detection techniques, open up new opportunities in toxicity testing. In this chapter, fundamentals of microfabrication and microfluidics are discussed with a focus on the broad and novel applications on toxicity studies enabled by these technologies. Emphasis is placed on microscale cell and tissue culture models for medium and high throughput systemic toxicity studies in vitro.

INTRODUCTION

Microfabrication refers to the production of structures with at least one dimension in the micrometer range and has been widely used in the manufacture of micro-electromechanical systems (MEMS) that have applications in various spheres, including automotive and biomedical devices, environmental monitoring, industrial automation, information technology and telecommunications. The field of microfluidic technology, also known as lab-on-a-chip (LOC) or μ-TAS (micro total analysis systems), has also been rapidly developed, as evidenced by the rapid increase in the number of patents and papers published in journals in the last 10 years (Fig. 1). Through various microfluidic technologies, microliter to nanoliter and even picoliter fluids can be handled with precise control. Microfluidic devices have a number of important and unique advantages, including: (a) minute consumption of reagents; (b) short paths for short reaction times; (c) highly paralleled operation with small footprints; (d) versatile controls for fluid transport and concentration manipulation; (e) relevant

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

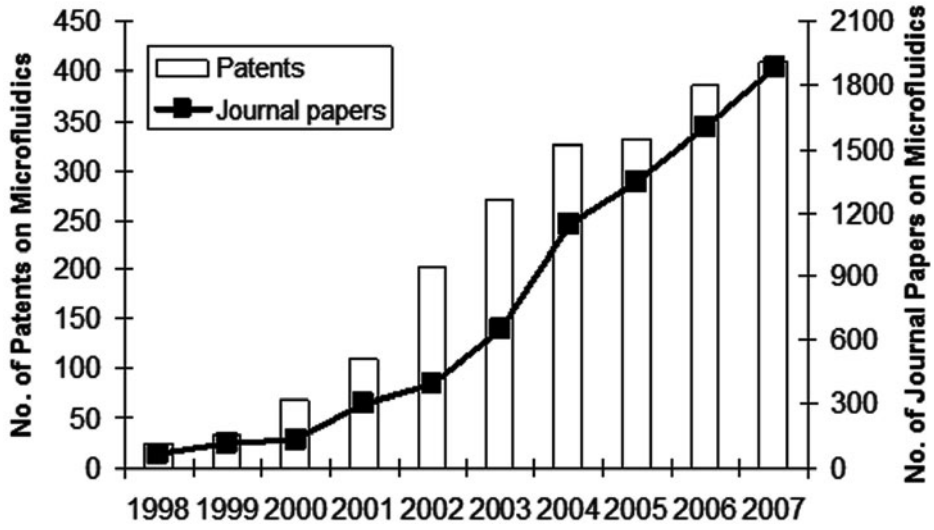

Figure 1. Numbers of patents and journal papers on microfluidics published in English from year 1998 through 2007. During the past decade, there was more than a 16-fold increase for patents, and more than a 28-fold increase for journal papers.

dimensions when dealing with cells and biomolecules; (f) widely applicable materials; and (g) low costs. Therefore, microfabricated devices, especially BioMEMS or biochips, are being widely adopted in the pharmaceutical and biotechnology industries.[1,2] Today, more than 120 companies are producing and commercializing miniaturized analytical devices, ranging from miniaturized sensors, DNA sequencers, high throughput (HTP) flow cytometry systems and high performance liquid chromatography (HPLC) chips to disposable electrode arrays, tissue arrays and lab compact disks.[3]

This chapter presents an overview of current microchip-based technologies, with in-depth discussions on their applications in toxicity testing. We will first introduce microfabrication techniques, including hard micromachining and soft lithography, followed by a discussion of on-chip detection methods. Then, the central theme of newly-developed chip-based technologies for HTP screening (HTS) will be explored in two categories: Microplate-based technology and microfluidic technology. Examples of microfabricated devices which are being applied in various aspects of toxicity testing are provided, including medium-to-high throughput platforms and miniaturized models for tissue-engineered systems.

MICROFABRICATION TECHNIQUES

Based on the target materials, microfabrication techniques can be divided into two categories (see Table 1); hard micromachining targets "hard" materials, such as silicon, glass, GaAs and metals, while soft micromachining (also known as soft lithography) uses polymers and gels to make microstructures useful for studies with biological cells and molecules.[1,4,5]

Table 1. Microfabrication techniques useful for making biochips and microfluidic devices

Hard Micromachining for Silicon, Glass, Semiconductor and Metal Substrates	
Thin-film deposition	An additive process through oxidation, including chemical vapor deposition, physical vapor deposition and electrodeposition; thickness control down to 20 Å; capable of producing microelectrodes.
Photolithography	Patterning of photoresist on a flat wafer by using UV light through designed masks; minimum feature sizes down to submicrons; rapid prototyping resolution around 20 μm, masks produced with rapidity and low cost; useful in producing most microfabricated devices for toxicity studies, either as building structures or molds.
Etching	A subtractive process through wet or dry etching; wet etching isotropic and selective; dry etching anisotropic but poorly selective, including ion milling, high-pressure plasma etching and reactive ion etching.
Substrate bonding	Forming 3D microstructures and/or closed systems between silicon-silicon, silicon-glass or glass-glass through hydrogen bonds or electrochemical reactions.
Soft Micromachining for Polymer and Plastic Substrates	
Micromolding	Microstructure transfer through replica molding, microtransfer molding, micromolding in capillaries and solvent-assisted micromolding; resolution down to 100 nm.
Hot embossing	Based on glass transition temperature of polymer substrate with low cost and high volume; compatible with relatively high aspect ratio features; feature size down to around 1 μm.
Microcontact printing	Based on self-assembled monolayers with great conformability to a substrate surface; feature sizes down to 20 nm; useful as ultra-thin resist in selective wet etching or deposition.

Hard Micromachining

Most of the hard micromachining techniques have their roots in the integrated circuit (IC) industry and are practiced in a clean room with high standards for particle control. The techniques mainly include: (a) thin-film deposition, (b) photolithography, (c) etching and (d) substrate bonding.[1,6,7]

Various thin-film deposition techniques, mainly oxidation, chemical vapor deposition (CVD), physical vapor deposition (PVD) and electrodeposition, can be used to produce thin films with a thickness precision down to 20Å.[1,2] These thin films have a wide number of applications; for example, as optical reflective/antireflective coatings, electrical insulation/conduction, magnetic memory disks, chemical gas/liquid sensors, mechanical tribological coatings and thermal barrier layers.[8] In particular, microfabricated electrodes generated with, for example, the PVD process, are important components of chip-based HTS technology.[9,10]

Photolithography is the technique most widely used to transfer a mask pattern onto the surface of a solid material such as a silicon wafer. As illustrated (Fig. 2), the process consists of the following main steps:[11]

1. A 2D pattern is first created with computer-aided design (CAD) software.
2. A photomask is then generated following the CAD design.
3. A thin and uniform layer of photoresist is spin-coated onto a silicon wafer.
4. The spin-coated wafer is then exposed to UV light under the photomask.
5. The wafer is washed to remove unpolymerized photoresist, then baked to conclude the process.

Photomasks carry the designed pattern in a light and dark field image on a flat medium and transfer the pattern to photoresist on silicon wafers by UV exposure. The conventional way of making a chrome-patterned mask is both time-consuming and expensive. However, with the advent of rapid prototyping for photolithography,[12] features that are equal to or larger than 20 μm in a transparency mask could be generated with a desktop computer and high resolution printer system in a few hours, at a cost of less than $1 per square inch.[4] This process has substantially accelerated photolithography with a much lower cost, helping to pave the road for the wide application of microfabrication in biomedical research.

Two types of photoresists are commonly used: positive tone and negative tone. Upon UV exposure, a positive tone photoresist becomes much more soluble due to rupture or scission of its main and side polymer chains, whereas a negative tone photoresist forms strengthened and less soluble polymer with random cross-linking of main chains or pendant side chains. For example, metallic micro-electrodes, which are often necessary when electrochemical assay is used for cell response studies,[9,10] can be produced by a commonly-used technique called lift-off. In this process, photoresist can mark a negative pattern on a substrate through photolithography. Negative pattern means that the spots where the electrodes are meant to be are not covered by photoresist, but defined by the photoresist covering the non-electrode area. This can actually be obtained with either positive tone or negative tone photoresist. Then a desired metal, such as platinum or

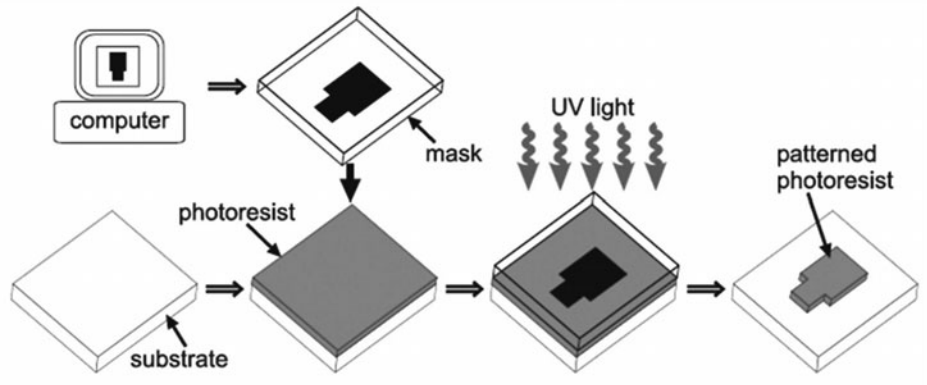

Figure 2. A schematic representation of the process of photolithography. Reprinted from Voldman J et al. Annu Rev Biomed Eng 1999; 1:401-425.[11] ©1999 Annual Reviews. www.annualreviews.org.

gold, can be deposited on the entire surface. After stripping off the photoresist bearing the metal, a thin film of metal with the desired pattern is left on the substrate.

In terms of structural material, thin-film deposition and photolithography are additive, while etching is subtractive. Etching can be categorized as wet and dry technologies. In wet etching, the isotropic and selective removal of material can be obtained. Silicon dioxide and silicon nitride are commonly used as masking materials for wet etching. Dry etching can produce a finer structure with higher anisotropicity, but the selectivity is poor.

Substrate bonding (silicon-silicon, silicon-glass and glass-glass) plays a key role in 3D microstructure formation and in microsystem packaging and encapsulation. Silicon-silicon fusion and silicon-glass electrostatic (or anodic) bonding are the two most important substrate bonding techniques.

Soft Micromachining

Many soft materials, such as various polymers, including plastics, rubber and gels, have low costs, proper physicochemical properties and good biocompatibility. In addition, they are amenable to a series of micromachining techniques under relatively mild conditions. Therefore, soft lithography is increasingly used in microfabrication for biomedical applications. Micromolding, hot embossing and microcontact printing are three commonly used soft micromachining techniques.

Molding plays a central role in microstructure transfer from a master to a substrate. The master for molding can be generated with hard micromachining techniques, such as photolithography. Several major micromolding techniques are used in soft lithography, including replica molding (REM), microtransfer molding (μTM), micromolding in capillaries (MIMIC) and solvent-assisted micromolding (SAMIM) (Fig. 3). REM is the most widely used technique,[2,4] which duplicates the structure from a master with a "negative tone." It has the capability for 3D pattern transfer in one step. REM may produce high fidelity and resolution in complex pattern transfer with an appropriate material, usually a prepolymer. The process is reliable, fast, simple and inexpensive, with a resolution of 100 nm or greater. Structures as complex as optical surfaces can also be formed by REM against elastomeric masters.[13]

Hot embossing is a low-cost and high-volume microfabrication technique.[14] In this process, a vacuum is applied and the temperature is elevated to just above the glass transition temperature (T_g) of a polymer substrate, followed by an applied pressure on the mold. In the presence of the embossing pressure, the system is cooled to just below the T_g to further stabilize the microstructures. Then a lower temperature is reached, followed by the removal of the embossing mold from the substrate. This technique is suitable for making high-aspect-ratio features with a dimension down to 1 μm.

Microcontact printing is a simple procedure, based on self-assembled monolayers (SAMs), where an elastic stamp bearing some "ink" is printed in conformability with a substrate surface, resulting in an additive SAM with patterns of submicron lateral dimensions transferred from the elastomeric stamp.[15] The patterned SAMs can be used as an ultrathin resist in selective wet etching[16,17] or as passivating layers in selective deposition.[18-20]

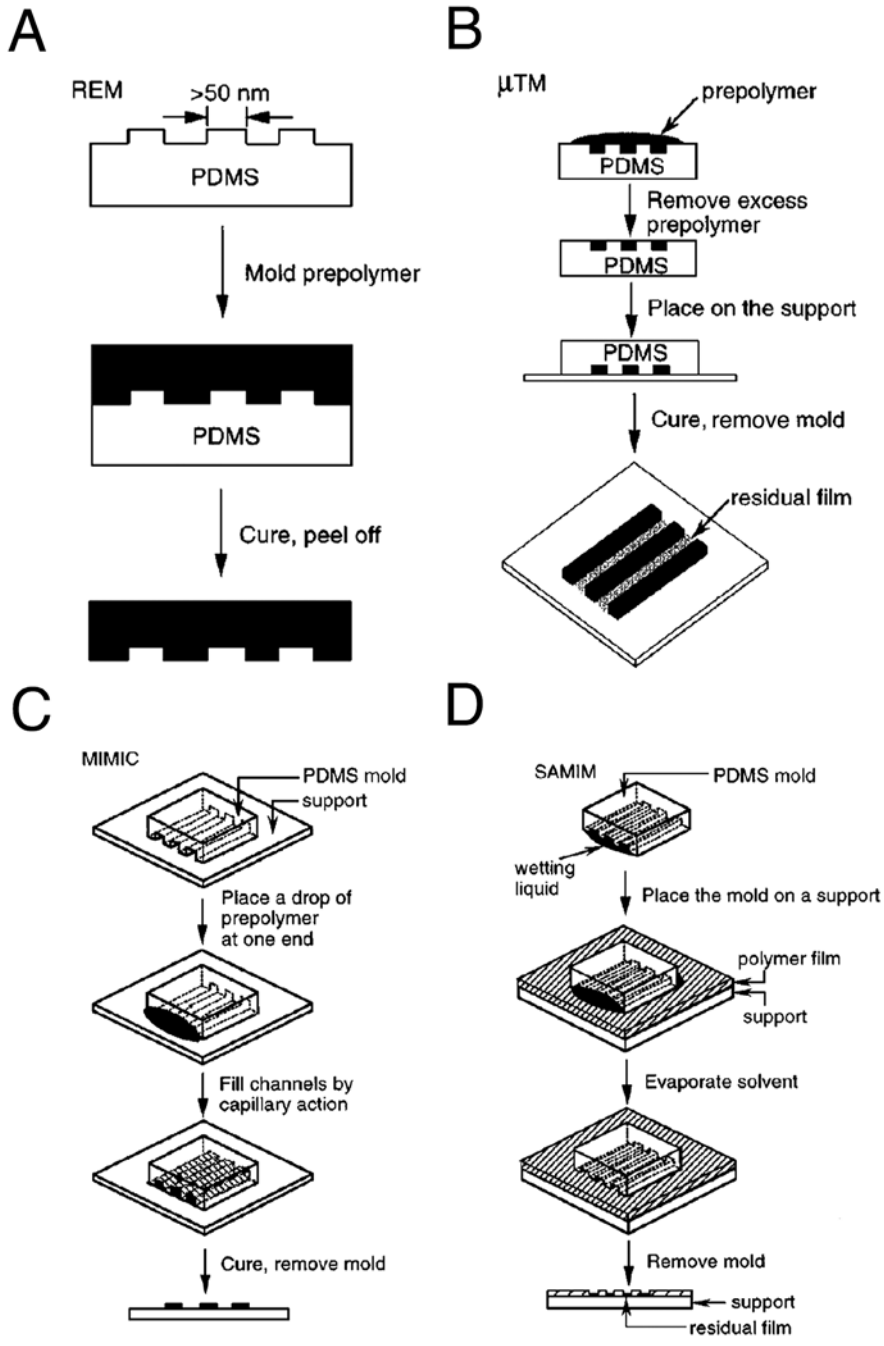

Figure 3. Micromolding techniques. A) replica molding; B) microtransfer molding; C) micromolding in capillaries; and D) solvent-assisted micromolding. Reprinted from Xia Y, Whitesides GM. Annu Rev Mat Sci 1998; 28:153-184,[4] ©1998 Annual Reviews. www.annualreviews.org.

DETECTION METHODS

Table 2 lists the optical and electrochemical methods relevant for the on-chip detection and quantification of cells, cell viability and/or cellular functions important to HTS applications. These methods are discussed below.

Table 2. On-chip detection methods for cell-based assays

Optical Methods	
Labeling cells with fluorescent dyes	MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)—produces a dark blue formazan product after reduction by active mitochondrial reductase
	Calcein AM (Acetoxymethyl ester of calcein)—releases calcein, a green fluorescent dye, after reacting with esterase in live cells
	Hoechst dyes—generate blue fluorescent signals after intercalating with nuclear DNA
	Ethidium homodimer-1—labels dead cells with red staining
Labeling cells with quantum dots (QD)	Fluorescent nanoparticles are photochemically stable and can be used for multiplex labeling with multiple emission colors from a single excitation wavelength light[32-35]
Using transformed cells with reporter genes	Non-invasive live-cell kinetics assays using cDNA encoding a reporter such as luciferase, galactosidase or variants of GFP
	FRET—reports the molecular proximity in living cells based on emission wavelength change due to energy transfer between two close fluorophores[44]
Using chromatophoric cells	Natural color changes induced in chromatophoric cells in the skin and scales of fish[21]
Electrochemical Methods	
Electrical impedance	The presence of the cells at the electrode/solution interface affects the local ionic environment and increases the electrode impedance, which can be calibrated to obtain a "cell index" that is proportional to the cell number
Electrical response	Electrogenic cells, such as neural and heart muscle cells, give various electrical signals in response to environmental stimuli
Electrical signals (pH, DO, etc.)	Changes in metabolic products and/or substrates in the culture media can be monitored with pH and/or DO (dissolved oxygen) electrodes

Optical Methods

Although some chromatophoric cells, such as those present in the skin and scales of fish, can give different color signals upon induction,[21] most mammalian cells cannot be easily detected optically in HTS. Therefore, conventional optical methods usually involve live/dead staining with green fluorescence such as calcein-AM or acridine orange for viable cells and red fluorescence such as ethidium homodimer-1 for dead cells to distinguish cell responses to toxicants. The fluorescence-labeling method was used to study the quantitative cytotoxic effects of anticancer drugs, such as Epirubicin against oral cancer-2 cells[22] and Paclitaxel against HeLa cells[23] in a microfluidic platform. The effects of the chemical modulators, arsenic trioxide and N-acetyl cysteine, on the treatment of breast cancer cells with Adriamycin were also studied with this method.[24] Furthermore, toxins, such as sodium azide and ricin, can be assayed for their cytotoxicity on a microchip.[25] Other examples of how this method can be used include its uses to study the effects of a gradient of fetal bovine serum on the attachment and viability of human mesenchymal stem cells,[26] to quantify pumping effects on cells[27] and to demonstrate a functional 3D cell culture component.[28] By labeling cells in a HTP fashion, stained cellular fluorescence intensity measurement in microchannels was used to demonstrate the efficacy of serial dilution channels[29] and the HTP cell staining of a gradient generator.[30]

In addition to its use for live/dead cell staining, the nuclear DNA intercalating dye, Hoeschst 33342, has been used to quantify cell growth in a microfluidic chip.[31] More recently, fluorescent nanoparticles, such as quantum dots, are also being widely exploited, to label cells or follow cellular activities.[32-35] However, labeling cells with fluorescent dyes or particles is invasive and disruptive to the cell culture and can only give final endpoint data rather than permitting continuous data collection. It is preferable to use label-free assays that are non-invasive and can generate dynamic data in real time and reveal the cytotoxic effects of drugs in an HTP fashion.

