The Tenth Anniversary of the Björn Ekwall Memorial Foundation

Erik Walum,[1] Hanna Tähti[2] and Ada Kolman[3,4]

[1]Glucox Biotech AB, Stockholm, Sweden; [2]FICAM, School of Medicine, University of Tampere, Finland; [3]Swedish Fund for Research without Animal Experiments, Älvsjö, Sweden; [4]with substantial contributions from all ten BEMF award winners

Summary — The Björn Ekwall Memorial Foundation (BEMF) was initiated by the Scandinavian Society for Cell Toxicology in 2001, to honour the memory of Dr Björn Ekwall (1940–2000) and to establish a prize, the Björn Ekwall Memorial Award. The prize is awarded to scientists who have significantly contributed to the field of cell toxicology, and whose work is contributing toward the replacement of animal experiments by alternative toxicity tests. Over the past 10 years, the Björn Ekwall Memorial Award has been presented annually. Björn Ekwall, an outstanding Swedish cell toxicologist, was one of the pioneers in the development and application of alternative methods to animal tests in toxicology. All his scientific work was devoted to *in vitro* toxicology, and in particular, to the use of cultured human cells for the screening of toxic chemicals. In the middle of the 1980s, he initiated the international Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) project, to evaluate the usefulness of *in vitro* tests for the estimation of human acute systemic toxicity. To prove his "basal cytotoxicity concept", he established the MEMO database, in which data on the acutely toxic human blood concentrations of drugs and chemicals were collated from the literature and from clinical studies. He also initiated another project, Evaluation-Guided Development of *In Vitro* Toxicity and Toxicokinetic Tests (EDIT). The ideas from the EDIT project, together with those from the MEIC project, became the basis for today's international EU projects, e.g. ACuteTox, Sens-it-iv and ReProTect. In this article, 10 years after the start of the BEMF, the scientific achievements of each of the award winners in the field of *in vitro* toxicology are presented, together with a brief synopsis of their careers.

Key words: *alternative methods, basal cytotoxicity, Björn Ekwall Memorial Foundation, EDIT,* in vitro *toxicology, MEIC, MEMO.*

Address for correspondence: *Erik Walum, Glucox Biotech AB, Wollmar Yxkullsgatan 7, SE-11850 Stockholm, Sweden.*
E-mail: *walum@glucoxbiotech.com*

The Björn Ekwall Memorial Foundation

The Björn Ekwall Memorial Foundation (BEMF) was established by the Scandinavian Society for Cell Toxicology (SSCT) in 2001. The intention was to honour the memory of Dr Björn Ekwall (1940–2000), an outstanding Swedish cell toxicologist and the founder of the SSCT, and to establish a prize bearing his name — the Björn Ekwall Memorial Award. The purpose of this prize is to reward scientists who have an interest in the reduction/replacement of animal experiments, and who have significantly contributed to the field of cell toxicology, e.g. by developing new *in vitro* tests or by mechanistic or validation studies. During the past 10 years, the Björn Ekwall Memorial Award has been presented every year, in connection with SSCT workshops or other relevant toxicological conferences. So far, 10 scientists have received the Award, among them some excellent cell toxicologists from England, Finland, Germany, Spain, Sweden and the USA.

Who Björn Ekwall Was and What He Accomplished

Björn Ekwall was born 1940, in Uppsala (Sweden), where he lived and worked almost all his life. There, at the Uppsala University Medical School, he obtained his MD (1969), defended his doctoral thesis in the Department of Anatomy (1980), and became an associate professor in the Division of Toxicology, Department of Pharmaceutical Biosciences (1989). All his scientific work was devoted to *in vitro* toxicology, and in particular, to the use of cultured human cells for the screening of toxic chemicals (1, 2).