Reporter gene methods can be tailored to detect and quantify both cell mass and specific cellular events or functions and are, therefore, gaining increasing popularity due to their relevance to human physiology/pathology, sensitivity and specificity. A reporter gene for an easily quantifiable protein, such as green fluorescent protein (GFP), is coupled with a regulatory DNA sequence or promoter.[36-38] Whereas assays based on luciferase or β-galactosidase require feeding the enzyme substrate into individual cells, assays based on GFP variants require only initiation. Hence, GFP and its variants are widely used, because of their capacities for automation and the real-time, non-invasive assessment of both chronic and acute cellular events.[37] Many GFP variants have been developed, with emission lights ranging from blue to yellow. They can also be multiplexed with *Discosoma* sp. red fluorescent protein (dsRed).[39] Reporter gene techniques can be responsive to targeted effects, such as gene expression and the activation of signal transduction pathways and are, therefore, suitable for use in disease-relevant assays. They can also provide dynamic and multiplexing information from cellular responses to external agents[40-42] and their simplicity and speed allow for miniaturization and HTS applications.[43]

Fluorescent reporters can be used in fluorescence resonance energy transfer (FRET), which reports molecular proximity in living cells, based on emission wavelength changes due to energy transfer between two proximal fluorophores.[44] In FRET assays, two tandem reporters, such as two GFP mutants, are fused with a peptide linker incorporating a specific

cellular function. For example, when the linker consists of a caspase-3 cleavage site (amino acid sequence DEVD), the activation of caspase-3 in live cells can be studied dynamically.[45,46]

Quantitative fluorescence microscopy is commonly used for detecting fluorescent cells in HTS. Live-cell kinetic assays for HTP drug screening can be carried out with commercial laser scanning imaging systems suitable for performing fluorescence microscopy and quantitative image analysis, such as KineticScan HCS reader from Cellomics, Pathway Bioimager from BD Biosciences and IN Cell Analyzer 3000 from GE Lifesciences. These imaging systems can be used to examine the contexts of living cells, quantify intracellular proteins and monitor the trafficking events of proteins or certain subcellular structures fused with fluorescent reporters.[47]

The toxic effects of chemicals on the development of endodermal cells were evaluated by using a stable mouse embryonic cell line with GFP expression, regulated by an alpha-fetoprotein enhancer, as a marker for endodermal cell differentiation.[48] The microscopic images of the fluorescent cells (Fig. 4) can be analyzed for fluorescence signals, providing high spatial and temporal resolution. However, they are limited to the late phase of compound characterization, because of their fragility, high cost and relatively low capacity for HTP application.[49] Furthermore, adequate validation is needed for quantitative fluorescence microscopy.[50] The accuracy of their quantification is another concern, because they are limited to planar images and the fluorescence intensity from the same sample varies according to different foci, especially for 3D cellular structures.

Commercially-available spectrofluorometers and fluorescence plate-readers that are commonly used in biochemical assays have not been widely used in real-time, cell-based HTS, because of their limited sensitivity and high background noise levels from cell culture media. However, these problems can be overcome by growing GFP cells in 3D fibrous matrices, to reach high density in modified multi-well plates (Fig. 5A) or perfusion microbioreactor arrays (Fig. 5B). These formats can be read by using conventional fluorescence plate-readers, potentially providing a simple, accurate and economical method for the non-invasive, real-time quantification of fluorescent cells for HTS on drug toxicity.

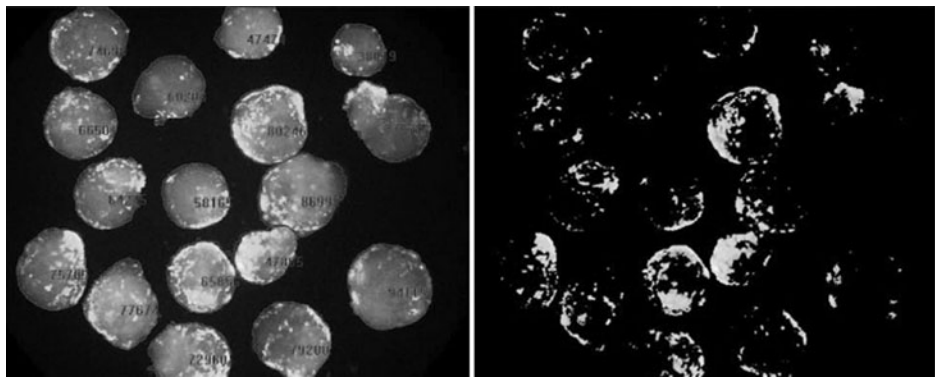

Figure 4. Fluorescent microscopic images of endodermal cells differentiated from mouse embryonic stem cells (ESCs) with GFP expression. Image analysis was used to study the embryotoxicity effects of chemicals on endodermal development of ESCs. Images on the left panel were processed and green fluorescent intensity above a certain value was visualized (right) and quantitatively analysed. Reprinted from Papavella M et al. Toxicol In Vitro 2002; 16(5):589-597, ©2002 Elsevier Science Ltd.

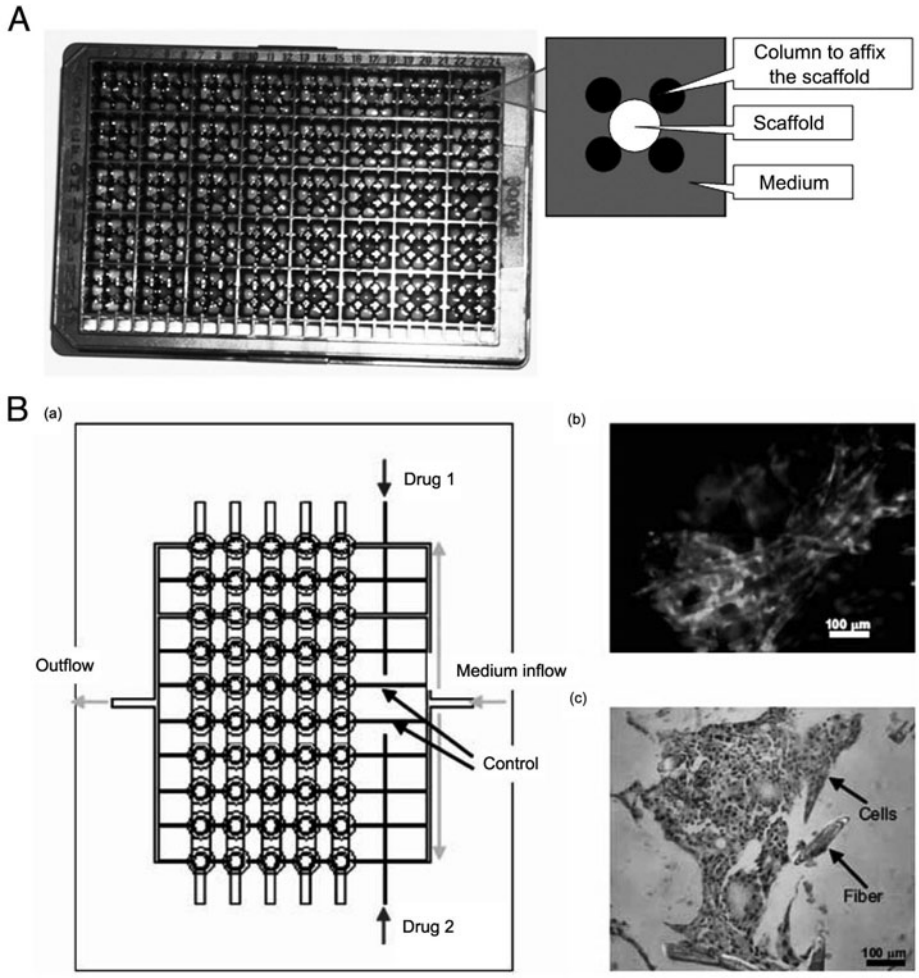

Figure 5. A) Modified 384-well plate with 40 cell culture units, each containing one fibrous scaffold for 3D culturing of cells with surrounding media as background that can be subtracted in reading the live cell fluorescence. B) Perfusion microbioreactor array for drug screening; (a) a schematic of the biochip with 5 × 10 microbioreactors, (b) photograph of GFP-cells grown in the fibrous scaffold in the microbioreactor, (c) hematoxylin and eosin staining of cell matrix slice showing uniform cell distribution. Reproduced from Yang ST et al. Curr Opin Drug Discov Dev 2008; 11:111-127.[82] ©2008 The Thomson Corporation and the authors.

Electrochemical Methods

Electrochemical methods that can provide real-time and dynamic information on cell activities usually involve the measurement of conductivity (or electrical impedance) changes due to contacts between cells and electrode surface, electrical responses of electrogenic cells, or chemical signals (e.g., pH) resulting from changes in metabolic products (e.g., lactic acid) or substrates (e.g., glucose).[51] A number of electrical cell-substrate impedance systems have been developed since the first reported detection of cells within an applied

electric field.[52] The electrical impedance increases with the amount of cells contacting the electrode surface (Fig. 6). Thus, an electrical impedance sensor array integrated onto the bottom of a microtiter plate can be used for the quantitative detection of living cells and for cytotoxicity assays.[41] Commercial impedance-based systems, such as the "real-time cell electronic sensing" (RT-CES) system from ACEA Bioscience, and xCELLigence from Roche, can also be used for the real-time, label-free monitoring of natural killer (NK) cell-mediated cytolysis.[53]

Cell toxicity can also be detected, based on the electrical responses of electrogenic cells and tissues, such as nerve and heart muscle cells. Nerve cells have high-specificity receptors sensitive to many neuroactive compounds. Changes in membrane potential influence the measurable capacity between a microelectrode and axon when nerve cells are grown on a field effect transistor.[54] An electrophysiological assay has been developed to test the spiking, bursting and oscillatory network activities of neural cells, as affected by drugs.[55] Similarly, biocompatible silicon chips with electrode arrays have been used to culture hippocampal rat brain tissue slices for the multi-site electroresponsive recording of fresh nervous tissues in real time, although the sensitivity of this array seems to be low.[56] The electrophysiological responses of heart muscle cells are more significant than those of neural cells. Delayed ventricular repolarization (QT interval prolongation)

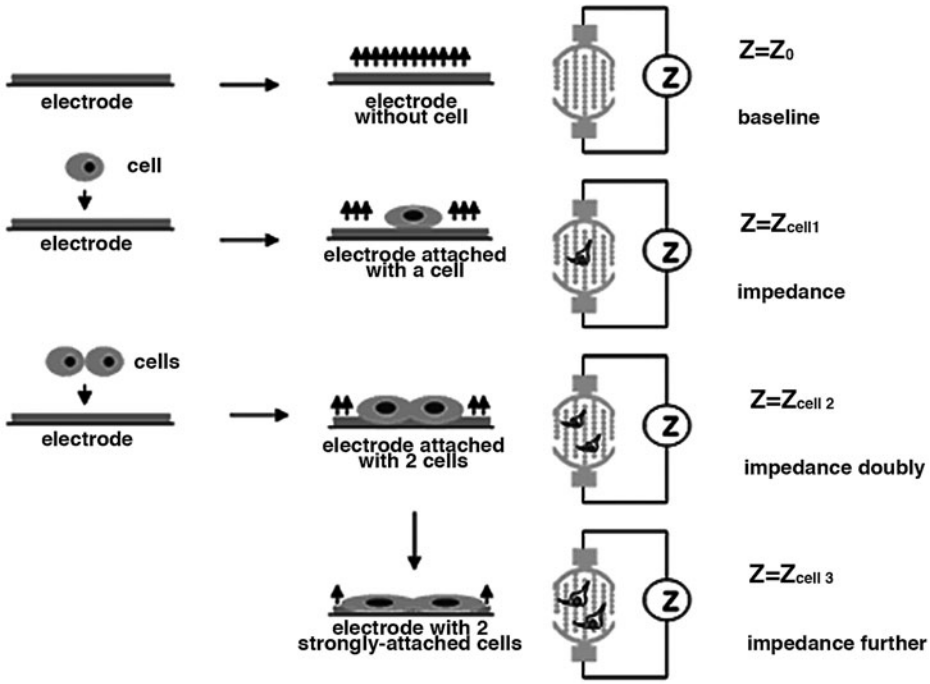

Figure 6. Principle of real-time cell electronic sensing (RT-CES) system based on electrical impedance measurements. The impedance is set as a baseline without any cell attachment on the electrode surface. With the same physiological status, the impedance presents cell number quantitatively. With the same number of cells attached, the impedance reflects a change of cell status such as viability or morphology. Reproduced from www.aceabio.com, with permission.

can cause death and is therefore a major concern in drug safety. The US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) require all drug candidates to undergo in vitro testing for their effects on QT interval prolongation. A micro-electrode assay (MEA) with multi-channel recording has been developed for automatically collecting data from cardiac cell networks (Fig. 7). Here, embryonic chicken ventricular cells are cultured on a polyethylenimine coated chip containing the electrode array and with a beating syncytium. In comparison with the conventional hERG assay, the MEA monitors a network of cells and can better represent the in vivo situation, thus reducing the risk of losing potential drug candidates due to "false-positive" results.[57]

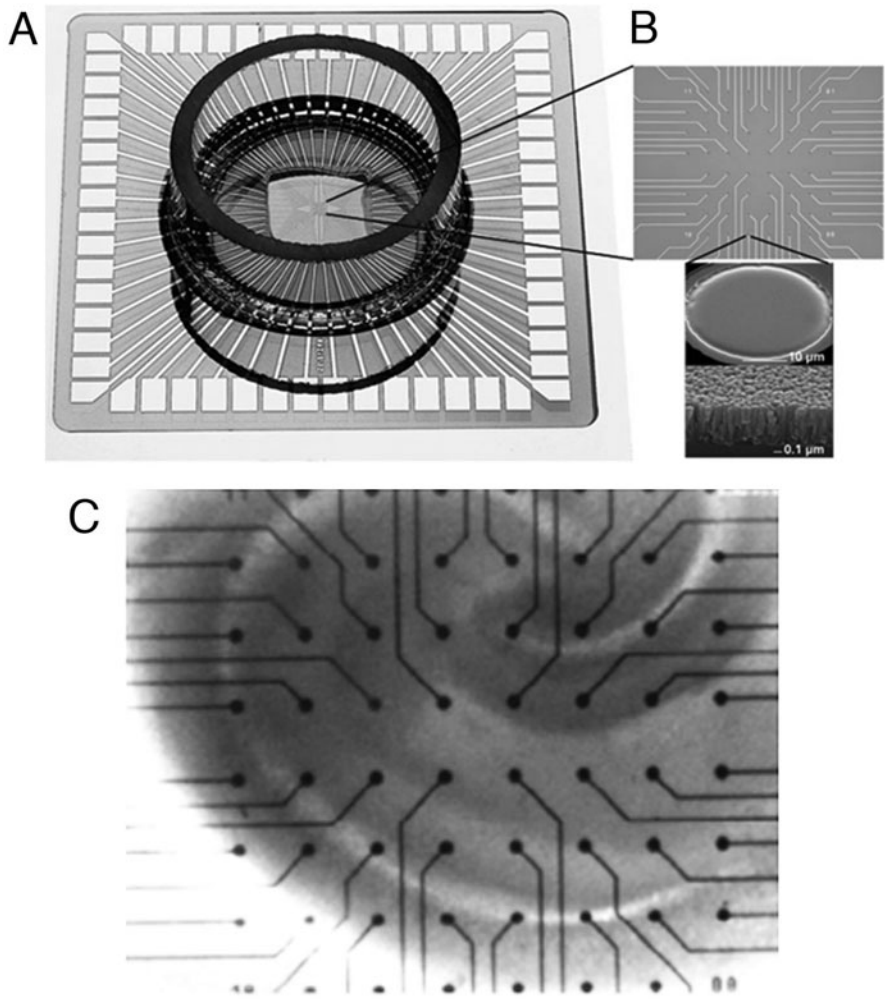

Figure 7. A micro-electrode assay (MEA) chip with multi-channel recording for collecting data from cardiac cell networks. A) MEA chip. B) Electrodes on MEA. C) Hippocampal slice mounted on a standard MEA. Reproduced from www.multichannelsystems.com, with permission.

Changes in metabolic products (e.g., lactic acid and CO_2) or substrates (e.g., glucose and dissolved oxygen), which are closely related to cell number and activities, can be monitored by measuring pH, dissolved oxygen or glucose levels, by using a specific ion or enzyme electrode. However, this approach has not been widely applied in HTP cell-based assays, partially because the metabolic activities can be affected by many environmental factors that are usually difficult to control in microbioreactors. A microphysiometer was developed to measure the acidification rate in the vicinity of cells.[58] The pH value in pH-sensing chambers has been used to indicate cellular biochemical responses due to the accumulation of lactic acid and CO_2. A miniaturized system for oxygen detection has also been reported for the study of heterogeneous pO_2 distributions around tissues.[59]

CELL-BASED HTS FOR TOXICITY TESTING

Rightly or wrongly, the current gold standard for toxicity studies is animal experiments. The capability of obtaining similar or even better information by using human cells with minimal sacrifice of animals is a Holy Grail for toxicology in vitro. Increasing evidence has suggested that 3D cell culture is required to demonstrate the in vivo physiology for many cell/tissue types, including tumor cells, hepatocytes, chondrocytes, neural cells and embryonic stem cells.[60-62] This is because 3D culture is critical to many important cell functions, including morphogenesis, cell metabolism, gene expression, differentiation and cell-cell interactions.[63] Colon cancer cells were found to be 1000 times more resistant to gemcitabine in 3D cultures than in 2D cultures.[64] The finding that gemcitabine is ineffective for killing colon cancer cells in 3D coincides with the lack of in vivo efficacy of this drug for colon cancer in clinical trials. Discrepancies in predicted drug treatment effectiveness in 2D and 3D cultures also implicate the advantage of using 3D culture systems for cell-based assays.[64-66]

Microfabrication can create 3D extracellular matrix (ECM) structures to cells as mechanical cues to mimic their in vivo counterparts. For example, tissue culture scaffolds can be generated with microfabrication, such as a multi-layer 3D scaffold with well-defined dimensions,[67] a vascular tissue scaffold[68] and layer-by-layer microfluidics for biomimetic 3D structures.[69] Specific tissues have been engineered with microfabrication and microfluidics techniques, such as functional liver cells,[70-76] bone cells,[77] cartilage culture[78] and neuronal cultures.[79,80] In addition, microfluidics can continuously supply nutrients and remove waste, to permit continuous experimentation in the long term, with controllable temporal parameters. Microfabricated devices and biochips, thus, offer opportunities for studying and using biological cells,[81] with the unique advantages of HTP experimentation and the ability to provide tailor-made microenvironments.[82] Interested readers are referred to some review articles on tissue engineering and cell biology in microfabricated devices.[83-87] This section will review cell-based assays involving the use of microplates and microfluidic devices, with applications ranging from early-stage drug screening to further pharmacokinetic (PK) and pharmacodynamic (PD) studies.

Microplate-Based Technology

With automatic liquid sample handling and readers, microplate-based HTP technology is widely used in the biotechnology and pharmaceutical industries for various

applications, including enzyme linked immunosorbent assay, polymerase chain reaction and DNA sequencing, as well as combinatorial chemical synthesis and the evaluation of drug effects on apoptosis, absorption, metabolism and toxicity in the drug discovery process.[88] Many commercial microplate systems, including peripheral accessories, such as incubators, robotic handlers and centrifuges, are available for cell-based and biochemical assays utilizing fluorescence, luminescence and colorimetric detection. Compared to biochemical assays, cell-based assays provide more-extensive functional information in early-stage screening and can better identify drug candidates with desired pharmacological characteristics.[89] Table 3 shows some examples of microplate-based HTP cell assays.

The transfected, primary, or stem cells used for HTS usually are cultured in multi-well tissue culture plates with modified surfaces that enhance protein binding and cell attachment. In toxicity studies, the quantification of cell viability and/or cell number is required. As discussed in the previous section, the majority of cell-based assays rely on live/dead cell staining to monitor cell responses to toxicants, which are usually quantified by imaging or microscopic visual count.[22-25,27-30,90] A new trend is to perform online non-invasive dynamic assays, which result in the saving of time and labor during manipulation and the gaining of more information with a time resolution. Disposable microplates integrated with electrode arrays or optical sensors have been developed for cell-based assays. A "real-time cell electronic sensing" (RT-CES) system (ACEA Biosciences), with electrode arrays

Table 3. Some examples of biochips and microplate-based cell assays used in HTP drug screening

Application or Endpoint	Examples
Cytotoxicity	RT-CES system with electrodes printed on the bottom of a microtiter plate for cytotoxicity assay[41]
Cardiotoxicity	QT interval prolongation assay—MEA using embryonic chicken ventricular cells cultured on chips containing electrode arrays for monitoring responses from cardiac cell networks[57]
Neurotoxicity	Silicon chips with electrode arrays for culturing hippocampal rat brain tissue slices for multi-site electroresponsive recording of fresh nervous tissues[56]
Embryotoxicity	Embryonic stem cell test (EST). The effects of chemicals on the development of endodermal cells evaluated by using a stable mouse embryonic cell line with GFP expression, regulated by an alpha-fetoprotein enhancer, as a marker for endodermal cell differentiation[48]
NK-mediated cytolysis	xCELLigence (Roche) for real-time, label-free monitoring of natural killer (NK) cell-mediated cytolysis[53]
Absorption	Caco-2 assay on 96-well transwell plates[197]
	BioCoat™ HTS Caco-2 Assay System (BD Biosciences) for intestine-like cell monolayer formation within 3 days (www.bdbiosciences.com)
Metabolism	Cryopreserved intact hepatocytes in HTS to determine drug metabolic stability[198]

integrated onto the bottom of a microtiter plate for the quantitative detection of living cells, has been developed for use in cytotoxicity assays.[41] This system has been shown to be useful for several cell-based assays and can also predict acute toxicity. A similar label-free assay system, xCELLigence, codeveloped by Roche and ACEA Biosciences, was used to directly monitor NK cell-mediated cytolysis. Corning® Epic™ system uses standard 384-well microplates with optical sensors to detect changes in the refraction index due to the binding of drugs to the biomolecules immobilized in each well.