Björn Ekwall is rightly referred to as one of the pioneers in the development of alternative methods to animal tests in toxicology. As a physician and skilled human toxicologist, his primary goal was to improve test predictions of human toxicity. His toxicology creed can be summarised as follows: a) most chemicals (including pharmaceuticals) cause toxicity by interfering with basal cell functions — the basal cytotoxicity concept (3);

"Well designed ongoing and future relevance-focused multicenter validation studies have a real chance to introduce in vitro *assays in general toxicity testing, provided that the tests themselves are good enough or can be made good enough. Such studies are economical and feasible, since an almost unlimited number of methods can be evaluated parallely against a single database of* in vivo *toxicity."*

Björn Ekwall (1992). Validation of *in vitro* cytotoxicity tests. In In Vitro *Alternative Methods to Animal Pharmaco-toxicology* (ed. J.V. Castell & M.J. Gómez-Lechón), pp. 361–390. Madrid, Spain: Farmaindustria.

b) human toxicity can be predicted from cell culture experiments — the *in vitro* focus (4); c) human cells are better predictors of human toxicity than animal cells — the human focus (4); d) by combining toxicity and toxicokinetic information from *in vitro* and computer models, it is possible to make accurate predictions of human toxicity — the elementary analysis concept (5); and e) compounds acting on the brain are severe acute systemic toxicants, due to the high sensitivity of the nervous system — the CNS focus (6). He proved his points by standardising human toxicity data from the literature, then constructing the MEMO database and applying the information in the international Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) evaluation study (4).

Björn Ekwall formulated the basal cytotoxicity concept for the first time, in 1983. In his opinion, the toxic effects of chemicals on man can be separated into three categories: a) basal cytotoxicity, i.e. chemical injury to structures and functions that are common to all human cells; b) organ-specific toxicity, i.e. injury to organotypic cell functions and structures; and c) extracellular toxicity. Furthermore, two hypotheses were proposed: the first hypothesis assumes that the majority of chemicals cause toxicity in man via basal cytotoxicity; the second hypothesis claims that basal cytotoxicity can be tested by the use of undifferentiated cell lines (3).

In the MEMO database, Björn Ekwall collected data on acutely toxic human blood concentrations of drugs and chemicals, together with the description of clinical and forensic cases of poisoning. Poisons centres in several countries were used as sources of data, supplemented with information from textbooks, international journals and databases. The final selection of data consisted of chemicals with known lethal human doses, sub-lethal and lethal blood concentrations from clinical and forensic medicine data, time between ingestion and death, target organ/organs, and toxic mechanisms of poisoning, as well as LD50 values for rodents. Later, the human data were summarised in a series of 50 MEIC Monographs.

The MEIC programme, initiated and coordinated by Björn Ekwall and conducted under the auspices of the SSCT, was a multi-centre programme to evaluate the relevance of *in vitro* cytotoxicity tests for acute human systemic toxicity. The programme was largely based on the basal cytotoxicity concept, which fundamentally changed the traditional approach to the field of *in vitro* toxicology. Together with other studies, the MEIC programme paved the way for the development of non-animal, cell-based assays for evaluating the acute systemic toxicity of chemicals in humans.

The purpose of the MEIC programme was to investigate whether the *in vitro* tests could be used for the estimation and prediction of acute toxicity in humans, and to find the best test battery for this purpose. Fifty reference chemicals were voluntarily tested in more than 60 non-animal assays, by 100 laboratories worldwide. Eight papers featuring the results of the MEIC study were published in *ATLA* (6–13). The MEIC results provided convincing evidence that, for the evaluation of acute systemic toxicity of chemicals in humans, animal tests could be replaced by *in vitro* tests, especially by those tests that use human cell lines (7, 11).

The MEIC scheme also considered the relevance of *in vitro* tests with human cells, to human problems. IC50 values were compared with human lethal blood concentrations (LCs) and it was found that an average IC50 value from 10 human cell line tests predicted human peak LCs better (R^2 = 0.74) than did the results from the *in vivo* tests (R^2 = 0.60–0.66, for rats and mice; 11). Human toxicity was clearly underpredicted for only four chemicals, indicating that the human cell line toxicity was highly relevant. Multivariate analysis was used to select an optimum combination of assays, resulting in a battery of three 24-hour human cell line tests (endpoints: protein, ATP and morphology/pH) with good direct prediction of human peak LCs (R^2 = 0.77; 13).

The development of long-term repeated dose and chronic exposure methods was also of significant interest to Björn Ekwall. His close co-worker in this field for many years was Paul Dierickx, of the Biochemical Toxicology Laboratory, Scientific Institute of Public Health, Brussels, Belgium, who died on 30 October 2005. Dr Dierickx made significant contributions to the MEIC and EDIT programmes. He was also involved in the ACuteTox project. Together with Dr Dierickx, and within the framework of the EDIT programme, Björn Ekwall investigated the long-term cytotoxicity of 27 chemicals on Hep G2 cells. A good correlation was found for all 27 chemicals (R^2 = 0.86) after 6 weeks of exposure, and the authors concluded that a good

alternative test could be developed for predicting long-term human toxicity (14).

Developments in Cell Toxicology after the MEIC Project

Björn Ekwall's opinion was that the predictive power of simple cytotoxicity test batteries could be considerably improved by the addition of new supplementary *in vitro* tests (cf. point d in the "toxicology creed" of Björn Ekwall, as outlined above). He felt that the development of such new tests would be facilitated by a close coupling of test development and evaluation. Therefore, he initiated a programme called Evaluation-guided Development of *In Vitro* Toxicity and Toxicokinetic Tests (EDIT). All EDIT subprojects were to be designed on a case-by-case basis. However, at the same time, the subprojects should follow a general pattern: the accumulated MEIC/EDIT data and experience from the previous MEIC evaluation would be used to suggest priority areas, i.e. the need for certain *in vitro* toxicity data/tests as supplements to existing *in vitro* models/batteries on human systemic toxicity. The EDIT programme was never completely realised, due to Björn Ekwall's untimely passing away. However, to a large extent, the ideas and structure of the EDIT programme were incorporated into new international projects such as ACuteTox, Sens-it-iv and ReProTect, under the auspices of the European Commission.

Cecilia Clemedson, a co-worker of Björn Ekwall, has been the scientific coordinator of the ACuteTox project (http://www.acutetox.eu). She has summarised the main goals of this integrated project, which comprised 35 partners, in a later section which outlines her career path.

The Sens-it-iv project (http://www.sens-it-iv.eu), an integrated project involving 28 partners, started in 2005 and ended in 2010. It focused on the development of alternative strategies to animal testing for the assessment of skin and/or respiratory sensitising potentials of chemicals. This included the development of predictive *in vitro* methods. The prescribed deliverables of the project are *in vitro* tests that are ready for prevalidation by the European Centre for the Validation for Alternative Methods (ECVAM).

The aim of the ReProTect project (http://www.reprotec.eu) was to develop a novel approach to the hazard and risk assessment of chemicals for their reproductive toxicity. The project began in 2004 and ended at the end of 2009. The consortium, which comprised 33 partners, developed a battery of tests which can be applied in a tiered strategy for reproductive toxicity testing.

The final outcome for regulatory use of these consortium studies depends on the results of the prevalidation and formal validation of the developed tests and testing strategies under the auspices of ECVAM.

Developments in Regulatory Testing

During the past decade, there have been a number of positive developments in the acceptance of *in vitro* alternative tests in EU and Organisation for Economic Co-operation and Development (OECD) guidelines. Alternative tests for skin corrosion (2004–2009), skin penetration (2004), phototoxicity (2004), skin irritation (2010), and eye irritation (2009) have been accepted in these guidelines. In many of them, cell toxicity is determined as an endpoint. Thus, Björn Ekwall's idea of cell toxicity as a fundamental phenomenon in toxic effects has been put into practice in these tests.