Almost all microplate-based cell assays use monolayer cultures grown on 2D plate surfaces. However, 2D cultures suffer from contact inhibition and loss of native cell morphology and functionality. Maintaining cells in their native functional state in order to accurately predict drug effects and reduce clinical trial failures is critical to the drug discovery process. As discussed, 3D cell models are better representations of real human tissues, so usually provide drug effects closer to those observed in in vivo studies. We have designed, built and tested a microplate-based, real-time, bioactivity assay based on 3D cultures of GFP-expressing mammalian cells (Fig. 5A). This HTP assay can monitor cellular responses to chemicals in a 3D environment, mimicking their in vivo counterpart and at the same time increasing the signal to noise ratio by at least one order of magnitude as compared to the conventional 2D culture system.[91] More importantly, this 3D cell-based assay gives a compound toxicity profile that is consistent with in vivo results and improves the predictability of cell-based assays for drug efficacy in clinical trials.

Microplate-based cell assays provide a convenient platform and clearly demonstrate the value of HTP cell assays. In current drug discovery practice, 96-well plates are the HTP universal format, while assays in plates with 384 or more wells are being verified to reduce the reagent cost and to further increase the efficiency.[92] However, there are constraints which limit the applicability of more-compact formats, such as 1536-well plates. The small volume of medium in wells is affected by surface tension forces, which lead to heterogeneous cell distribution. Evaporation may also reduce the quality of cell culture. In comparison with the complexity of human body, microplate cultures are also limited to very simple configurations. The maintenance of in vivo micro-environments, especially taking physiological hydrodynamic forces into account, can only be addressed with the help of the microfluidic techniques discussed below.

Microfluidic Technology

Microfluidic devices are composed of different functional components for various liquid handling processes, including pumping, valving, mixing and concentration manipulation. These microfluidic components play critical roles in on-chip cell cultures, which have many applications, including toxicity testing. Here, we will discuss microfluidic components and devices, as well as their applications in cell-based assays (Table 4).

Micro Valves and Pumps

Valves are common parts in a fluidic conduit. By multilayer soft lithography, Quake et al[93] developed a microfluidic system containing on-off valves controlled by using air-actuated control lines on top of flow lines based on elastomer pneumatic valving. Tunable hydrogels were also used to make biomimetic valves.[94,95] Another example of a biomimetic valve is a passive "lymph" valve, which permits or stops flow under

Table 4. Some examples of microfluidic devices used for cell-based assays

Applications	Examples
Cytotoxicity	Microfluidic HTP serial dilution of Paclitaxel for cancer cell cytotoxicity tests[199]
	Microfluidic linear dilution useful for cytotoxicity HTS[29]
	Epirubicin against oral cancer-2 cells[22] and Paclitaxel against HeLa cells[23] in HTP microfluidic devices
	HepG2 cell apoptosis induced by doxorubicin in a microfluidic device[152]
	3D perfusion microbioreactor system for continuous culture of primary rat liver cells[75]
	A microfluidic array of 576 chambers used to study five toxins at various concentrations against 3 cell types[165]
Cell-cell interactions	Macrophages and osteoblasts[141]
	Embryonic stem cells with fibroblasts and/or hepatocytes[166]
	Red blood cells and vascular endothelium[167]
	Micro-scale cell culture analog as a PBPK model for systemic toxicity study[100,168,169]
Cell migration	Chemoattractant effect of epidermal growth factor on human metastatic breast cancer cells in a microfluidic chemotaxis chamber[170]
	Neutrophils or neutrophil-like cells in microfluidic chemokine or peptide gradients[171,172]
Cell differentiation	Differentiation from myoblasts to myotubes in a long-term perfusion culture[173]
	Human neural stem cell differentiation to astrocytes under a gradient of a cocktail of growth factors[174]
	Uniform embryoid body formation[175]
	A microbioreactor array used to study human ESC differentiation under different cell densities and flow configurations[177]

different flow directions, based on the elastomer and the lymph design.[96] In addition to pressure systems, a valving mechanism can be produced with magnetic forces[97] and passive valves based on capillarity action are used to control parallel flows.[98]

Valves can be further exploited for actuation as a pump. An array of parallel control lines over the flow lines of the on-off valve can work in sequential deflection to drive fluid flow.[93] The unidirectional lymph valves discussed above can be put together in a series for pumping.[96] In many examples, the diaphragmatic actuation of elastomer is used to work as a pump or even as a logical structure.[99-102] Furthermore, novel micropumps that utilize cellular energy, such as the intrinsic pulsatile mechanical

functions of cardiomyocytes,[103,104] have also been used for on-chip pumping. Beebe et al[105] developed a magnetically-driven oscillation bar to pump liquid in one direction, which operates in a similar way to that whereby a swimming fish uses its tail fin to generate vortices and pressure differences. Readers are referred to a number of review articles on micropumps.[106-108]

Fluids can also be transported in microfluidic channels by using an external pressure source, such as a syringe, a micromachined or a peristaltic pump. Microfluid flow can be powered by electrical (electroosmotic flow or electrohydrodynamic flow),[109,110] magnetic (magnetohydrodynamic flow),[111,112] centrifugal,[113,114] or surface tension directed forces.[115-118] Water transport can also used, based on processes such as evaporation,[119] pervaporation and osmosis.[120]

Microfluidic Mixers

Microfluidic mixers are important in many applications, including on-chip microreactors, drug delivery systems, sequencing, single cell or organism studies and HTP analyses. Since laminar flow dominates in microchannels, complete mixing needs to be achieved with specific on-chip components for passive or active mixing. On the whole, passive mixing is achieved by molecular diffusion and chaotic advection, which increases the contact surface and decreases the diffusion paths between different fluids[121] through various microchannel designs. Examples of such mixers include a T-mixer or Y-mixer (Fig. 8A),[122-124] parallel lamination mixers with multiple streams driven by either pressure[125,126] or electro-osmosis,[127,128] serial lamination mixers with multiple splitting and joining stages, both horizontally and vertically (Fig. 8B)[129] and other special microchannel geometries (Fig. 8C).[130-132] There are also active micromixers, which depend on disturbance in pressure,[133,134] or electrohydrodynamic,[135] electrokinetic,[136] magneto hydrodynamic,[137] or acoustic fields.[138]

Microfluidic Gradient Generators

Microfluidic gradient generators involve microfluidic channels capable of highly-parallel fluid manipulation for generating concentration gradients critical to cell-based assays. These can replace the costly automatic liquid dispensing systems commonly used with the microplate systems discussed above. A gradient generator usually involves multiple stages of parallel channels combined from the upstream direction and diverging with an increasing number of serpentine channels downstream, in order to generate stable concentration gradients at the outlet (Fig. 9A).[139-142] This method is based on diffusive mixing at both the junctions of merging channels and the diverging individual channels. Similar microfluidic networks can be used to generate nonlinear and complex gradients.[143,144] A universal microfluidic gradient generator based on series of microdividers has also been developed, specifically for nonlinear spatial gradient generation (Fig. 9B).[145] Generating a static gradient is necessary for certain applications, such as cell-signaling and chemotaxis studies. This can be achieved through high-resistance membranes[146] or hydrogels between a source and a sink (Fig. 9C).[147] In addition to concentration gradient generators, oxidative microgradients have also been generated by using an array of microelectrodes.[148] Other microfluidic devices are capable of generating temperature gradients.[149,150]

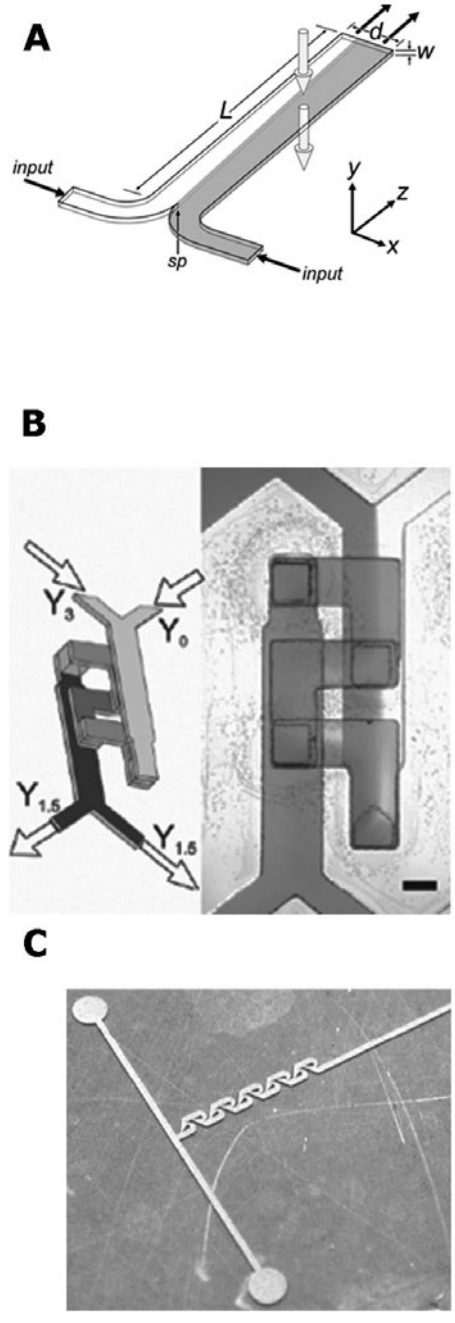

Figure 8. Representative types of micromixers. A) T-sensor for diffusion-based mixing, courtesy of Dr. Paul Yager. B) Micromixer with multiple splitting and joining stages in three dimensions. Reproduced from Neils C et al. Lab Chip 2004; 4:342-350,[129] ©2004 The Royal Society of Chemistry. C) Micromixer with modified Tesla structures. Reproduced from Hong CC et al. Lab Chip 2004; 4:109-113.[130] ©2004 The Royal Society of Chemistry.

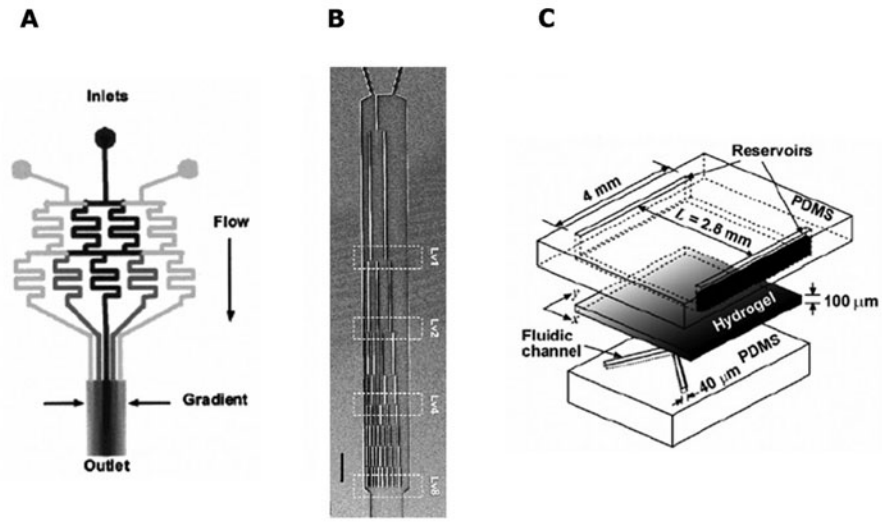

Figure 9. Different formats of microfluidic gradient generators. A) Microfluidic system to produce tunable gradients based on diffusive mixing. Reprinted in part with permission from Jeon NL et al. Langmuir 2000; 16:8311-8316,[140] ©2000 American Chemical Society. B) Photograph of a microfluidic device able to generate universal gradients by design through microdividers. Reprinted in part with permission from Irimia D et al. Anal Chem 2006; 78:3472-3477,[145] ©2006 American Chemical Society. C) Microfluidic system to generate complex static gradients along microchannels of arbitrary designs from a source gradient through the hydrogel above. Reprinted, in part, with permission from Wu H et al. J Am Chem Soc 2006; 128:4194-4195.[147] ©2006 American Chemical Society.

Microfluidic Devices for Cell-Based Assays

Table 4 shows some examples of the microfluidic cell-based assays used in drug screening. Of all the microfluidic cell-based systems, "liver-on-a-chip" has been the most extensively evaluated, due to the need for in vitro tools for hepatoxicity testing.[151] Based on fluorescence signals for high-content assays, a microfluidic device was used to study HepG2 cell apoptosis induced by doxorubicin, by examining several apoptotic events on the chip, including morphological changes, plasma membrane phosphatidylserine externalization and mitochondrial membrane potential collapse.[152]

For high-density 3D cell cultures, mass transport can be a limiting factor in providing sufficient nutrient to cells. Perfusion is thus beneficial for prolonged cell growth in cell and tissue cultures.[153] Microfluidic flow, therefore, becomes the technique of choice for microscale perfusion culture. Griffith et al[75] developed a 3D perfusion microbioreactor system for the continuous culture of micro-tissue units from primary rat liver cells (Fig. 10), with phenotypes substantially closer to native liver than cultures obtained by other in vitro methods, which promises to be a useful model for studying drug-drug interactions, acute and chronic liver toxicity, viral hepatitis infection and cancer metastasis. The 3D cell organization of this system was originated from multicellular spheroids prepared in a spinner flask. 3D tissue structures in microfluidic channels can also be prepared by the encapsulation of cells in hydrogels[22,25,28,30,90,154-158] or biological extracellular matrix (ECM), such as Matrigel[30,159] and collagen.[160] An enzymatically-cross-linked gelatin was also

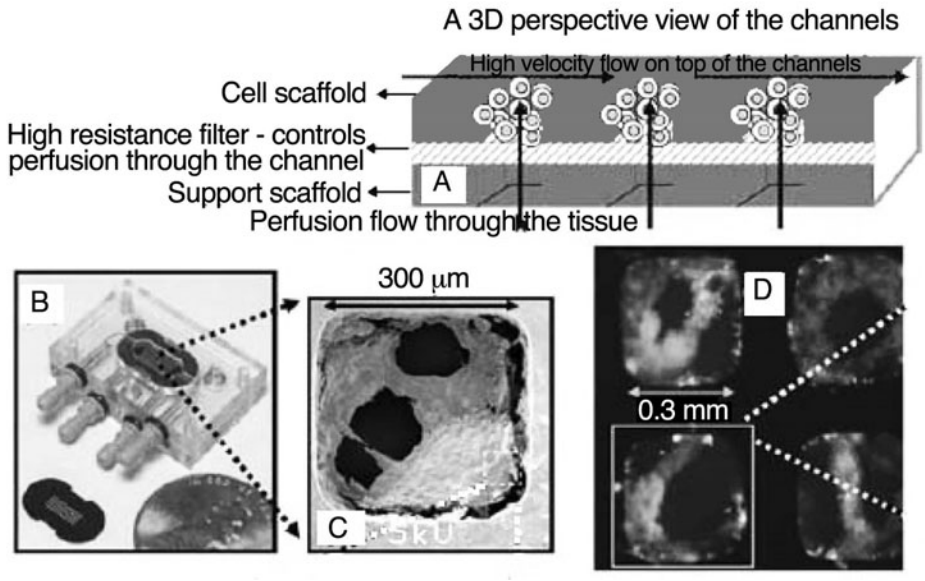

Figure 10. Microfluidic 3D culture of liver cells in microfabricated scaffolds with (A) illustrating a microchannel with cell aggregates, (B) showing the image of the device, which could cultivate cells to high density 3D structures (C) with high viability (D). Reproduced from Sivaraman A et al. Curr Drug Metab 2005; 6:569-591.[75] ©2005 Bentham Science Publishers Ltd.

used to form microfluidic channels containing cells in the wall structure.[161] Furthermore, polymeric fibrous matrices can be employed in 3D cell cultures. Yang et al[82] incorporated highly porous poly(ethylene terephthalate) (PET) nonwoven fibrous matrices into an array of perfusion microbioreactors, with forced interstitial flows through the matrix to permit high mass transfer rates and to promote high-density cell expansion.

Many efforts have been directed towards increasing the throughput of microfluidic cell-based assays through well-controlled fluid handling without complex robotics. An 8 × 8 nanoliter scale microbioreactor array with an on-chip concentration generator has been described.[139,162,163] An array of 256 nanoliter microbioreactors has also been developed for characterizing gene expression in living cells genetically modified with fluorescent reporter proteins under specific promoters.[164] Figure 11 shows a microfluidic array of 576 chambers used in cytotoxicity studies for five toxins at different concentrations against three cell types.[165]

Microfluidic devices have also been developed for studying cell-cell interactions in cocultures, which can generate valuable knowledge for guiding the design of in vitro toxicity study tools with the capacity to reveal systematic phenotypic changes induced by of drugs or toxins. For example, in a two-level microfluidic coculture system, cytokines and other factors released from up-stream macrophages flew through a gradient generator before passing down-stream to an osteoblast culture, where cell-cell interaction was studied with a bone resorption marker and in terms of osteoblast viability.[141] Static and sequentially dynamic patterned cocultures of embryonic stem cells with NIH-3T3 fibroblasts and/or AML12 hepatocytes were produced by using microfabricated parylene-C stencils, thus providing a means to temporally and spatially control cell-cell interactions.[166] Furthermore, the cellular interaction between red blood cells and the vascular endothelium was also

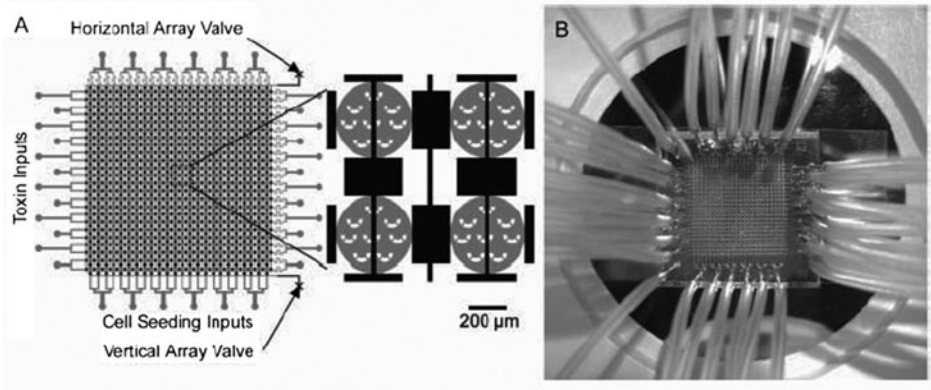

Figure 11. A microfluidic array of 576 chambers for high-throughput cytotoxicity studies. A) A schematic of the microfluidic cytotoxicity array; B) An image of the chip with fluid interconnects. Reproduced from Wang Z et al. Lab Chip 2007; 7:740-745.[165] ©2007 The Royal Society of Chemistry.

identified with cellular ATP release and NO secretion increased by drug stimulation.[167] Finally, a microscale cell culture analog (μCCA), also called "animal-on-a-chip," was developed and used as a physiologically-based PK (PBPK) model.[100,168,169] This system had several interconnected microfluidic compartments, each for a different organotypic cell culture, to recapitulate drug metabolism, transport, distribution and toxicity (Fig. 12). It harnesses the complexity of toxicity at the systemic level in vitro in order to increase predictive efficacy at an early stage and represents one of the most important directions for future toxicity evaluation systems.

Microfluidic devices have been used to study cell differentiation into specifically functional cell types. Various organotypic cells are useful in these cell-based toxicity studies. In one example, the whole process of differentiation from myoblasts to myotubes was examined with morphological and biochemical markers in a long-term perfusion culture on a chip.[170] In another microfluidic device, human neural stem cell growth and differentiation into astrocytes were investigated under a gradient of a cocktail of growth factors.[171] Microfluidic devices were also used to study how to generate uniform embryoid bodies from embryonic stem cells (ESCs).[172] Cellular microarrays and microfluidics were used for the HTP analysis of signals regulating stem cell fate and function.[173] Figallo et al[174] designed a microbioreactor array and used it to study human ESC differentiation under different conditions of cell density and flow configuration. These systems can also be used in cell-based toxicity studies, as in vitro models closely mimicking in vivo conditions.

Besides toxicity testing in microfluidic channels, another relevant application of microfluidic devices is for chemotaxis assays. The chemoattractant effect of epidermal growth factor on human metastatic breast cancer cells has been examined by using a microfluidic chemotaxis chamber capable of generating multiple growth factor gradients.[175] In another example, a microfluidic device that was capable of establishing stable chemical gradients and allowing fast gradient changes, was used to measure neutrophil migratory responses. Temporary depolarization of neutrophils was observed under fast reversal of the gradient direction of a chemokine.[176] Finally, a hydrogel-based microfluidic gradient generator has been used to study neutrophil-like cells that migrate towards higher concentrations of formyl-Met-Leu-Phe.[177]

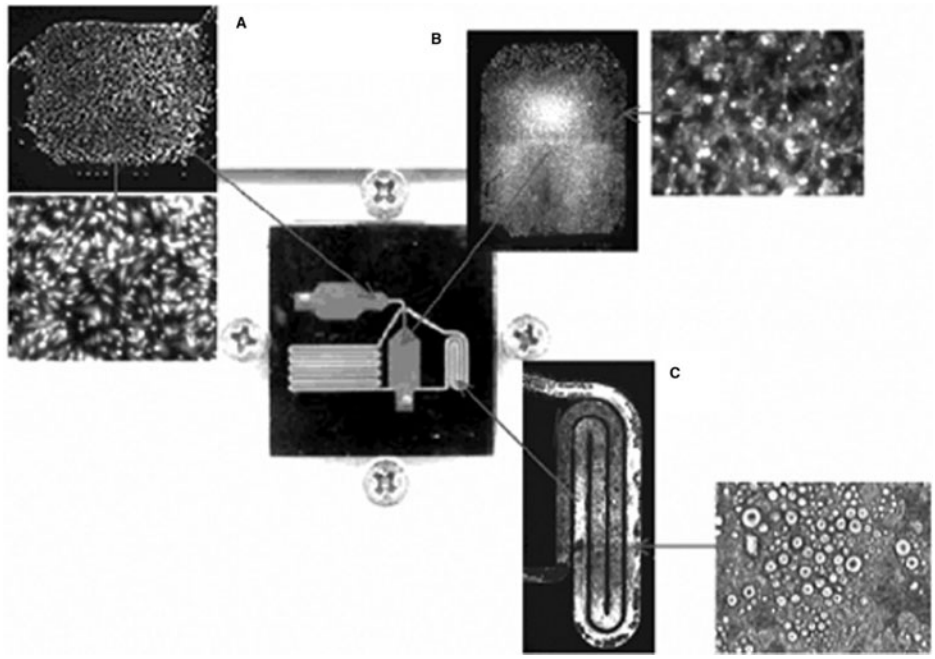

Figure 12. Photographs and microscopic pictures of the microscale cell culture analog, featuring connected chambers representing different organs, with (A) indicating lung epithelial cells (L2 cell line) in the lung chamber, (B) representing liver cells (HepG2/C3A cell line) in the liver chamber and (C) adipocytes differentiated from 3T3-L1 cell line in the fat chamber. Reprinted from Viravaidya K, Shuler ML. Biotechnol Prog 2004; 20:590-597.[169] ©2004 American Chemical Society and American Institute of Chemical Engineers.