An additional recent activity, which was influenced by Björn Ekwall's work, is a multi-laboratory validation study which evaluated the extent of reduction in animal use and mortality. This is achieved by using *in vitro* basal cytotoxicity test methods to predict the starting doses for the acute oral toxicity test. This study demonstrated that data from *in vitro* cytotoxicity tests can be used to reduce the numbers of animals required for acute oral toxicity determinations, and could lead to an official guideline on the use of cytotoxicity tests for dose-setting in systemic toxicity assays (15). Furthermore, recent preliminary work by the Institute for *In Vitro* Sciences (IIVS) and ECVAM suggests that cytotoxicity assay can be used to identify non-toxic industrial chemicals.

The EU legislation for regulatory testing contains principles that were upheld by Björn Ekwall. The newly-accepted Directive for the protection of experimental animals used for scientific purposes (*Directive 2010/163/EU*; 16), implements the principles of the Three Rs (*Refinement*, *Reduction* and *Replacement*), and emphasises the replacement of animal experiments with alternative methods, e.g. by promoting validation. Both the 7th Amendment to the EU Cosmetics Directive (17), and the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation (18), include the goal to replace regulatory toxicity testing in animals with non-animal approaches, whenever possible.

Future Challenges

Human cell-based models are especially important, because they permit the effects of chemicals on humans to be evaluated directly, with no need for inter-species extrapolation, which is often confounded by species differences. In the future, stem cell-derived cells and new genetically-defined cell

lines will provide an excellent opportunity to develop better functional models for the most important human tissues. A key feature of cell-based methods is that automation and machine vision systems can be applied to them. Together with intelligent kinetic modelling, these developments may considerably improve the evaluation of human health risks.

Cytotoxicity assessments are currently limited by their inability to measure multiple mechanistic parameters that would capture a wide spectrum of potential cytopathological changes. By examining target organ-specific effects, it is possible to discover, for example, whether relevant hepatic functions are altered by a xenobiotic. The development of robust *in vitro*-based multiparametric screening assays, covering a wider spectrum of key effects, will strengthen their predictive capacity for human toxicity and accelerate the drug development process. Technological progress in the fields of genomics, proteomics, metabonomics and cytomics, plays a very important role in the detection of novel biochemical pathways and biomarkers of target-organ toxicity. Although biochemical mechanisms of drug-induced toxicity are complex, a risk assessment profile/decision-making guide can be obtained for each drug candidate, by combining the most relevant screening methods into a panel of assays.

During the last few years, scientific and technological development has been rapid in the areas of cell research, functional genomics, bioinformatics, high-throughput screening technology, and in the understanding of the human genome. This rapid development has resulted in the availability of a wide range of new tools for performing rapid and cost-effective studies on the effects of chemicals on cells. Stem cell technologies have made it possible to improve the availability of human cell and tissue models. The need to evaluate the safety of an increasingly large number of chemicals has led many authorities to present new outlines for the future of toxicity testing, in which a shift from animal tests toward studies at the cellular level is proposed (e.g. the US National Research Council document, *Toxicity Testing in the 21st Century: A Vision and a Strategy* [19]). It is noteworthy that Björn Ekwall had already presented similar ideas to these, at the beginning of the 1980s!

The Björn Ekwall Memorial Award-winners

The Björn Ekwall Memorial Award-winners have made significant contributions to the scientific field of *in vitro* toxicology, and thus have considerably speeded up progress in this area. Brief synopses of the scientific careers of the first 10 winners of the Award are presented below.

2002: Maria José Gómez-Lechón (Spain) — for work in the field of *in vitro* hepatotoxicity and the development of several hepatic cellular models

Maria José Gómez-Lechón was born on 24 February 1952, in Valencia, Spain. Her present position is as a senior scientist at the Research Centre of the La Fe University Hospital, Valencia. In 1983, she obtained her PhD in Biology from the University of Valencia.

Maria José Gómez-Lechón has broad expertise in the culture of hepatic cellular models (primary cultures of human and animal species, three-dimensional [3-D] cultures in collagen, and co-cultures of hepatocytes with non-parenchymal cells), in developing genetically-engineered hepatocellular models with the transient or permanent expression of biotransformation enzymes, and in obtaining cells with the differentiated hepatic phenotype from progenitor cells. She specialises in cell and molecular biology, with particular emphasis on drug metabolism and molecular mechanisms of hepatotoxicity of xenobiotics *in vitro* (biotransformation and identification of metabolic profiles of drugs, induction/inhibition of cytochrome P450 isoenzymes, identification of the cytochrome P450 isoenzymes involved in the production of specific metabolites, and toxic action mechanisms of drugs).

Dr Gómez-Lechón has been involved in several scientific research projects, and thus has contributed to the development and acceptance of alternatives to laboratory animal models. She participated in the MEIC project, in 21 projects funded by Spanish organisations, 14 projects under the aegis of the EU, and in more than 70 R&D projects involving national and international pharmaceutical companies.

Maria José Gómez-Lechón's most important scientific achievements are related to drug metabolism, with particular emphasis on her studies of the regulation of expression of P450 enzymes and of the molecular mechanisms involved in drug-induced hepatotoxicity. In addition, she has played an important role in the development of assays for the risk assessment and prediction of drug hepatotoxicity, and for the prediction of chronic drug-induced hepatotoxicity (steatosis/cholestasis), with a keen focus on the implementation of new technologies (cytomics and metabonomics) in the field of *in vitro* toxicology (20, 21). The number of drugs able to induce hepatic injury is very high, and the incidence of hepatic injury is increasing as a result of exposure to a higher number of chemical agents. Consequently, evaluation of potential hepatotoxicity constitutes a major goal in new drug development. However, there are no suitable strategies that effectively permit the early pre-

diction of drug toxicity. Therefore, future plans propose strategies which are based on the identification of biomarkers that are the representative 'fingerprint' of each toxicity mechanism. This strategy, when applied to early drug development, will help to identify drugs that are potentially hepatotoxic. It is based on the use of multi-parametric assays (simultaneous assessment of key cell functions) by cytomics, in cells that have been exposed to hepatotoxin models, and on the analysis of intracellular and extracellular metabolites (metabonome) by mass spectrometry (22). The analysis strategy will integrate the use of a new cell model generated by genetic manipulation ('artificial hepatocyte'), which is capable of reproducing the metabolic idiosyncrasy of the human liver and will help to detect hepatotoxins that are able to undergo bioactivation (23). The development of a test for lymphocytic activation, based on the assessment by flow cytometry of the specific expression of CD69 (early activation antigen) after the exposure of patients' lymphocytes to liver surface antigens and/or to a suspected drug *in vitro*, in order to distinguish between allergic and autoimmune hepatitis, is currently in progress.

Dr Gómez-Lechón has served as Vice-President of ESTIV (European Society of Toxicology *In Vitro*; 1999–2002); Vice-President of ETCS (European Tissue Culture Society; 2004–2008), President of the Spanish Branch (1992 to date); promotional and active member of the Executive Board of REMA (Spanish Network of Alternative Methods; 1998); member of the board of the Spanish Society of Cellular Biology (1997–1999); and founding member of the World Federation Preventive and Regenerative Medicine (2006).

2003: Per Kjellstrand (Sweden) — for work in the field of *in vitro* screening of complex materials for acute toxicity evaluation

Per Kjellstrand was born on 14 July, 1941. He received his PhD from Lund University, Sweden, in 1977, and became associate professor in animal physiology there in 1979. He left the university in 1987 to take up a position as a principal scientist and later, from 1993, as senior scientific advisor in the Cell Biology Group at Gambro AB, Lund. Per Kjellstrand passed away on 30 October, 2010.