FUTURE PERSPECTIVES

The throughput and quality of data generation can be increased by the parallel operation of microbioreactors with well-controlled parameters, such as pH, dissolved oxygen (DO), optical density (OD) and major metabolic activities, etc.[178-191] Non-invasive optical sensors play a key role in the development of such microbioreactors. DO in the culture media can be monitored with ruthenium dye[192] and Alamar Blue.[193] In addition to monitoring, a microliter bioreactor array of eight independent units has recently been developed, with the control of both pH and DO.[183] It is envisaged that, before long, the use of microbioreactors for bioprocess development will be routine in the pharmaceutical industry. Furthermore, the authors believe that the need for creating highly in vivo like microenvironments may, one day, make control capabilities for parameters such as pH and DO also become critical in toxicity testing. It is vital that cell-based assays are performed under physiologically relevant conditions, which can vary greatly with different types of tissues. For example, the pH in the stomach is normally as low as 1.0 and more and more evidence indicates that delicately controlled DO levels may have significant effects on various tissues and diseases, such as embryonic stem cells,[194] cartilage tissue development[195] and even tumor progression.[196] Incorporating DO and pH control capabilities into current microplates and microfluidic devices for HTS, however, remains a challenge.

CONCLUSION

Microfabrication and microfluidic technologies permit the development of unprecedented tools for exploitation in HTS. The conceivable advantages of using chip-based microdevices include some of the common benefits of miniaturization—minimal reagent consumption, short processing time, small space requirement, low cost and portability. This chapter introduces important microfabrication techniques and microfluidic devices with applications in the pharmaceutical industry. For cell-based toxicity assays, the unique strengths of using microfluidic devices are increasingly being developed and employed, including the capability of creating in vivo-like microenvironments with relevant dimensions for cells and versatile fluid and concentration handling and manipulation. With effectively integrated detection methods, microchip-based technologies can be used to perform HTP experiments to speed up the drug discovery process. In addition, new advances in microbioreactors with controllable pH and DO, originally developed for applications in tissue engineering and bioprocessing, can be incorporated into future cell-based assay platforms.

An important trend in toxicity testing involves the concept of creating systemic interactions in vitro through different and interconnected organotypic cultures. These can also be used in testing drug absorption, distribution, metabolism and elimination. This poses unmet challenges in our understanding of both fundamental biology and the application of advanced engineering techniques. No doubt, microfabrication, microfluidics and the related detection methods are at the core of the technologies required to serve this concept. Therefore, in line with the theme of this book, we sincerely hope that this chapter serves as a primer that will help its readers, beginners and experts alike, to imagine the great possibilities ahead of us with the new technologies and to stimulate the desire to unleash the creativity to make them come true.

REFERENCES

1. Ziaie B, Baldi A, Lei M et al. Hard and soft micromachining for BioMEMS: review of techniques and examples of applications in microfluidics and drug delivery. Adv Drug Deliv Rev 2004; 56:145-172.
2. Madou MJ. Fundamentals of Microfabrication: The Science of Miniaturization, 2nd ed. Boca Raton: CRC Press, 2002.
3. Haber C. Microfluidics in commercial applications; an industry perspective. Lab Chip 2006; 6:1118-1121.
4. Xia Y, Whitesides GM. Soft lithography. Annu Rev Mat Sci 1998; 28:153-184.
5. Whitesides GM, Ostuni E, Takayama S et al. Soft lithography in biology and biochemistry. Annu Rev Biomed Eng 2001; 3:335-373.
6. Jaeger CJ. Introduction to Microelectronic Fabrication. Upper Saddle River, New Jersey: Prentice Hall, 2002.
7. Campbell SA. The Science and Engineering of Microelectronic Fabrication. New York: Oxford University Press, 2001.
8. Smith DL. Thin-Film Deposition: Principles and Practice. New York: McGraw-Hill, 1995.
9. Wu CC, Saito T, Yasukawa T et al. Microfluidic chip integrated with amperometric detector array for in situ estimating oxygen consumption characteristics of single bovine embryos. Sensor Actuator B 2007; 125:680-687.
10. Ehret R, Baumann W, Brischwein M et al. Monitoring of cellular behaviour by impedance measurements on interdigitated electrode structures. Biosens Bioelectron 1997; 12:29-41.
11. Voldman J, Gray ML, Schmidt MA. Microfabrication in biology and medicine. Annu Rev Biomed Eng 1999; 1:401-425.
12. Qin D, Xia Y, Whitesides GM. Rapid prototyping of complex structures with feature sizes larger than 20 μm. Adv Mater 1996; 8:917-919.
13. Xia Y, Kim E, Zhao XM et al. Complex optical surfaces formed by replica molding against elastomeric masters. Science 1996; 273:347-349.

14. Becker H, Heim U. Hot embossing as a method for the fabrication of polymer high aspect ratio structures. Sensor Actuator A 2000; 83:130-135.
15. Wilbur JL, Kumar A, Kim E et al. Microfabrication by microcontact printing of self-assembled monolayers. Adv Mater 1994; 6:600-604.
16. Biebuyck HA, Larsen NB, Delamarche E et al. Lithography beyond light: microcontact printing with monolayer resists. IBM J Res Dev 1997; 41:159-170.
17. Xia Y, Zhao XM, Kim E et al. A selective etching solution for use with patterned self-assembled monolayers of alkanethiolates on gold. Chem Mater 1995; 7:2332-2337.
18. Drelich J, Miller JD, Kumar A et al. Wetting characteristics of liquid drops at heterogeneous surfaces. Colloids and Surfaces A 1994; 93:1-13.
19. Jackman RJ, Wilbur JL, Whitesides GM. Fabrication of submicrometer features on curved substrates by microcontact printing. Science 1995; 269:664-666.
20. Jeon NL, Nuzzo RG, Xia Y et al. Patterned self-assembled monolayers formed by microcontact printing direct selective metallization by chemical vapor deposition on planar and nonplanar substrates. Langmuir 1995; 11:3024-3026.
21. Danosky TR, McFadden PN. Biosensors based on the chromatic activities of living, naturally pigmented cells: digital image processing of the dynamics of fish melanophores. Biosens Bioelectron 1997; 12:925-936.
22. Wu MH, Huang SB, Cui ZF et al. Development of perfusion-based micro 3-D cell culture platform and its application for high throughput drug testing. Sensor Actuator B 2008; 129:231-240.
23. Lee PJ, Gaige TA, Ghorashian N et al. Microfluidic tissue model for live cell screening. Biotechnol Prog 2007; 23:946-951.
24. Liu D, Wang L, Zhong R et al. Parallel microfluidic networks for studying cellular response to chemical modulation. J Biotechnol 2007; 131:286-292.
25. Koh WG, Pishko MV. Fabrication of cell-containing hydrogel microstructures inside microfluidic devices that can be used as cell-based biosensors. Anal Bioanal Chem 2006; 385:1389-1397.
26. Park JY, Hwang CM, Lee SH et al. Gradient generation by an osmotic pump and the behavior of human mesenchymal stem cells under the fetal bovine serum concentration gradient. Lab Chip 2007; 7:1673-1680.
27. Kim JY, Park H, Kwon KH et al. A cell culturing system that integrates the cell loading function on a single platform and evaluation of the pulsatile pumping effect on cells. Biomed Microdevices 2008; 10:11-20.
28. Wu MH, Huang SB, Cui Z et al. A high throughput perfusion-based microbioreactor platform integrated with pneumatic micropumps for three-dimensional cell culture. Biomed Microdevices 2008; 10:309-319.
29. Walker GM, Monteiro-Riviere N, Rouse J et al. A linear dilution microfluidic device for cytotoxicity assays. Lab Chip 2007; 7:226-232.
30. Frisk T, Rydholm S, Liebmann T et al. A microfluidic device for parallel 3-D cell cultures in asymmetric environments. Electrophoresis 2007; 28:4705-4712.
31. Yu H, Alexander CM, Beebe DJ. A plate reader-compatible microchannel array for cell biology assays. Lab Chip 2007; 7:388-391.
32. Beske OE, Goldbard S. High-throughput cell analysis using multiplexed array technologies. Drug Discov Today 2002; 7(18 Suppl):S131-135.
33. Bruchez M Jr, Moronne M, Gin P et al. Semiconductor nanocrystals as fluorescent biological labels. Science 1998; 281:2013-2016.
34. Chan WCW, Nile S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 1998; 281:2016-2018.
35. Han M, Gao X, Su JZ et al. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat Biotechnol 2001; 19:631-635.
36. Daunert S, Barrett G, Feliciano JS et al. Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. Chem Rev 2000; 100:2705-2738.
37. Gribbon P, Sewing A. Fluorescence readouts in HTS: no gain without pain? Drug Discov Today 2003; 8:1035-1043.
38. Sundberg SA. High-throughput and ultra-high-throughput screening: solution- and cell-based approaches. Curr Opin Biotech 2000; 11:47-53.
39. Wolff M, Wiedenmann J, Nienhaus GU et al. Novel fluorescent proteins for high-content screening. Drug Discov Today 2006; 11:1054-1060.
40. Barrett KL, Willingham JM, Garvin AJ et al. Advances in cytochemical methods for detection of apoptosis. J Histochem Cytochem 2001; 49:821-832.
41. Xing JZ, Zhu L, Gabos S et al. Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. Toxicol In Vitro 2006; 20:995-1004.
42. Zucco F, De Angelis I, Testai E et al. Toxicology investigations with cell culture systems: 20 years after. Toxicol In Vitro 2004; 18:153-163.
43. Hertzberg RP, Pope AJ. High-throughput screening: new technology for the 21st century. Curr Opin Chem Biol 2000; 4:445-451.

44. Takanishi CL, Bykova EA, Cheng W et al. GFP-based FRET analysis in live cells. Brain Res 2006; 1091:132-139.
45. Mahajan NP, Harrison-Shostak DC, Michaux J et al. Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. Chem Biol 1999; 6:401-409.
46. Xu X, Gerard ALV, Huang BCB et al. Detection of programmed cell death using fluorescence energy transfer. Nucleic Acids Res 1998; 26:2034-2035.
47. Abraham VC, Taylor DL, Haskins JR. High content screening applied to large-scale cell biology. Trends Biotechnol 2004; 22:15-22.
48. Paparella M, Kolossov E, Fleischmann BK et al. The use of quantitative image analysis in the assessment of in vitro embryotoxicity endpoints based on a novel embryonic stem cell clone with endoderm-related GFP expression. Toxicol In Vitro 2002; 16:589-597.
49. Haney SA, LaPan P, Pan J et al. High-content screening moves to the front of the line. Drug Discov Today 2006; 11:889-894.
50. Ramm P. Image-based screening: a technology in transition. Curr Opin Biotechnol 2005; 16:41-48.
51. Ziegler C. Cell-based biosensors. Fresenius J Anal Chem 2000; 366:552-559.
52. Giaever I, Keese CR. Monitoring fibroblast behavior in tissue culture with an applied electric field. Proc Natl Acad Sci USA 1984; 81:3761-3764.
53. Matthew A, Cooper TM. Current Biosensor Technologies in Drug Discovery. Drug Discovery World 2006; Summer:68-82
54. Fromherz P, Offenhausser A, Vetter T et al. A neuron-silicon junction: a Retzius cell of the leech on an insulated-gate field-effect transistor. Science 1991; 252:1290-1293.
55. Gross GW, Harsch A, Rhoades BK et al. Odor, drug and toxin analysis with neuronal networks in vitro: extracellular array recording of network responses. Biosens Bioelectron 1997; 12:373-393.
56. Jahnsen H, Kristensen BW, Thiebaud P et al. Coupling of organotypic brain slice cultures to silicon-based arrays of electrodes. Methods 1999; 18:160-172.
57. Meyer T, Boven KH, Gunther E et al. Micro-electrode arrays in cardiac safety pharmacology: a novel tool to study QT interval prolongation. Drug Safety 2004; 27:763-772.
58. Rabinowitz JD, Rigler P, Carswell-Crumpton C et al. Screening for novel drug effects with a microphysiometer: a potent effect of clofilium unrelated to potassium channel blockade. Life Sci 1997; 61:PL87-PL94.
59. Braun RD, Lanzen JL, Snyder SA et al. Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. Am J Physiol 2001; 280:H2533-H2544.
60. Mueller-Klieser W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am J Physiol 1997; 273:C1109-1123.
61. Abbott A. Biology's new dimension. Nature 2003; 424:870-872.
62. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol 2006; 7:211-224.
63. Mueller-Klieser W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am J Physiol 1997; 273:C1109-C1123.
64. Smitskamp-Wilms E, Pinedo HM, Veerman G et al. Postconfluent multilayered cell line cultures for selective screening of gemcitabine. Eur J Cancer 1998; 34:921-926.
65. Kobayashi H, Man S, Graham CH et al. Acquired multicellular-mediated resistance to alkylating agents in cancer. Proc Natl Acad Sci USA 1993; 90:3294-3298.
66. Ries LA, Wingo PA, Miller DS et al. The annual report to the nation on the status of cancer, 1973-1997, with a special section on colorectal cancer. Cancer 2000; 88:2398-2424.
67. Yang Y, Basu S, Tomasko DL et al. Fabrication of well-defined PLGA scaffolds using novel microembossing and carbon dioxide bonding. Biomaterials 2005; 26:2585-2594.
68. Borenstein J, Whyte JL, Badamgarav E et al. Physician practice patterns in the treatment of isolated systolic hypertension in a primary care setting. J Clin Hypertens 2002; 4:93-100.
69. Tan W, Desai TA. Layer-by-layer microfluidics for biomimetic three-dimensional structures. Biomaterials 2004; 25:1355-1364.
70. Powers MJ, Domansky K, Kaazempur-Mofrad MR et al. A microfabricated array bioreactor for perfused 3D liver culture. Biotechnol Bioeng 2002; 78:257-269.
71. Powers MJ, Janigian DM, Wack KE et al. Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. Tissue Eng 2002; 8:499-513.
72. Ostrovidov S, Jiang J, Sakai Y et al. Membrane-based PDMS microbioreactor for perfused 3D primary rat hepatocyte cultures. Biomed Microdevices 2004; 6:279-287.
73. Leclerc E, Sakai Y, Fujii T. Cell culture in 3-dimensional microfluidic structure of PDMS. Biomed Microdevices 2003; 5:109-114.
74. Park J, Berthiaume F, Toner M et al. Microfabricated grooved substrates as platforms for bioartificial liver reactors. Biotechnol Bioeng 2005; 90:632-644.

75. Sivaraman A, Leach JK, Townsend S et al. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. Curr Drug Metab 2005; 6:569-591.
76. Kane BJ, Zinner MJ, Yarmush ML et al. Liver-specific functional studies in a microfluidic array of primary mammalian hepatocytes. Anal Chem 2006; 78:4291-4298.
77. Leclerc E, David B, Griscom L et al. Study of osteoblastic cells in a microfluidic environment. Biomaterials 2006; 27:586-595.
78. Petersen EF, Spencer RG, McFarland EW. Microengineering neocartilage scaffolds. Biotechnol Bioeng 2002; 78:801-804.
79. Taylor A, Rhee S, Tu C et al. Microfluidic multicomponent device for neuroscience research. Langmuir 2003; 19:1551-1556.
80. Oliva AA Jr, James CD, Kingman CE et al. Patterning axonal guidance molecules using a novel strategy for microcontact printing. Neurochem Res 2003; 28:1639-1648.
81. El-Ali J, Sorger PK, Jensen KF. Cells on chips. Nature 2006; 442:403-411.
82. Yang ST, Zhang X, Wen Y. Microbioreactors for high-throughput cytotoxicity assays. Curr Opin Drug Discov Dev 2008; 11:111-127.
83. Pearce TM, Williams JC. Microtechnology: meet neurobiology. Lab Chip 2007; 7:30-40.
84. Li N, Tourovskaia A, Folch A. Biology on a chip: microfabrication for studying the behavior of cultured cells. Crit Rev Biomed Eng 2003; 31:423-488.
85. Khademhosseini A, Langer R, Borenstein J et al. Microscale technologies for tissue engineering and biology. Proc Natl Acad Sci USA 2006; 103:2480-2487.
86. Folch A, Toner M. Microengineering of cellular interactions. Annu Rev Biomed Eng 2000; 2:227-256.
87. Andersson H, van den Berg A. Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. Lab Chip 2004; 4:98-103.
88. Majors RE. New developments in microplates for biological assays and automated sample preparation. LC-GC Europe 2005; 18:70-76.
89. Croston GE. Functional cell-based uHTS in chemical genomic drug discovery. Trends Biotechnol 2002; 20:110-115.
90. Kim MS, Yeon JH, Park JK. A microfluidic platform for 3-dimensional cell culture and cell-based assays. Biomed Microdevices 2007; 9:25-34.
91. Zhang X. 3-D cell-Based High-Throughput Screening for Drug Discovery and Cell Culture Process Development. Columbus, Ohio: Chemical and Biomolecular Engineering, The Ohio State University, 2008.
92. Müller UR, Nicolau DV. Microarray Technology and its Applications. Berlin/New York: Springer; 2005.
93. Unger MA, Chou HP, Thorsen T et al. Monolithic microfabricated valves and pumps by multilayer soft lithography. Science 2000; 288:113-116.
94. Eddington DT, Beebe DJ. Flow control with hydrogels. Adv Drug Deliv Rev 2004; 56:199-210.
95. Beebe DJ, Moore JS, Yu Q et al. Microfluidic tectonics: a comprehensive construction platform for microfluidic systems. Proc Natl Acad Sci USA 2000; 97:13488-13493.
96. McDonald JC, Whitesides GM. Poly(dimethylsiloxane) as a material for fabricating microfluidic devices. Acc Chem Re 2002; 35:491-499.
97. Jackson WC, Tran HD, O'Brien MJ et al. Rapid prototyping of active microfluidic components based on magnetically modified elastomeric materials. J Vac Sci Technol B 2001; 19:596-599.
98. Ahn CH, Choi JW, Beaucage G et al. Disposable smart lab on a chip for point-of-care clinical diagnostics. P IEEE 2004; 92:154-173.
99. Urbanski JP, Thies W, Rhodes C et al. Digital microfluidics using soft lithography. Lab Chip 2006; 6:96-104.
100. Sin A, Reardon CF, Shuler ML. A self-priming microfluidic diaphragm pump capable of recirculation fabricated by combing soft lithography and traditional machining. Biotechnol Bioeng 2003; 85:359-363.
101. Grover WH, Skelley AM, Liu CN et al. Monolithic membrane valves and diaphragm pumps for practical large-scale integration into glass microfluidic devices. Sensor Actuator B 2003; 89:315-323.
102. Grover WH, Ivester RH, Jensen EC et al. Development and multiplexed control of latching pneumatic valves using microfluidic logical structures. Lab Chip 2006; 6:623-631.
103. Tanaka Y, Morishima K, Shimizu T et al. Demonstration of a PDMS-based bio-microactuator using cultured cardiomyocytes to drive polymer micropillars. Lab Chip 2006; 6:230-235.
104. Tanaka Y, Morishima K, Shimizu T et al. An actuated pump on-chip powered by cultured cardiomyocytes. Lab Chip 2006; 6:362-368.
105. Atencia J, Beebe DJ. Magnetically-driven biomimetic micro pumping using vortices. Lab Chip 2004; 4:598-602.
106. Laser DJ, Santiago JG. A review of micropumps. J Micromech Microeng 2004; 11:R35-64.
107. Nguyen NT, Huang XY, Toh KC. MEMS-micropumps: a review. ASME Trans—J Fluid Eng 2002; 124:384-392.
108. Woias P. Micropumps-past, progress and future prospects. Sensor Actuator B 2005; 105:28-38.

109. Ajdari A. Pumping liquids using asymmetric electrode arrays. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics 2000; 61:R45-48.
110. Brown AB, Smith CG, Rennie AR. Pumping of water with ac electric fields applied to asymmetric pairs of microelectrodes. Phys Rev E Stat Nonlin Soft Matter Phys 2001; 63:016305.
111. Lemoff AV, Lee AP. An AC magnetodynamic micropump. Sensor Actuator B 2000; 63:178-185.
112. Pamme N. Magnetism and microfluidics. Lab Chip 2006; 6:24-38.
113. Duffy DC, Gillis HL, Lin J et al. Microfabricated centrifugal microfluidic systems: characterization and multiple enzymatic assays. Anal Chem 1999; 71:1832-1837.
114. Lai S, Wang S, Luo J et al. Design of a compact disk-like microfluidic platform for enzyme-linked immunosorbent assay. Anal Chem 2004; 76:1832-1837.
115. Bouaidat S, Hansen O, Bruus H et al. Surface-directed capillary system; theory, experiments and applications. Lab Chip 2005; 5:827-836.
116. Juncker D, Schmid H, Drechsler U et al. Autonomous microfluidic capillary system. Anal Chem 2002; 74:6139-6144.
117. Walker GM, Beebe DJ. A passive pumping method for microfluidic devices. Lab Chip 2002; 2:131-134.
118. Zhao B, Moore JS, Beebe DJ. Surface-directed liquid flow inside microchannels. Science 2001; 291(5506):1023-1026.
119. Goedecke N, Eijkel J, Manz A. Evaporation driven pumping for chromatography application. Lab Chip 2002; 2:219-223.
120. Eijkel J, Bomer JG, van den Berg A. Osmosis and pervaporation in polyimide submicron microfluidic channel structures. Appl Phys Lett 2005; 87:114103.
121. Nguyen NT, Wu Z. Micromixers—a review. J Micromech Microeng 2005; 15:R1-16.
122. Schilling E, Kamholz AE, Weigl BH et al. The T-sensor. Seattle: University of Washington, 2001: Available at: http://faculty.washington.edu/yagerp/microfluidicstutorial/tsensor/tsensor.htm, 2008.
123. Hinsmann P, Frank J, Svasek P et al. Design, simulation and application of a new micromixing device for time resolved infrared spectroscopy of chemical reactions in solution. Lab Chip 2001; 1:16-21.
124. Kamholz AE, Weigl BH, Finlayson BA et al. Quantitative analysis of molecular interaction in a microfluidic channel: the T-sensor. Anal Chem 1999; 71:5340-5347.
125. Bessoth FG, de Mello AJ, Manz A. Microstructure for efficient continuous flow mixing. Anal Commun 1999; 36:213-215.
126. Koch M, Witt H, Evans A et al. Improved characterization technique for micromixers. J Micromech. Microeng 1999; 9:156-158.
127. Hadd AG, Raymond DE, Halliwell JW et al. Microchip device for performing enzyme assays. Anal Chem 1997; 69:3407-3412.
128. Jacobson SC, McKnight TE, Ramsey JM. Microfluidic devices for electrokinematically driven parallel and serial mixing. Anal Chem 1999; 71:4455-4459.
129. Neils C, Tyree Z, Finlayson B et al. Combinatorial mixing of microfluidic streams. Lab Chip 2004; 4:342-350.
130. Hong CC, Choi JW, Ahn CH. A novel in-plane passive microfluidic mixer with modified Tesla structures. Lab Chip 2004; 4:109-113.
131. Nichols KP, Ferullo JR, Baeumner AJ. Recirculating, passive micromixer with a novel sawtooth structure. Lab Chip 2006; 6:242-246.
132. Wang H, Iovenitti P, Harvey E et al. Optimizing layout of obstacles for enhanced mixing in microchannels. Smart Mater Struct 2002; 11:662-667.
133. Glasgow I, Aubry N. Enhancement of microfluidic mixing using time pulsing. Lab Chip 2003; 3:114-120.
134. Lin CH, Tsai CH, Pan CW et al. Rapid circular microfluidic mixer utilizing unbalanced driving force. Biomed Microdevices 2007; 9:43-50.
135. Moctar M, Pfeffer E. Effects of abomasal infusions of casein on N excretion and urea turnover in sheep. Z Tierphysiol Tierernahr Futtermittelkd 1981; 46:33-38.
136. Oddy MH, Santiago JG, Mikkelsen JC. Electrokinetic instability micromixing. Anal Chem 2001; 73:5822-5832.
137. Bau HH, Zhong J, Yi M. A minute magneto hydrodynamic (MHD) mixer. Sensor Actuator B 2001; 79:207-215.
138. Rife JC, Bell MI, Horwitz JS et al. Miniature valveless ultrasonic pumps and mixers. Sensor Actuator A 2000; 86:135-140.
139. Hung PJ, Lee PJ, Sabounchi P et al. Continuous perfusion microfluidic cell culture array for high-throughput cell-based assays. Biotechnol Bioeng 2005; 89:1-8.
140. Jeon NL, Dertinger S, Chiu DT et al. Generation of solution and surface gradients using microfluidic systems. Langmuir 2000; 16:8311-8316.
141. Wei CW, Cheng JY, Young TH. Elucidating in vitro cell-cell interaction using a microfluidic coculture system. Biomed Microdevices 2006; 8:65-71.

142. Zaari N, Rajagopalan P, Kim SK et al. Photopolymerization in microfluidic gradient generators: microscale control of substrate compliance to manipulate cell response. Adv Mater 2004; 16:2133-2137.
143. Dertinger S, Chiu DT, Jeon NL. Generation of gradients having complex shapes using microfluidic networks. Anal Chem 2001; 73:1240-1246.
144. Lin F, Saadi W, Rhee SW et al. Generation of dynamic temporal and spatial concentration gradients using microfluidic devices. Lab Chip 2004; 4:164-167.
145. Irimia D, Geba DA, Toner M. Universal microfluidic gradient generator. Anal Chem 2006; 78:3472-3477.
146. Abhyankar VV, Lokuta MA, Huttenlocher A et al. Characterization of a membrane-based gradient generator for use in cell-signaling studies. Lab Chip 2006; 6:389-393.
147. Wu H, Huang B, Zare RN. Generation of complex, static solution gradients in microfluidic channels. J Am Chem Soc 2006; 128:4194-4195.
148. Park J, Bansal T, Pinelis M et al. A microsystem for sensing and patterning oxidative microgradients during cell culture. Lab Chip 2006; 6:611-622.
149. Cheng JY, Hsieh CJ, Chuang YC et al. Performing microchannel temperature cycling reactions using reciprocating reagent shuttling along a radial temperature gradient. Analyst 2005; 130:931-940.
150. Mao H, Yang T, Cremer PS. A microfluidic device with a linear temperature gradient for parallel and combinatorial measurements. J Am Chem Soc 2002; 124:4432-4435.
151. Baudoin R, Corlu A, Griscom L et al. Trends in the development of microfluidic cell biochips for in vitro hepatotoxicity. Toxicol In Vitro 2007; 21:535-544.
152. Ye N, Qin J, Liu X et al. Characterizing doxorubicin-induced apoptosis in HepG2 cells using an integrated microfluidic device. Electrophoresis 2007; 28:1146-1153.
153. Pei M, Solchaga LA, Seidel J et al. Bioreactors mediate the effectiveness of tissue engineering scaffolds. FASEB J 2002; 16:1691-1694.
154. Wu MH, Urban JP, Cui Z et al. Development of PDMS microbioreactor with well-defined and homogenous culture environment for chondrocyte 3-D culture. Biomed Microdevices 2006; 8:331-340.
155. Ling Y, Rubin J, Deng Y et al. A cell-laden microfluidic hydrogel. Lab Chip 2007; 7:756-762.
156. Choi NW, Cabodi M, Held B et al. Microfluidic scaffolds for tissue engineering. Nat Mater 2007; 6:908-915.
157. Zguris JC, Itle LJ, Koh WG et al. A novel single-step fabrication technique to create heterogeneous poly(ethylene glycol) hydrogel microstructures containing multiple phenotypes of mammalian cells. Langmuir 2005; 21:4168-4174.
158. Braschler T, Johann R, Heule M et al. Gentle cell trapping and release on a microfluidic chip by in situ alginate hydrogel formation. Lab Chip 2005; 5:553-559.
159. Frisk T, Rydholm S, Andersson H et al. A concept for miniaturized 3-D cell culture using an extracellular matrix gel. Electrophoresis 2005; 26:4751-4758.
160. Toh YC, Zhang C, Zhang J et al. A novel 3D mammalian cell perfusion-culture system in microfluidic channels. Lab Chip 2007; 7:302-309.
161. Paguirigan A, Beebe DJ. Gelatin based microfluidic devices for cell culture. Lab Chip 2006; 6:407-413.
162. Lee PJ, Hung PJ, Rao VM et al. Nanoliter scale microbioreactor array for quantitative cell biology. Biotechnol Bioeng 2006; 94:5-14.
163. Hung PJ, Lee PJ, Sabounchi P et al. A novel high aspect ratio microfluidic design to provide a stable and uniform microenvironment for cell growth in a high throughput mammalian cell culture array. Lab Chip 2005; 5:44-48.
164. King KR, Wang S, Irimia D et al. A high-throughput microfluidic real-time gene expression living cell array. Lab Chip 2007; 7:77-85.
165. Wang Z, Kim MC, Marquez M et al. High-density microfluidic arrays for cell cytotoxicity analysis. Lab Chip 2007; 7:740-745.
166. Wright D, Rajalingam B, Selvarasah S et al. Generation of static and dynamic patterned cocultures using microfabricated parylene-C stencils. Lab Chip 2007; 7:1272-1279.
167. Genes LI, Tolon NV, Hulvey MK et al. Addressing a vascular endothelium array with blood components using underlying microfluidic channels. Lab Chip 2007; 7:1256-1259.
168. Viravaidya K, Sin A, Shuler ML. Development of a microscale cell culture analog to probe naphthalene toxicity. Biotechnol Prog 2004; 20:316-323.
169. Viravaidya K, Shuler ML. Incorporation of 3T3-L1 cells to mimic bioaccumulation in a microscale cell culture analog device for toxicity studies. Biotechnol Prog 2004; 20:590-597.
170. Tourovskaia A, Figueroa-Masot X, Folch A. Differentiation-on-a-chip: a microfluidic platform for long-term cell culture studies. Lab Chip 2005; 5:14-19.
171. Chung BG, Flanagan LA, Rhee SW et al. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. Lab Chip 2005; 5:401-406.
172. Torisawa YS, Shiku H, Yasukawa T et al. Multi-channel 3-D cell culture device integrated on a silicon chip for anticancer drug sensitivity test. Biomaterials 2005; 26:2165-2172.

173. Underhill GH, Bhatia SN. High-throughput analysis of signals regulating stem cell fate and function. Curr Opin Chem Biol 2007; 11:357-366.
174. Figallo E, Cannizzaro C, Gerecht S et al. Micro-bioreactor array for controlling cellular microenvironments. Lab Chip 2007; 7:710-719.
175. Saadi W, Wang SJ, Lin F et al. A parallel-gradient microfluidic chamber for quantitative analysis of breast cancer cell chemotaxis. Biomed Microdevices 2006; 8:109-118.
176. Irimia D, Liu SY, Tharp WG et al. Microfluidic system for measuring neutrophil migratory responses to fast switches of chemical gradients. Lab Chip 2006; 6:191-198.
177. Cheng SY, Heilman S, Wasserman M et al. A hydrogel-based microfluidic device for the studies of directed cell migration. Lab Chip 2007; 7:763-769.
178. Akay G, Erhan E, Keskinler B. Bioprocess intensification in flow-through monolithic microbioreactors with immobilized bacteria. Biotechnol Bioeng 2005; 90:180-190.
179. Boccazzi P, Zhang Z, Kurosawa K et al. Differential gene expression profiles and real-time measurements of growth parameters in Saccharomyces cerevisiae grown in microliter-scale bioreactors equipped with internal stirring. Biotechnol Prog 2006; 22:710-717.
180. Harms P, Kostov Y, French JA et al. Design and performance of a 24-station high throughput microbioreactor. Biotechnol Bioeng 2005; 93:6-13.
181. Kostov Y, Harms P, Randers-Eichhorn L et al. Low-cost microbioreactor for high-throughput bioprocessing. Biotechnol Bioeng 2001; 72:346-352.
182. Lamping SR, Zhang H, Allen B et al. Design of a prototype miniature bioreactor for high throughput automated bioprocessing. Chem Eng Sci 2003; 58:747-758.
183. Lee HL, Boccazzi P, Ram RJ et al. Microbioreactor arrays with integrated mixers and fluid injectors for high-throughput experimentation with pH and dissolved oxygen control. Lab Chip 2006; 6:1229-1235.
184. Maharbiz MM, Holtz WJ, Howe RT et al. Microbioreactor arrays with parametric control for high-throughput experimentation. Biotechnol Bioeng 2004; 86:485-490.
185. Schulz CM, Ruzicka J. Real-time determination of glucose consumption by live cells using a lab-on-valve system with an integrated microbioreactor. Analyst 2002; 127:1293-1298.
186. Schulz CM, Scampavia L, Ruzicka J. Real-time monitoring of lactate extrusion and glucose consumption of cultured cells using a lab-on-valve system. Analyst 2002; 127:1583-1588.
187. Szita N, Boccazzi P, Zhang Z et al. Development of a multiplexed microbioreactor system for high-throughput bioprocessing. Lab Chip 2005; 5:819-826.
188. Zanzotto A, Szita N, Boccazzi P et al. Membrane-aerated microbioreactor for high-throughput bioprocessing. Biotechnol Bioeng 2004; 87:243-254.
189. Zhang Z, Boccazzi P, Choi HG et al. Microchemostat-microbial continuous culture in a polymer-based, instrumented microbioreactor. Lab Chip 2006; 6:906-913.
190. Zhang Z, Perozziello G, Boccazzi P et al. Microbioreactors for bioprocess development. JALA 2007; 12:143-151.
191. Zhang Z, Szita N, Boccazzi P et al. A well-mixed, polymer-based microbioreactor with integrated optical measurements. Biotechnol Bioeng 2006; 93:286-296.
192. Wodnicka M, Guarino RD, Hemperly JJ et al. Novel fluorescent technology platform for high throughput cytotoxicity and proliferation assays. J Biomol Screen 2000; 5:141-152.
193. O'Brien J, Wilson I, Orton T et al. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267:5421-5426.
194. Ezashi T, Das P, Roberts RM. Low O_2 tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci USA 2005; 102:4783-4788.
195. Malda J, Martens DE, Tramper J et al. Cartilage tissue engineering: controversy in the effect of oxygen. Crit Rev Biotechnol 2003; 23:175-194.
196. Vaupel P. The role of hypoxia-induced factors in tumor progression. Oncologist 2004; 9(Suppl 5):10-17.
197. Marino AM, Yarde M, Patel H et al. Validation of the 96 well Caco2 cell culture model for high throughput permeability assessment of discovery compounds. Int J Pharm 2005; 297:235-241.
198. Li AP, Lu C, Brent JA et al. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability and drug-drug interaction potential. Chem Biol Interact 1999; 121:17-35.
199. Bang H, Lim SH, Lee YK et al. Serial dilution microchip for cytotoxicity test. J Micromech Microeng 2004; 14:1165-1170.

CHAPTER 12

THE USE OF GENOMICS IN MODEL IN VITRO SYSTEMS

Daniel A. Casciano
Department of Applied Science, University of Arkansas at Little Rock, Little Rock, Arkansas, USA
Email: dcasciano@sbcglobal.net

Abstract: Traditional toxicological tests generally provide descriptive information regarding the potential toxicity of chemicals, drugs and physical agents and are limited in their ability to assess risk to humans because they use model systems that are nonhuman in origin. Upon completion of the sequencing of the human genome, new tools were established that identify early biomarkers of toxicity and disease not only in model organisms but also in man. Gene expression profiling led to the development of a new subdiscipline of toxicology termed toxicogenomics. This new subdiscipline combines the emerging technologies of genomics, proteomics and bioinformatics to identify and characterize mechanisms of action of known and suspected toxicants. This chapter describes some advances in the area of toxicogenomics and discusses several models to study chemical-induced liver injury.

INTRODUCTION

The completion of the sequencing of the human genome not only provides us with the potential knowledge to understand all of the possible disease consequences to which humans may be susceptible, but also provides us with the technologies for deciphering these consequences. This accomplishment marks the first step toward using the information for diagnosis, treatment and prevention of human disease, as well as for determining the etiology of human diseases. One such genomic technology, DNA microarray, is a tool that is used to evaluate simultaneously the relative expression of thousands of genes. This technology has developed rapidly and has been suggested as the presently preferred technology to identify early biomarkers of toxicity and disease in model organisms and in man.[1]

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

Toxicogenomics is a new subdiscipline of toxicology that combines the emerging technologies of genomics (gene structure and function), proteomics and bioinformatics to identify and characterize mechanisms of action of known and suspected toxicants. One goal of this subdiscipline is to identify specific sets of genes that may be candidate biomarkers of specific toxic effects. The responses may be a result of exposure to certain chemicals or classes of chemicals, or may be more related to a specific organ's response to the insult. The enormous worth of these technologies is that they provide similar tools that can be applied to model in vitro and in vivo systems used as human surrogates and also directly to humans. This provides toxicologists with a greater degree of confidence in the use of the systems presently available to estimate human risk of exposure to chemicals and/or to allow development of new surrogates that may have greater relevance for predicting human responses.

Gene expression profiling using DNA microarrays has been widely applied to elucidate a variety of biological processes since their introduction in 1995.[2] The field of toxicology was among the first to recognize the promise of this new technology for understanding mechanisms of toxicity and for identifying biomarkers of exposure and effect, as well as for defining the fundamental cellular processes involved in disease. As one can imagine, these tools have been applied to in vitro and in vivo systems already in use to define toxicity. For example, Hu et al[3] have identified gene expression profiles that discriminate indirect-acting genotoxins from direct-acting genotoxins in the L5178Y $TK^{+/-}$ mouse lymphoma cell system, by using the protocol suggested for identifying mutations at the thymidine kinase locus. Early understanding of the relevance of positive genotoxic responses is expected to result in a more efficient and less costly process of drug development and perhaps provide us with better tools to determine the safety of newly developed nutriceuticals, dietary supplements and nutrients in food. Ackerman et al[4] examined molecular pathways affected by a DNA damaging agent in the TK6 human lymphoblastoid cell line. They found minimal effects on gene expression when the cells were exposed to low concentrations of the genotoxic polycyclic aromatic hydrocarbon benzo[a]pyrene diol epoxide, while relatively large changes occurred at doses associated with cellular toxicity and mutation. Several of the genes affected included those involved in apoptotic pathways, detoxification and mutation.

Although toxicogenomic techniques have been used in a variety of in vitro systems, many of the in vivo studies published to date have been limited to understanding normal and altered gene expression in the liver. Desai et al[5] assessed basal gene expression in the liver of rats as a function of time of day, because of the possibility that circadian rhythms would confound the interpretation of the toxicogenomic data. They found that more than 60 genes were significantly altered, representing genes in drug metabolism, signal transduction and the immune response. Boormann et al[6] were also interested in the effects on gene expression in rat livers of time of day and day versus night. Their DNA microarray analysis identified differential expressions in their comparisons. They discovered numerous periodically expressed genes, including period genes, clock-controlled genes and genes involved in metabolic pathways. Both of these studies demonstrated a prominent circadian rhythm component in gene expression in the rat, which should be a critical factor in planning toxicogenomic experiments. Interestingly, Desai and Fuscoe[7] and Desai et al[8] developed a novel MitoChip microarray that can be used for transcriptional profiling to understand the basis of mitochondrial involvement in disease and toxicity. The array contains 542 oligonucleotides that represent genes from the nuclear and mitochondrial genomes associated with mitochondrial structure

and function. The authors are validating the MitoChip by evaluating the expression of mitochondrial genes in the livers of p53 haplodeficient and wild type C3B6F1 female mice exposed to several HIV therapeutic drugs. They have found that a majority of the mitochondrial genes were differentially expressed during antiretroviral treatment. These results appear to confirm our present understanding of the pathology associated with rodents and humans exposed to antiretroviral drugs.[9]

Cornwell et al[10] studied hepatic gene expression in rats treated with fibric acid analogs. This class of drugs is used to treat humans suffering from dyslipidemias. Although fibrates are known to have beneficial effects and are relatively safe, there are known adverse effects, including liver and muscle toxicity. Cornwell et al[10] exposed rats to multiple doses of several fibrates and found that the expression of 1288 genes were related to dose or length of treatment and correlated well with the observed hepatocellular hypertrophy. The gene list included changes that were consistent with increased mitochondrial and peroxisomal β-oxidation, increased fatty acid transport, increased hepatic uptake of LDL-cholesterol, decreased uptake of glucose, decreased gluconeogenesis and glycolysis. The authors concluded that the results indicated that the hepatomegaly response was due to PPARα activation, although signaling through other receptors or through nonreceptor pathways cannot be excluded.

Huang et al[11] investigated gene expression changes associated with hepatotoxicity. They exposed rats to several hepatotoxicants, including acetaminophen, methotrexate and methapyrilene and evaluated altered gene expression at 684 target genes. Using principal component analysis, they were able to distinguish clear differences in expression for each compound. They concluded from their study that expression profiling could be used to distinguish different hepatotoxicity endpoints, predict the development of toxicity and, most importantly, develop hypotheses regarding mechanisms of toxicity.

IN VITRO MODELS TO STUDY CHEMICAL-INDUCED LIVER INJURY

Liver injury is the most commonly reported clinic liability associated with the development of pharmaceutical agents. The remainder of this chapter will be focused on several ex vivo and in vitro liver models to assess this phenomenon and the presentation of some data derived by using a variety of gene expression profiling techniques. For an excellent early review of model liver injury systems, including the isolated perfused liver, liver slices and isolated hepatic cells, see reference 12. The present review will mainly be concerned with isolated primary cultured rat and human hepatocytes, as well as cell lines that act as surrogates for in vitro human hepatocytes.

Currently, primary cultures of hepatocytes are the in vitro model of choice to study drug metabolism and the hepatotoxicity of chemicals, especially new drugs. These systems are useful, because they retain, under defined culture conditions, many of the functions of the tissue of origin. Casciano,[13,14] Harris et al[15,16] and Harris and Casciano[17] discussed several rat and mouse liver perfusion techniques, as well as other methods used in their laboratory to study metabolic activation, DNA damage and repair, gene expression and in vitro toxicity, in rodent and human hepatocyte cultures.

The initial efforts of isolation and culturing of rat hepatocytes involved the two-step in situ technique, as modified by Bonney et al.[18,19] This technique, a modification of that of Seglen,[20] used canulation of the inferior vena cava instead of the portal vein. Perfusing retrograde via the vena cava was preferred, because it was easier to canulate and

reproducibly resulted in the isolation of a much larger number of viable cells. Retrograde perfusion usually resulted in 1-1.5 × 10^9 viable cells from a 10 g rat liver, whereas only approximately 3 × 10^8 viable cells were isolated when perfusion was through the portal vein. The former number represents about 50% of the hepatocytes of a liver, assuring the recovery of a large proportion of the heterogeneous cell population of the liver. Recovery of smaller numbers usually represents those that have survived the collagenase perfusion process, which itself may represent a selection mechanism that most probably results in an inadequate cellular representation of the cellular populations that reside in the liver.

The cells are usually cultured in plastic vessels. Casciano[13] found that a higher percentage of viable cells attached to the plastic substratum if the cells were placed in ice-cold medium rather than in the more commonly-used medium at 37˚C. It was suggested that the cells retained residual protease (a constituent of the collagenase) on their surface and that the cold environment, as well as the serum contained in the medium, inhibited further proteolysis, thus allowing the cellular membrane to repair and resulting in a higher percent attachment. It is well established that primary cultures of rat and human hepatocytes cultured on plastic surfaces and allowed to form a 2-dimensional monolayer, lose much of their initial CYP450 activity in the first 24-hr period, but also have a tendency to lose other liver-specific functions.[21,22] Several novel culture conditions have been described that allow rat hepatocyte primary cultures to retain stable differentiated liver functions, such as Phase I and Phase II metabolizing enzymes and responsiveness to chemicals and drug, for greater periods of times.[23,24] These culture conditions included the use of an extracellular matrix or other complex substrata (e.g., collagen and/or Matrigel). Davila and Morris[23] found that, when rat hepatocytes were overlaid with Matrigel (a solubilized basement membrane preparation extracted from a mouse sarcoma tumor rich in extracellular matrix proteins), the cells acquired a 3D configuration and were reorganized as acinar structures with the cells becoming more cuboidal and with a distinctive canalicular network. Using these culture conditions, they found that basal levels of a variety of liver genes are maintained and can be stabilized or induced by xenobiotics to levels attained in the liver in vivo.

Although in vitro rodent hepatocyte systems are of use in toxicology, the species of greatest value in predicting liver metabolism and toxicity in the human is the human primary cultured hepatocyte system. Currently, primary human hepatocytes are the system of choice to predict human risk before new molecular entities are evaluated in Phase I clinical trials by the pharmaceutical industry. Since these cells are generally in limited supply and suffer to the same extent as other primary cells in culture with regard to the retention of the functions of the tissue of origin, as well as from variability in genotype and phenotype amongst donors, it is necessary to carefully define culture conditions that result in the highest retention of basal functions and stability in culture.

GENE EXPRESSION IN MODEL IN VITRO HEPATIC SYSTEMS

To determine whether a specific in vitro cell system is useful for predicting a particular organ response, one should measure functions that are somewhat specific to that organ in vivo. Harris et al[14] investigated differentially expressed genes in Fischer 344 (F344) rats exposed to aflatoxin B_1 (AFB1) and in hepatocytes isolated from control rats and treated with AFB1 in primary cell culture. They were also interested in identifying additional mechanisms of AFB1-induced carcinogenesis and toxicity. Table 1 indicates the genes

Table 1. In vitro hepatocyte gene expression changes induced by aflatoxin B_1

Genes with Increased Expression	Genes with Decreased Expression
Serum amyloid A-2	Transferrin*
Cytochrome P-450 4F1*	Cytochrome P450 3A-like
Cytochrome P450 3A*	
Alpha-2-microglobulin	
Glutathione S-transferase Yb2*	
C4b- binding protein alpha chain	
Corticosteroid binding globulin*	

*gene changes also detected in vivo

whose mRNA was increased in cultured primary rat hepatocytes and F344 rats after AFB1 treatment, which included the genes for corticosteroid binding globulin (CBG), cytochrome P450 4F1 (CYP4F1), alpha-2 microglobulin, C4b-binding protein (C4BP), serum amyloid A-2 and glutathione S-transferase (GST). There were reduced mRNA levels for transferrin and a small CYP3A-like cDNA after AFB1 exposure. When liver mRNA from AFB1-exposed male F344 rats was evaluated for transferrin, CBG, GST, CYP3A and CYP4F1 expression, a decrease in transferrin mRNA and increases in CBG, GST, CYP3A and CYP4F1 mRNAs were also seen.

Representatives of both Phase I and Phase II drug metabolizing enzymes were found to be responsive in both AFB1-treated animals and primary hepatocytes, indicating that this in vitro system captures important biological responses attributable to the liver in the intact animal. CYP3A has been reported to metabolically activate AFB1 to a DNA reactive species in rodents and humans.[25-28] Harris et al[15] found that AFB1 caused an increase in CYP3A mRNA levels in both primary rat hepatocytes and AFB1-treated rats, indicating that CYP3A P450s also play a major role in AFB1 activation under these experimental conditions. It was also intriguing that they found that CYP4F1 was upregulated by AFB1 in both model systems. Chen and Hardwick[29] identified CYP4F1 as a member of the CYP4 superfamily. They showed that CYP4F1 mRNA was constitutively expressed in rat liver and upregulated in an AFB1-induced transplantable tumor. In this same study, they also showed that CYP4F1 mRNA was not present in tumors induced by other liver carcinogens. The function of CYP4F1 is unknown, but it is intriguing that induction of this mRNA has been observed in acute AFB1 exposure in cultured primary rat hepatocytes and after chronic exposure in F344 male rats (Fig. 1). Perhaps AFB1 serves as a substrate for this enzyme in addition to CYP3A and contributes to the metabolic activation of this potent rodent and human mutagenic carcinogen.

Another interesting response to AFB1 reported by Harris et al[14] was the induction of CBG in vitro and in vivo. CBG content is regulated in the blood by the concentration of the corticosteroids contained therein. The dynamic equilibrium dictates the free corticosteroid in the blood: When corticosteroid concentrations are high, corticosteroid is bound by CBG so it is not available to tissues. When corticosteroid concentrations are low, corticosteroid is released from CBG and becomes available to tissues. Since AFB1 and corticosteroids are planar molecules with similar ring structures, it is interesting to

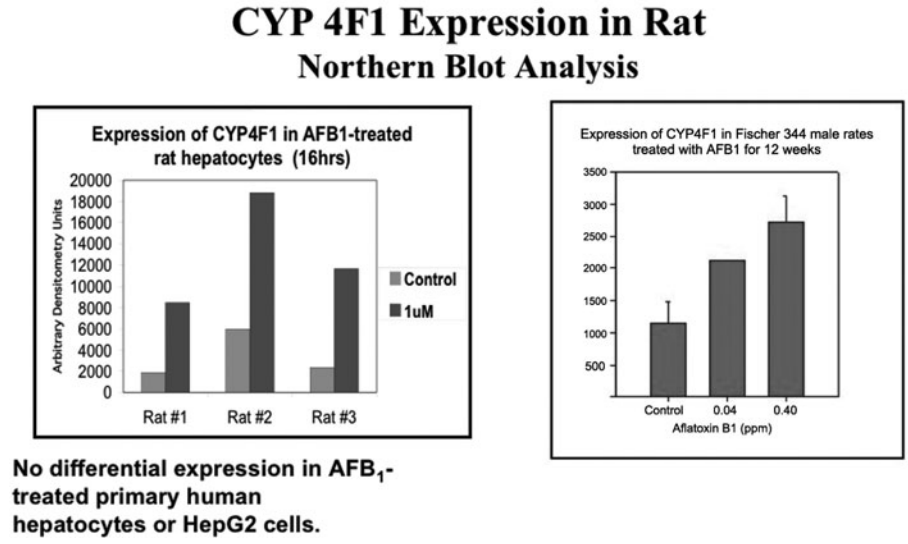

Figure 1. Left panel: CYP4F1 expression in rat hepatocytes isolated from three different F344 rats treated with 1 μM AFB1 for 16 hours. Right panel: CYP4F1 mRNA levels in control and AFB1-treated F344 rat liver. RNA was from male rats fed 0, 0.04 or 0.4 ppm AFB1 intermittently for 12 weeks (4 weeks on, 4 off, 4 on).

hypothesize that AFB1 or an AFB1 metabolite upregulates CBG and binds to the protein, making it unavailable for transfer into the nucleus to cause mutagenic damage to DNA, or that CBG binds AFB1 or a metabolite and the adducted molecule is excreted into the blood, thus serving as a detoxification mechanism.

GENE EXPRESSION COMPARISONS IN CULTURED PRIMARY HUMAN HEPATOCYTES AND HepG2 CELLS

Several in vitro cell culture systems have been proposed as useful surrogates to predict possible adverse outcomes when humans are exposed to chemicals or drugs. Primary human hepatocytes in culture and the hepatocarcinoma cell line HepG2 are two systems that have been used to measure metabolism, gene expression and toxicity. HepG2 cells were derived from a liver hepatoma of a 15-year-old Caucasian male.[30] Wang et al[31] have shown that this cell line retains some Phase I and Phase II metabolic functions, although the metabolism is generally lower than that seen in primary cultures of human hepatocytes. Primary cultured human hepatocytes are considered to be the in vitro system of choice for the study of human metabolism. However, there are some issues that require further investigation, including the degree of variability of hepatocytes derived from individual donors; the extent of differential gene expression between human hepatocytes and HepG2 cells and the identification of differences in responses to external stimuli between the two cellular models.

Figure 2 shows a phase-contrast microphotograph of HepG2 cells in culture at varying times in the growth cycle. The various panels indicate HepG2 at 2, 22 and 72

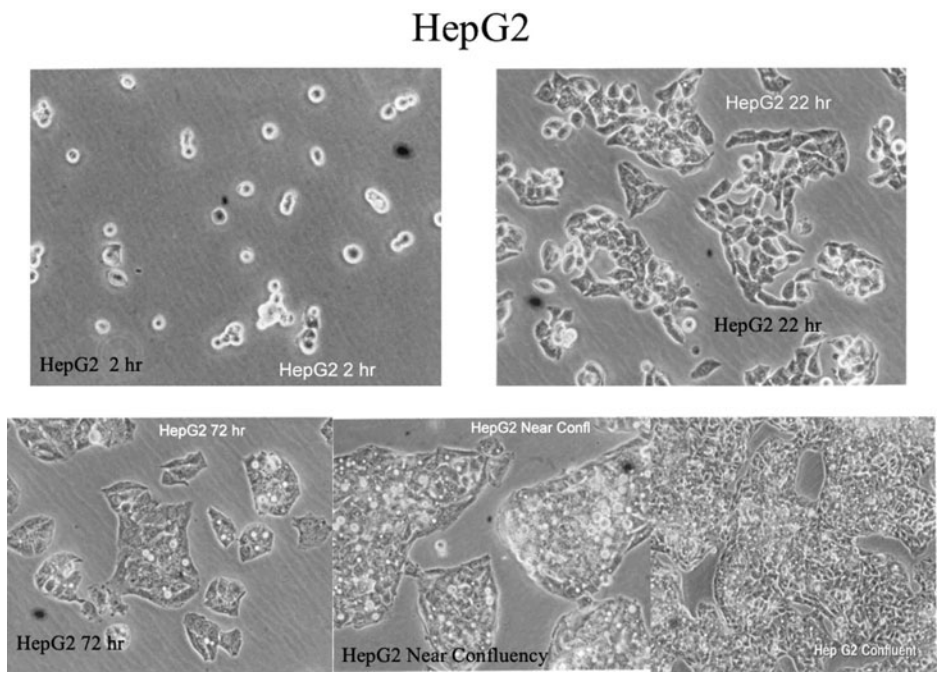

Figure 2. Phase-contrast microscope images of viable HepG2 cells at various phases of the growth cycle (100X).

hours after culture initiation and when they are near confluence. As can be seen, this cell line appears to grow in patches or as aggregates and the cells never completely cover the surface of the substratum, but may exhibit contact inhibition at near confluence. It is probable that different gene expression profiles would be found at these different growth phases, thus distinguishing HepG2 cells in different stages of a growth cycle. For instance, during cell replication, the genes expressed would be mainly those associated with mitosis; at contact inhibition, one would find genes expressed in the resting cell population that may resemble the resting liver and not an abnormal in vitro cell line. Therefore, the same cell line would look very different because of regulation of gene expression at different phases of the growth cycle and if one didn't know this, one would not conclude that the expression profiles were from the same cell. Therefore, it is important to define the culture conditions and cellular kinetics when one compares HepG2 cell cultures with static, terminally-differentiated primary hepatocyte cultures. Also obvious is the fact that HepG2 cells replicate, in stark contrast to the hepatocytes they are supposed to mimic. Therefore, one would expect dramatic differences in gene expression responses because of comparison of an abnormal cancer cell with a normal hepatocyte cell and take into considerations the genes that are associated with replicating cells as opposed to terminally-differentiated cells.

For gene expression studies, it is important to identify the culture conditions that best reflect the basal expression and response in vivo. Harris[32] investigated basal gene expression in human hepatocytes cultured on collagen and Matrigel (Fig. 3). The expression profiles obtained were compared with gene expression profiles derived from

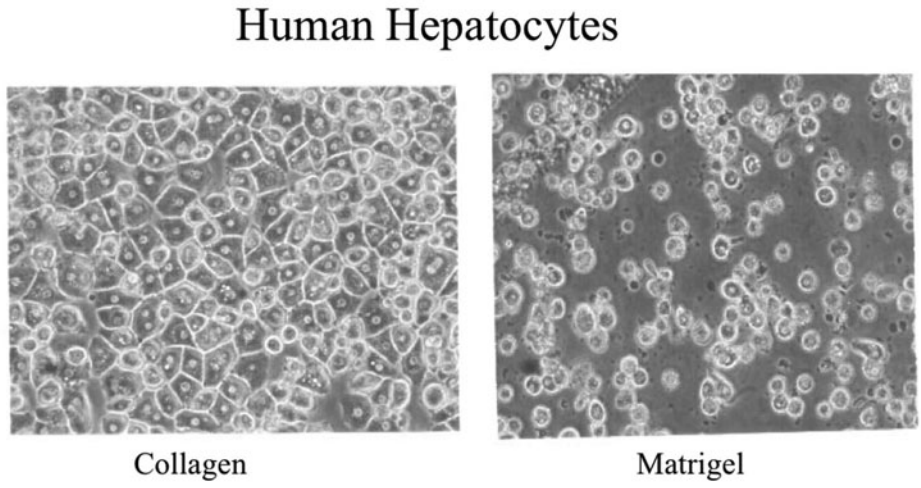

Figure 3. Phase-contrast microscope images of viable human hepatocytes plated in collagen (left panel, 100X) or Matrigel (right panel, 100X).

hepatocytes freshly isolated from the human liver. The results indicated that the cells on the Matrigel substratum more closely resembled the in vivo profile. The following discussion is, therefore, based on data derived from human hepatocytes cultured on Matrigel. There were 10 genes with expression patterns that consistently increased in human hepatocytes cultured on collagen, but not on Matrigel. Three of the genes coded for molecular chaperones, indicating that the cells cultured on collagen were compensating for a cellular stress not evident in hepatocytes plated on Matrigel. Interestingly, when Harris[32] compared primary rat hepatocytes cultured on collagen or Matrigel, there were no apparent differences in gene expression.

Harris et al[16] also investigated basal gene expression profiles in cultured primary human hepatocytes and HepG2 cells. Their initial efforts were directed toward evaluating the expression profile from 5 different male donors. The donors varied in age from 38 to 56 years of age and several of them had some confounding factors derived from alcohol or drug use. Figure 4 indicates the expression of totals of 1690, 1939 and 951 genes (out of the 31,104 genes spotted on the arrays) that were detectable in three of the donors. Of the identified genes, 867 were detected in primary hepatocytes from these donors, while 631 genes were unique to a single donor. For instance, 160 genes derived from donor 1 were not detectable in either donor 2 or donor 3. It appeared that the gene expression profiles from donors 1 and 2 were the most similar, as they shared 651 genes in addition to the 867 genes that were identified in all three donors. Although some of the observed variations could have been due to unknown experimental factors, such as hepatocyte isolation and plating, individual array effects, target labeling efficiency and/or the choice of cutoff for nondetectable expression, it is suggested that individual human variation in gene expression profiles should be factored into the experimental design and data interpretation.

When the basal gene expression of cultured primary human hepatocytes was compared with expression profiles in HepG2 cells, the data depicted in Figure 5 were generated.

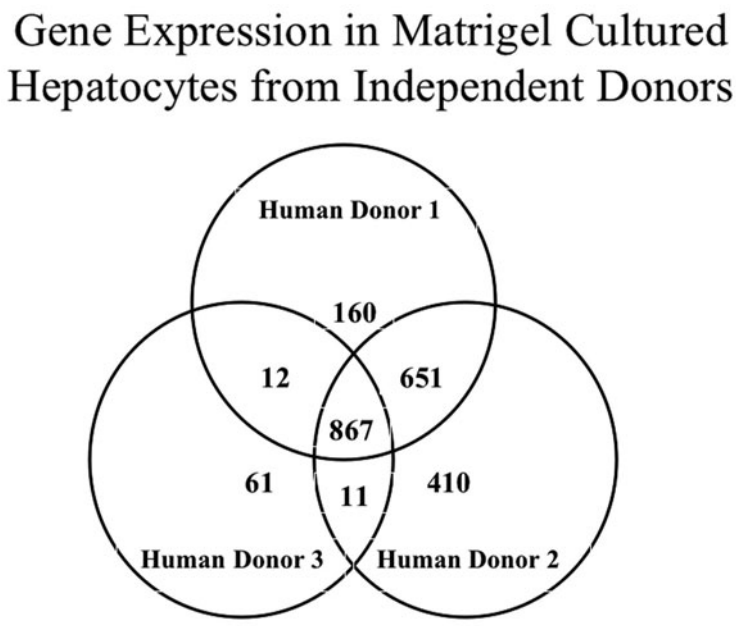

Figure 4. Venn diagram illustrating shared and variable gene expression in cultured primary human hepatocytes from three donors, cultured in Matrigel for 16 hours.

The expression of 2974 genes was identified in the HepG2 cells, including the 867 genes expressed in the cultured human hepatocytes (Fig. 4). Another 920 genes were detected as being exclusively in HepG2 cells. It is of interest to note that only a few genes were detected in primary human hepatocytes alone and not in HepG2 cells (Fig. 5). Although HepG2 cells appear to express a majority of the genes that were identified in cultured primary human hepatocytes, 31% of the HepG2 transcriptome examined was unique to these cells. HepG2-specific gene pathways included RNA processing, nucleotide metabolism, transcription factors, histone associated proteins, cell cycle control, cell signaling, DNA repair, growth factors and receptors, cellular defense, oncogenes and tumor suppressor genes, metabolism, aminopeptidases, protein transport, protein kinases and phosphatases, transporters, cytoskeletal proteins, cell adhesion, neuronal genes, steroid lipid metabolism and expressed sequence tags (ESTs) with unknown functions. Presumably, a large number of the genes solely expressed in HepG2 cells represent functional changes associated with the transformed phenotype of this cell line and the data of Harris et al[15] would suggest that this may not be a good model for the normal human hepatocyte.

CONCLUSION

Toxicogenomics is a relatively new discipline in the field of toxicology. DNA microarrays and bioinformatics tools are being used to increase understanding of the effects of toxicants on in vivo and in vitro models and to better evaluate the relevance

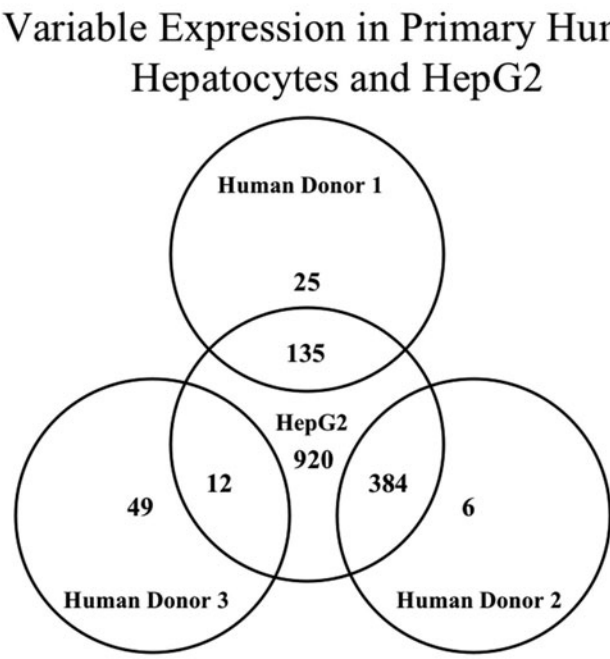

Figure 5. Venn diagram illustrating genes uniquely expressed in HepG2 cells and in cultured primary human hepatocytes from three donors.

of the in vitro models commonly used in toxicological studies. These tools are already successfully being introduced into the regulatory arena[1] and their continued refinement will result in the identification of human biomarkers of exposure to chemicals and drugs, thus providing regulators and toxicologists with the confidence required to determine the safety of these molecular entities.

ACKNOWLEDGEMENTS

The author gratefully acknowledges Dr. Angela Harris for her outstanding contributions to the author's toxicogenomic knowledge base.

REFERENCES

1. Casciano DA, Woodcock J. Empowering microarrays in the regulatory setting. Nature Biotechnol 2006; 24:1103.
2. Casciano DA, Fuscoe JC. Preface to mutation research special issue on toxicogenomics. Mut Res 2004; 549:1-3.
3. Hu T, Gibson DP, Carr GJ et al. Identification of a gene expression profile that discriminates indirect-acting genotoxins from direct-acting genotoxins. Mut Res 2004; 549:5-27.
4. Ackerman GS, Rozenzweig BA, Domon OE et al. Gene expression profiles and genetic damage in benzo[a] pyrene diol epoxide-exposed TK6 cells. Mut Res 2004; 549:43-64.

5. Desai VG, Moland CL, Branham WS et al. Changes in gene expression as a function of time of day in the liver of rats. Mut Res 2004; 549:115-129.
6. Boorman GA, Blackshear PE, Parker JS et al. Hepatic gene expression changes throughout the day in the Fischer rat: Implications for toxicogenomic experiments. Toxicol Sci 2005; 86:185-193.
7. Desai VG, Fuscoe JC. Transcriptional profiling for understanding the basis of mitochondrial involvement in disease and toxicity using the mitochondria-specific MitoChip. Mut Res 2007; 616:210-212.
8. Desai VG, Lee T, Delongchamp RR et al. Nucleoside reverse transcriptase (NRTIs)-induced expression profile of mitochondria related genes in the mouse liver. Mitochondrion 2008; 8:181-195.
9. Poirier MC, Div RL, Al-Hari L et al. Long-term mitochondrial toxicity in HIV-infected infants born to HIV-infected mothers. J Acquired Immune Def Syndromes 2003; 33:175-183.
10. Cornwell PD, DeSouza AT, Ulrich RG. Profiling of hepatic gene expression in rats treated with fibric acid analogs. Mut Res 2004; 549:131-145.
11. Huang Q, Jin X, Gaillard ET et al. Gene expression profiling reveals multiple toxicity endpoints induced by hepatotoxicants. Mut Res 2004; 549:147-168.
12. Guillouzo A. Liver models in in vitro toxicology. Env Health Persp 1998; 106:511-532.
13. Casciano DA. Development and utilization of primary hepatocyte culture systems to evaluate metabolism, DNA binding and repair of xenobiotics. Drug Metab Rev 2000; 32:1-14.
14. Casciano DA. The potent hepatocarcinogens methapyrilene: A hypothesis regarding its hepatotoxicity. In: Sahu S, ed. Hepatotoxicity: From Genomics to In Vitro and In Vivo Models. Chichester: J Wiley and Sons, 2007:578-587.
15. Harris AJ, Shaddock JG, Manjanatha MG et al. Identification of differentially expressed genes in aflatoxin B_1-treated cultured primary rat hepatocytes and Fischer 344 rats. Carcinogenesis 1998; 19:101-109.
16. Harris AJ, Dial SL, Casciano DA. Comparison of basal gene expression profiles and effects of hepatocarcinogens on gene expression in cultured primary human hepatocytes and HepG2 cells. Mut Res 2004; 549:79-99.
17. Harris AJ, Casciano DA. Use of DNA arrays in understanding hepatic test systems. In: Sahu S, ed. Hepatotoxicity: From genomics to in vitro and in vivo models, Chichester: J Wiley and Sons Ltd, 2007:489-506.
18. Bonney RJ, Walker PR, Potter VR. Isozyme patterns in parenchymal and nonparenchymal cells isolated from regenerating and regenerated liver. Biochem J 1974; 32:947-954.
19. Bonney RJ, Becker JE, Walker PR et al. Adult liver parenchymal cells in primary culture characteristics and standards. In Vitro 1974; 10:130-142.
20. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biology 1976; 13:29-83.
21. McMillan JM, Shaddock JG, Casciano DA et al. Effects of dexamethasone on drug metabolizing enzyme activities in primary rat hepatocyte cultures. Mut Res 2001; 249:81-92.
22. LeCluse E. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci 2001; 13:343-368.
23. Davila JC, Rodrigues RJ, Melchert RB et al. Predictive value of in vitro model systems in toxicology. Ann Rev Pharmacol Toxicol 1998; 38:63-96.
24. Davila JC, Morris DL. Analysis of cytochrome P450 and phase II conjugating enzyme expression in adult male rat hepatocytes. In Vitro Cell Dev Biol 1999; 35:120-130.
25. Yanagimoto T, Susmu I, Sawada M et al. Molecular cloning and functional expression of a mouse cytochrome P-450 (Cyp3a-13): examination of Cyp3a-13 enzyme to activate aflatoxin B_1 (AFB_1). Biochem Biophys Acta 1994; 1201:405-410.
26. Aoyama T, Yamano S, Guzelian PS et al. Five of 12 forms of vaccine virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1. Proc Natl Acad Sci USA 1990; 87:4790-4793.
27. Shimada T, Guengerich FP. Evidence for cytochrome P-450NF, the nifedine oxidase, being the principal enzyme involved in the bioactivation of aflatoxin in human liver. Proc Natl Acad Sci USA 1989; 86:462-465.
28. Metcalfe SA, Colley PJ, Neal GE. A comparison of the effects of pretreatment with Phenobarbital and 3-methylcholanthrene on the metabolism of aflatoxin B_1 by liver rat microsomes and isolated hepatocytes in vitro. Chem Biol Interact 1981; 35:145-157.
29. Chen L, Hardwick JP. Identification of a new P450 subfamily, CYP4F1, expressed in rat hepatic tumors. Arch Biochem Biophys 1993; 300:18-23.
30. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma protein and hepatitis B surface antigen. Science 1980; 209:497-499.
31. Wang K, Shindoh H, Inoue T et al. Advantages of in vitro cytotoxicity testing by using primary rat hepatocytes in comparison with established cell lines. J Toxicol Sci 2002; 27; 229-237.
32. Harris AJ. Modulation of gene expression in chemical carcinogenesis: Analysis of aflatoxin B1induced gene expression in primary human hepatocytes. Thesis. Little Rock: The University of Arkansas for Medical Sciences, 2001..

CHAPTER 13

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

Michael Balls,*[,1] Robert D. Combes[2] and Nirmala Bhogal[3]

[1]*FRAME, Nottingham, UK;* [2]*Independent Consultant, Norwich, UK;* [3]*Independent Consultant, Nottingham, UK*
**Corresponding Author: Michael Balls—Email: michael.balls@btopenworld.com*

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

New Technologies for Toxicity Testing, edited by Michael Balls, Robert D. Combes, and Nirmala Bhogal.

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.

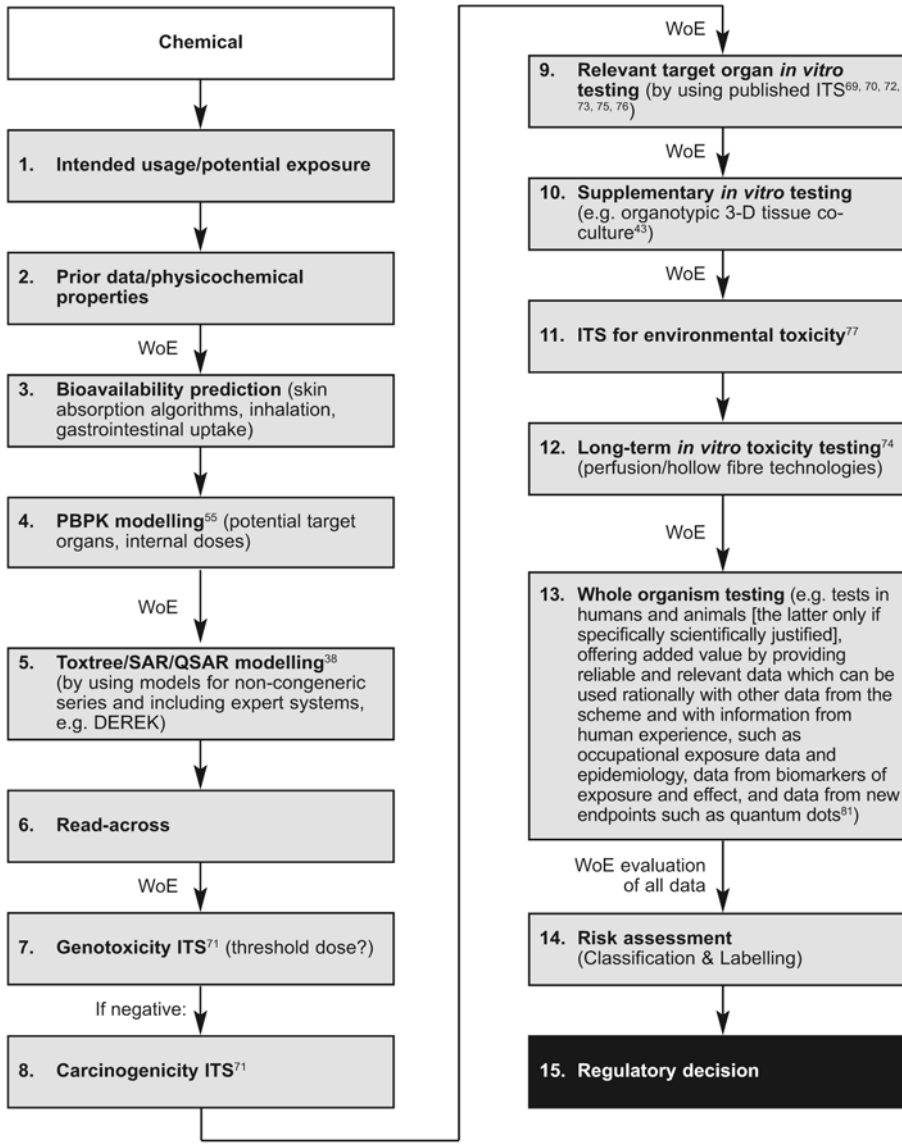

Figure 1. An ITS for general chemicals. This ITS scheme could be used, for example, for compliance with the EU REACH system and US HPV system, to provide the required comprehensive set of information for new chemicals and so-called 'missing' information for existing chemicals. It is based on proposals 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]

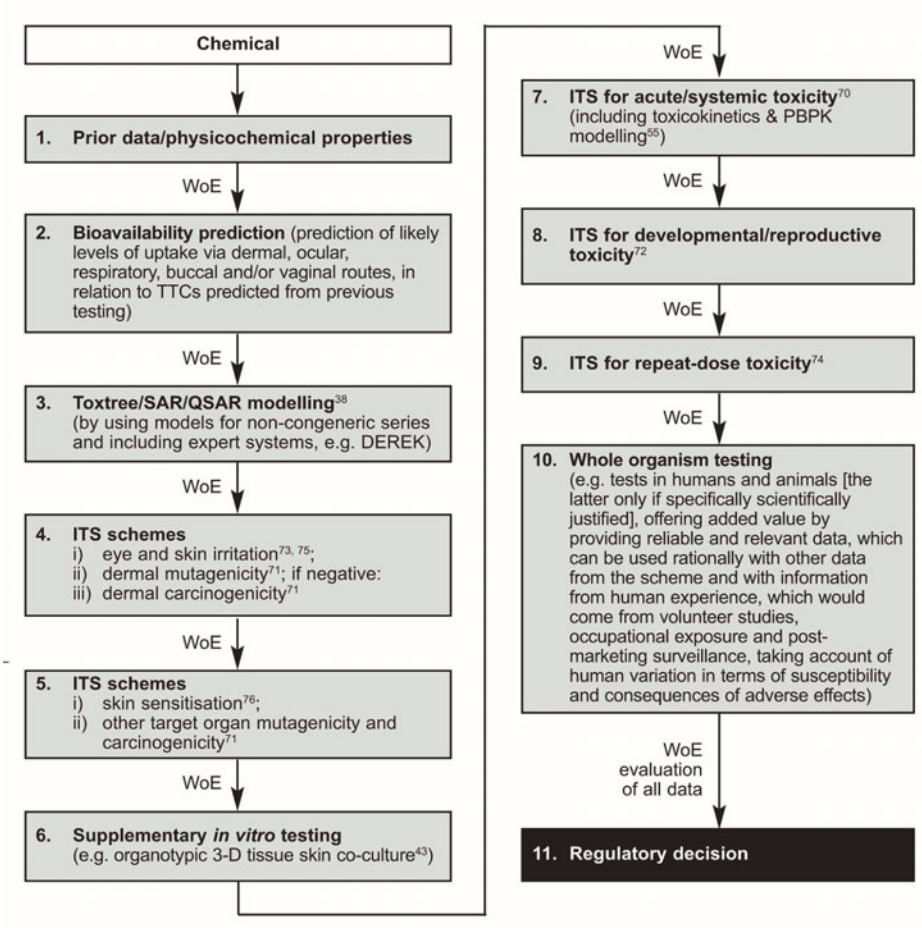

Figure 2. An ITS for cosmetic ingredients. This ITS scheme could be used, for example, for compliance with legislation such as the EU Cosmetics Directive. It is based on proposals made during the FRAME/Liverpool John Moores University/Defra REACH project,[69–77] and some of the other chapters in this 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

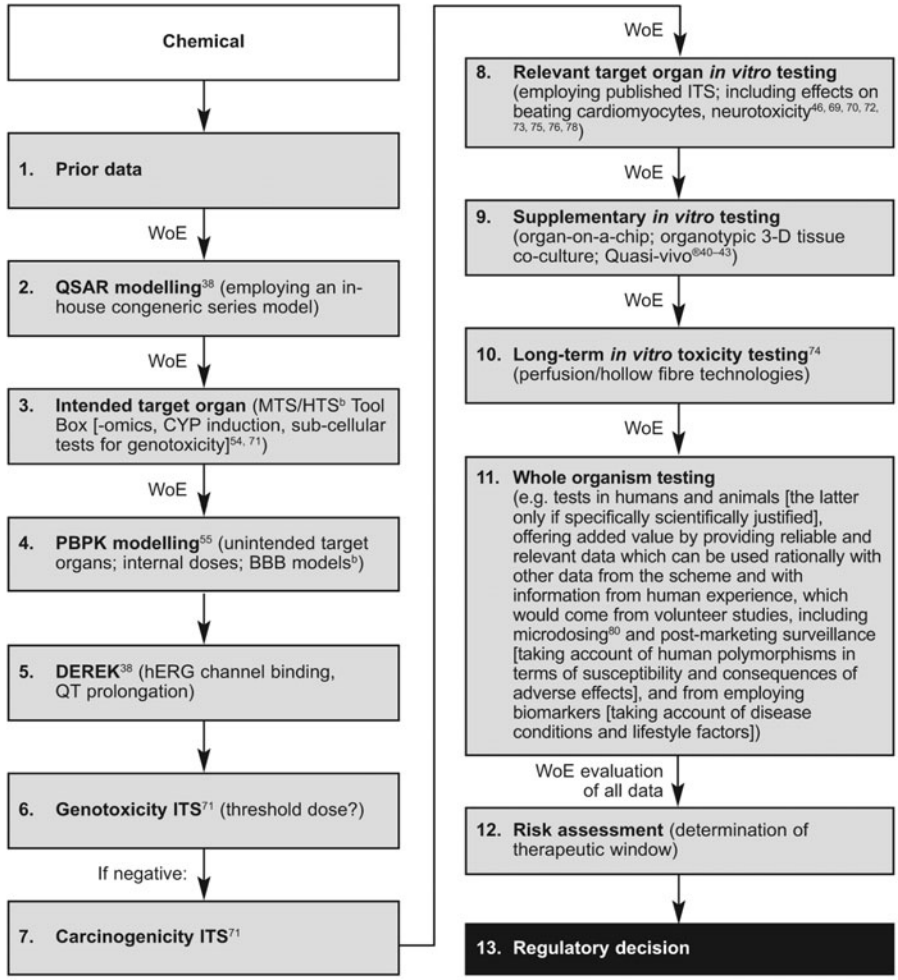

Figure 3. An ITS for candidate pharmaceuticals. This ITS scheme could be used for compliance with legislation such as the EU, US and Japanese regulations on pharmaceuticals. It is based on proposals made during the FRAME/Liverpool John Moores University/Defra REACH project, [69–77] and some of the other 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]

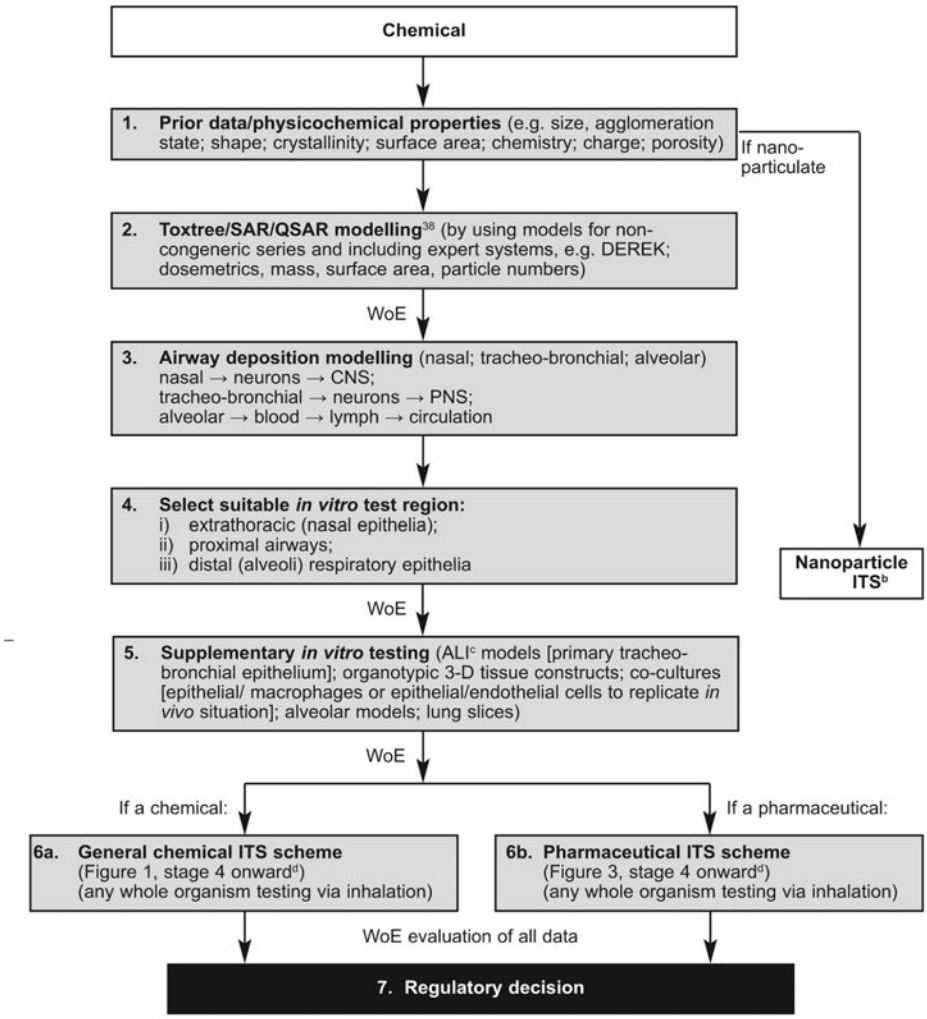

Figure 4. An ITS for inhaled chemicals. This ITS scheme is based on the report of a FRAME workshop,[82] with input from Dr Kelly BéruBé. Animal models cannot reliably reflect the responses and effects that occur in humans and in any case, such studies are very expensive and time-consuming and can only 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

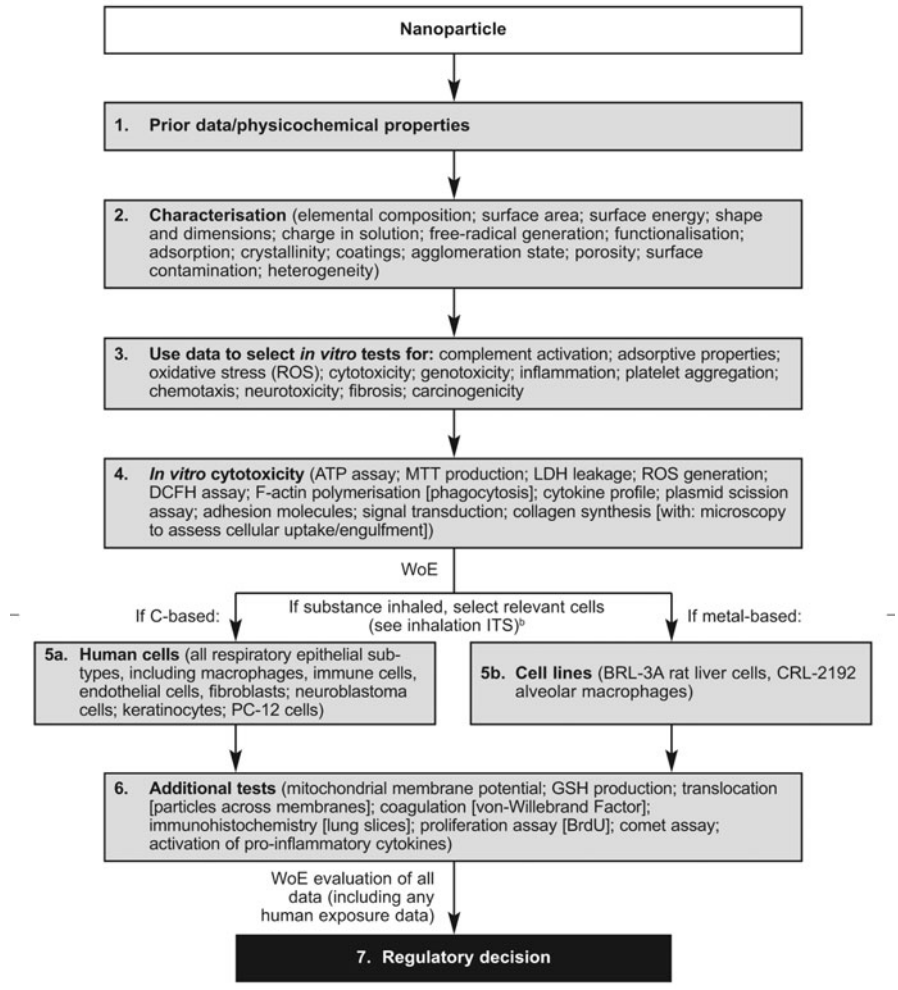

Figure 5. An ITS for nanoparticles. This ITS scheme is based on those in the chapter by Schrand et al[32] and in BéruBé et al.[82] The toxicity testing of nanoparticles presents a significant new challenge, which is unlikely to be met via conventional laboratory animal tests, which were designed for 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

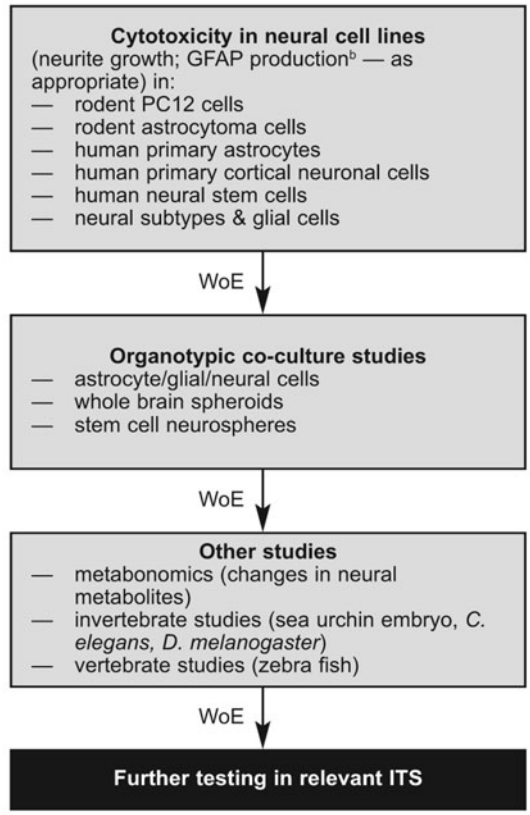

Figure 6. An ITS for neurotoxicity. This ITS scheme is based on those of Coecke et al.[79] Neurotoxicity is one of the most serious effects of exposure to high doses of chemicals and is a major cause of acute toxicity. Developmental neurotoxicity to humans is very difficult to assess by using animal 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.

REFERENCES

As a service to the readers of this chapter, pdf versions of the quoted references published in Altern Lab Anim—ATLA have been made freely available on the FRAME web site, at: http://bit.ly/FRAME-pharmbiotechnol.

1. ECHA. European Chemicals Agency. About the Agency. Helsinki: European Chemicals Agency, 2011: Available at: http://echa.europa.eu/home_en.asp.
2. . HSE. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). London: Health and Safety Executive, 2011.
3. EPA. Summary of the Toxic Substances Control Act. Washington, DC: US Environmental Protection Agency, 2011: Available at: http://www.epa.gov/regulations/laws/tsca.html.

4. Takemoto K. Practical approach in chemicals legislation – Japan's experience. Tokyo: Ministry of the Environment, 2010: Available at: http://www.un.org/esa/dsd/resources/res_pdfs/csd-18/12may/launching-publication-chemicals/presentation_Japan.pdf.
5. OECD. Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: Organisation for Economic Co-operation and Development, 2011. Available at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.
6. Combes RD, Gaunt I, Balls M. A scientific and animal welfare assessment of the OECD health effects test guidelines for the safety testing of chemicals under the European Union REACH system. Altern Lab Anim—ATLA 2004; 32(3):163-208.
7. UN. Globally harmonized systems of classification and labelling of chemicals (GHS). 3rd ed. Geneva: United Nations, 2009.
8. GAO. Comparison of U.S. and recently enacted European Union approaches to protect against the risks of toxic chemicals. GAO-07-825. Washington, DC: US Government Accountability Office, 2007.
9. ECHA. Proposals to identify Substances of Very High Concern: Annex XV reports for commenting by Interested Parties. Helsinki: European Chemical Agency, 2011: Available at: http://echa.europa.eu/consultations/authorisation/svhc/svhc_cons_en.asp.
10. Knight A, Bailey J, Balcombe J. Animal carcinogenicity studies: 2. Obstacles to extrapolation of data to humans. Altern Lab Anim—ATLA 2006; 34(1):29-38.
11. Bremer S, Pellizzer C, Hoffmann S et al. The development of new concepts for assessing reproductive toxicity applicable to large scale toxicological programmes. Curr Pharm Des 2007; 13(29):3047-3058.
12. Hartung T. Toxicology for the twenty-first century. Nature 2009; 460(7252):208-212.
13. Bhogal N, Grindon C, Combes R et al. Toxicity testing—creating a revolution based on new technologies. Trends Biotechnol 2005; 23(6):299-307.
14. National Research Council. Toxicity testing in the 21st century: a vision and a strategy. Washington, DC: National Academies Press, 2007.
15. MHRA. How we regulate. London: Medicines and Healthcare Products Regulatory Agency, 2011: Available at: http://www.mhra.gov.uk/Howweregulate/index.htm.
16. EMA. What we do. London: European Medicines Agency, 2011: Available at: http://www.ema.europa.eu/ema/index.jsp?curl=pages/about_us/general/general_content_000091.jsp&mid=WC0b01ac0580028a42&jsenabled=true.
17. FDA. Protecting and promoting your health. Silver Spring: US Food and Drug Administration, 2011: Available at: http://www.fda.gov/Drugs/default.htm.
18. JPMA. Pharmaceutical administration and regulations in Japan. Tokyo: Japan Pharmaceutical Manufacturers Association, 2010.
19. ICH. Process of harmonisation. Geneva: International Conference on Harmonisation 2011. Available at: http://www.ich.org/about/process-of-harmonisation.html.
20. ICH Secretariat. The value and benefits of ICH to drug regulatory authorities—advancing harmonization for better health. Geneva: International Conference on Harmonisation, 2010.
21. Pollard CE, Abi-Gerges N, Bridgland-Taylor MH et al. An introduction to QT interval prolongation and nonclinical approaches to assessing and reducing risk. Br J Pharmacol 2010; 159(1):12-21.
22. Issa AM, Phillips KA, Van Bebber S et al. Drug withdrawals in the United States: a systematic review of the evidence and analysis of trends. Curr Drug Safety 2007; 2 (3):177-185.
23. FDA. Challenge and opportunity on the critical path toward new medical products. Silver Spring: US Food and Drug Administration, 2004.
24. IMI. Home page. Brussels: Innovative Medicines Initiative, 2011: Available at: http://www.imi.europa.eu/ .
25. Balls M, Clothier R. A FRAME Response to the European Commission Consultation on the Draft Report on Alternative (Nonanimal) Methods for Cosmetics Testing: Current Status and Future Prospects—2010. Altern Lab Anim—ATLA 2010; 38(5):345-353.
26. van der Zee B. Cosmetics industry criticised as EU set to admit delay in animal testing ban. London: Guardian, 2010.
27. Adler S, Basketter D, Creton S et al. Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010. Arch Toxicol 2011; 85:367-485.
28. Hudson M, Balls M. Ethics and regulation in the use of laboratory animals. In: Ballo, A, ed. Implant Dentistry Research Guide: Basic, Translational and Experimental Clinical Research. Hauppauge: Nova Science Publishers, in press.
29. Bhogal N, Combes R. TGN1412: time to change the paradigm for the testing of new pharmaceuticals. Altern Lab Anim—ATLA 2006; 34(2):225-239.
30. Department of Health. Interim report of the Expert Scientific Group on Phase 1 Clinical Trials. London: Department of Health, 2006.
31. Bhogal N, Seabra R. Why animal studies cannot suitably assess nanomedicines. Pharm Technol 2010; 22(1):38-41.

32. Schrand AM, Dai L, Schlager JJ et al. Toxicity testing of nanomaterials. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011: 59-76.
33. Bhogal N, Combes R, Balls M. Preclinical drug development planning. In: Gad S, ed. Preclinical Development Handbook. Hoboken: Wiley 2007;1-63.
34. Bhogal N, Balls M. Translation of new technologies: from basic research to drug discovery and development. Curr Drug Discov Technol 2008; 5(3):250-262.
35. Balls M, Bennett, A, Kendall D. Translation of new technologies in biomedicines: shaping the road from basic research to drug development and clinical application. In: Kayser O, Warzeka H, eds. Pharmaceutical biotechnology—a comprehensive handbook. Weinheim: Wiley-VCG, in press.
36. Ekins S, Mestre J, Testa B. In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. Br J Pharmacol 2007; 152(1):9–20.
37. Ekins S, Mestre J, Testa B. In silico pharmacology for drug discovery: applications to targets and beyond. Br J Pharmacol 2007; 152(1):21–37.
38. Combes RD. In silico methods for toxicity prediction. In: Balls M, Combes R, Bhogal N eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011: 97-118.
39. Cronin MTD, Madden JC, eds. In Silico Toxicology: Principles and Applications. Cambridge: Royal Society of Chemistry, 2010.
40. Marx U. Trends in cell culture technology. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011: 27-47.
41. Sbrana T, Ahluwalia A. Engineering Quasi-Vivo® in vitro organ models. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Sceince + Business Media, 2011:139-154.
42. Wen Y, Zhang X, Yang ST. Medium to high throughput screening: microfabrication and chip-based toxicology. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011: 183-212.
43. Shakesheff KM, Rose FRAJ. Tissue engineering in the development of replacement technologies. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011:48-54.
44. Agresti JJ, Antipov E, Abate AR et al. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. Proc Natl Acad Sci USA 2010; 107(9):4004-4009.
45. Stacey G. Current developments in cell culture technology. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011:1-14.
46. Stummann TC, Bremer S. Embryonic stem cells in safety pharmacology and toxicology. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011: 15-26.
47. Takahashi T, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131(5):861-872.
48. Yu J, Hu K, Smuga-Otto K et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009; 324(5928):797-801.
49. Anson B, Nuwaysir E, Wang WB et al. Industrialised production of human iPSCV-derived cardiomyocytes for use in drug discovery and toxicity testing. Biopharm Internat 2009; 24(3):58-67.
50. Sullivan GJ, Hay DC, Park IH et al. Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 2010; 51(1):329-335.
51. Solanki A, Lee KB. A step closer to complete chemical reprogramming for generating iPS cells. Chembiochem 2010; 11(6):755-757.
52. Thomas N. High-content screening—a decade of evolution. J Biomol Screen 2010; 15(1), 1-9.
53. Taylor DL. A personal perspective on high-content screening (HCS): from the beginning. J Biomol Screen 2010; 15(7):720-725.
54. Casciano DA. The use of genomics in model in vitro systems. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011:213-256.
55. Lipscomb JC, Haddad S, Poet T et al. Physiologically-based pharmacokinetic (PBPL) models in toxicity testing and risk assessment. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin/New York: Landes Bioscience/Springer Science + Business Media, 2011:77-96.
56. Thomas S. Physiologically-based simulation modelling for the reduction of animal use in the discovery of novel pharmaceuticals. Altern Lab Anim—ATLA 2009; 37(5):497-511.
57. Noble D. Modeling the heart—from genes to cells to the whole organ. Science 2002; 295(5560):1678-1682.

58. Holden AV. Development and application of human virtual excitable tissues and organs from premature birth to sudden cardiac death. Altern Lab Anim—ATLA 2010; 38(suppl. 1):87-99.
59. Kleiman M, Sagi Y, Bloch N et al. (2009) Use of virtual patient populations for rescuing discontinued drug candidates and for reducing the number of patients in clinical trials. Altern Lab Anim—ATLA 2009; 37(suppl. 1):39-45.
60. Vasan RS. Biomarkers of cardiovascular disease—molecular basis and practical considerations. Circulation 2006; 113(19):2335-2362.
61. Corrias A, Jie X, Romero L et al. Arrhythmic risk biomarkers for the assessment of drug cardiotoxicity: from experiments to computer simulations. Phil Trans Roy Soc A 2010; 368(1921):3001-3025.
62. Dieterle F, Sistare F, Goodsaid F et al. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. Nat Biotechnol 2010; 28(5):455-462.
63. Anon. New developments in clinical imaging. NGP Magazine, 2008:13. Available at: http://www.ngpharma.com/article/New-Developments-in-Clinical-Imaging.
64. Benoit G. Bioinformatics. In: Cronin B, ed. Annual Review of Information Science and Technology. Medford: Information Today for ASIST 2005; 39:179-218.
65. Butcher EC, Berg EL, Kunkel EJ. Systems biology in drug discovery. Nat Biotechnol 2004; 22(10):1253-1259.
66. Hoffmann S, Hartung T. Toward an evidence-based toxicology. Hum Exp Toxicol 2006; 25(9):497-513.
67. Balls M. Integrated testing strategies and the prediction of toxic hazard. In: Cronin MTD, Madden JC, eds. In Silico Toxicology: Principles and Applications. Cambridge: Royal Society of Chemistry, 2010; 584-605.
68. Combes RD, Balls M. Integrated testing strategies for toxicity employing new and existing technologies. Altern Lab Anim—ATLA 2011; 39(3):213-225.
69. Grindon C, Combes R, Cronin MTD et al. Integrated testing strategies for use in the EU REACH system. Altern Lab Anim—ATLA 2006; 34(4):407-427.
70. Combes R, Grindon C, Cronin MTD et al. Integrated decision-tree testing strategies for acute systemic toxicity and toxicokinetics with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36(1):45-63.
71. Combes R, Grindon C, Cronin MTD et al. Proposed integrated decision-tree testing strategies for mutagenicity and carcinogenicity in relation to the EU REACH legislation. Altern Lab Anim—ATLA 2007; 35(2):267-287.
72. Grindon C, Combes R, Cronin MTD et al. Integrated decision-tree testing strategies for developmental and reproductive toxicity with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36(1):65-80.
73. Grindon C, Combes R, Cronin MTD et al. An integrated decision-tree testing strategy for eye irritation with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36(1):81-92.
74. Grindon C, Combes R, Cronin MTD et al. An integrated decision-tree testing strategy for repeat dose toxicity with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36(1):93-101.
75. Grindon C, Combes R, Cronin MTD et al. Integrated decision-tree testing strategies for skin corrosion and irritation with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2007; 35(6):673-682.
76. Grindon C, Combes R, Cronin MTD et al. An integrated decision-tree testing strategy for skin sensitisation with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2007; 35(6):683-697.
77. Grindon C, Combes R, Cronin MTD et al. Integrated decision-tree testing strategies for environmental toxicity with respect to the requirements of the EU REACH legislation. Altern Lab Anim—ATLA 2008; 36(suppl. 1):29-42.
78. Bal-Price AK, Hogberg HT, Buzanska L et al. Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. Neurotoxicol Teratol 2010; 32(1):36-41.
79. Coecke S, Eskes C, Gartlon J et al. The value of alternative testing for neurotoxicity in the context of regulatory needs. Environ Toxicol Pharmacol 2006; 21(2):153-167.
80. Combes RD, Berridge T, Connelly J et al. Early microdose drug studies in human volunteers can minimise animal testing: Proceedings of a workshop organised by Volunteers in Research and Testing. Eur J Pharm Sci 2003; 19(1):1-11.
81. Dave SR, White CC, Kavanagh TJ et al. Luminescent quantum dots for molecular toxicology. In: Balls M, Combes R, Bhogal N, eds. New Technologies in Toxicity Testing. Austin, TX, USA: Landes Bioscience 2011: 118-138.
82. BéruBé K, Aufderheide M, Breheny D et al. In vitro models of inhalation toxicity and disease. The Report of a FRAME Workshop. Altern Lab Anim—ATLA 2009; 37(1):89-141.
83. Combes R, Balls M. Intelligent testing strategies for chemicals testing—A case of more haste, less speed? Altern Lab Anim—ATLA 2005; 33(3):289-297.

84. Kinsner-Ovaskainen A, Akkan Z, Casati S et al. Overcoming barriers to validation of non-animal partial replacement methods/integrated testing strategies: The Report of an EPAA–ECVAM Workshop. Altern Lab Anim—ATLA 2009; 37(4):437-444.
85. Knight DJ, Poncipe C. Use of literature and surrogate safety data for notifications, HPV chemicals and biocides. Spec Chem Mag 2002; 22(10):28-29.
86. BéruBé K. Alternatives for lung research: stuck between a rat and a hard place. Altern Lab Anim—ATLA 2011; 39(2):121-130.
87. Russmann S, Kullak-Ublick GA, Grattagliano I. Current concepts of mechanisms in drug-induced hepatotoxicity. Curr Med Chem 2009; 16:3041-3053.
88. Aithal GP. Mind the gap. Altern Lab Anim—ATLA 2010; 38(suppl. 1):1-4.
89. Doehmer J. The use and value of in vitro technologies in metabolism studies. Altern Lab Anim—ATLA 2009; 37(suppl. 1):29-32.
90. Lee WM, Senior JR. Recognising drug induced liver injury: current problems, possible solutions. Toxicol Pathol 2005; 33:155-164.
91. Marchant CA, Fisk L, Note RR et al. An expert system approach to the assessment of hepatotoxic potential. Chem Biodivers 2009; 6:2107-2114.
92. Mandenius CF, Andersson TB, Alves PM et al. Toward preclinical predictive drug testing for metabolism and hepatotoxicity by using in vitro models derived from human embryonic stem cells and human cell lines—a report on the Vitrocellomics EU-project. Altern Lab Anim—ATLA 2011; 39:147-171.
93. Anon. The DILI-sim initiative. Research Triangle Park: Hamner–UNC Institute for Drug Safety Sciences, 2011: Available at: http://www.thehamner.org/content/DILI-sim_WebEx_4_7_2011.pdf.
94. Anon. Drug-induced liver injury. Liverpool: MRC Centre for Drug Safety Studies, 2011: Available at: http://www.liv.ac.uk/drug-safety/research/researchareas/liver-injury.htm.
95. Daly AK, Donaldson PT, Bhatnagar P et al. HLA-B*5701 genotype is a majordeterminant of drug-induced liver injury due to flucloxacillin. Nat Genet 2009; 41:816-819.
96. Gamazon ER, Huang RS, Cox NJ et al. Chemotherapeutic drug susceptibility associated SNPs are enriched in expression quantitative trait loci. Proc Natl Acad Sci USA 2010; 107:9287-9292.
97. Yang TP, Beazley C, Montgomery SB et al. Genevar: A database and Java application for the analysis and visualization of SNP-gene associations in eQTL studies. Bioinformatics 2010; 26:2474-2476.
98. Balls M. Modern alternative approaches to the problem of drug-induced liver injury. Altern Lab Anim—ATLA 2011; 39:103-107.
99. O'Connell TM, Watkins PB. The application of metabonomics to predict drug-induced liver injury. Clin Pharmacol Ther 2010; 88:394-399.
100. Chen M, Vijay V, Shi Q et al. FDA-approved drug labeling for the study of drug-induced liver injury. Drug Discov Today 2011; in press.

INDEX

E

F

G

H

I

L

M

N

O

P

Q

R

S

T

U

V