Early on, Per Kjellstrand recognised the necessity to develop rapid and reproducible methods for the detection of the toxic activities of large sets of compounds. In the 1980s, he introduced cell-based techniques for the high-throughput toxicity screening of complex materials, which now have replaced the traditional animal acute toxicity tests in the industrial laboratory. The methods for detection of toxic activity of dialysis fluids and equipment, which are now used worldwide, were developed by Per Kjellstrand's team. He was also one of the first researchers to take part in the MEIC project.

This is the (edited) abstract of Per Kjellstrand's Björn Ekwall Memorial Award Lecture, entitled *Dynamics of Glucose Degradation*, presented at the 21st Annual SSCT Workshop on *In Vitro* Toxicology, presented in Tampere, Finland, in 2003:

"Energy from the sun, through photosynthesis, constitutes the main basis for life on our planet. In plants, glucose is one of the main molecules to store that energy. The energy is then transported through food chains to different forms of animal life. Glucose is also important, since its degradation products constitute building blocks for other molecules central to life processes. It is not clear why, among all the carbohydrates, glucose has become one of the most important molecules in the evolution process of life. When dealing with glucose and its degradation products from a toxicological point of view, we have to remember the approximately 3,500 million years of evolution in which it has played such a central role. Glucose is not just a molecule like any other.

In medicine, glucose is used as parenteral nutrition and as an osmotic agent in peritoneal dialysis. In both cases, it is delivered in 1 or 2 litre plastic bags. These bags containing the fluids are sterilised by heat. Glucose degradation during the heat sterilisation has been shown to make the fluids toxic. Degradation may also take place during the storage of the fluids, which further increases its toxicity. Recently, 3,4-dideoxy-glucosone-3-ene (3,4-DGE), the biologically active degradation product responsible for the toxicity, was identified. This presentation deals with the dynamics of the process in which 3,4-DGE is involved, from the mixing of the dry substances, through the complicated process during storage until the fluids are used by the patients."

The results of this study, performed by Per Kjellstrand's team at Gambro, have been summarised by Erixon *et al.* (24). It was concluded that the heat sterilisation of peritoneal dialysis fluids promoted the formation of large quantities of 3,4-DGE, rendering the fluid highly cytotoxic, which led to inhibition of cell growth. During storage, most of the 3,4-DGE is reversibly converted in a temperature-dependent manner to a less cytotoxic pool, consisting mainly of 3-DG. Cytotoxicity seems to be dependent exclusively on 3,4-DGE. In order to avoid higher levels of 3,4-DGE concentrations, peritoneal dialysis fluids should not be used too soon after sterilisation, and should not be stored at temperatures above room temperature.

2004: Hanna Tähti (Finland) — for work in the field of *in vitro* neurotoxicity and the development of *in vitro* models for corneal, retinal and blood–brain barriers

Hanna Tähti, born on 5 September 1939, in Sääksmäki, Finland, received her PhD in physiological zoology from the University of Helsinki, Finland, in 1978. She began research in the field of toxicology in 1973, and became an adjunct professor of toxicology in 1986 at the medical faculty of the University of Tampere, Finland. She also worked as a toxicologist in the pharmaceutical industry (1986–1988), but returned to the university, where she held various academic posts and was appointed as professor of environmental toxicology in 2000. She retired at the end of 2004. At present, she is active as an emerita professor and as a part-time special expert in FICAM, the Finnish Centre for Alternative Methods at the University of Tampere. She has been a EUROTOX Registered Toxicologist since 1998.

The special research area of Hanna Tähti and her research group has been *in vitro* neurotoxicology. Cell membrane receptor functions and membrane fluidity changes have been studied as targets of neurotoxic effects in synaptosomes and in various human neuronal cell lines. Both neural and glial effects were characterised and evaluated in human-derived cell lines. In the EU Biomed project (BMH4-2324; 1997–2001) for *in vitro* eye toxicology, retinal and corneal *in vitro* models were developed for use in evaluating the adverse effects of drugs on the eye.

After the establishment of the Cell Research Centre (CRC) in the Medical School of the University of Tampere in 1999, the specific target of her research was to find human cell-based methods for the evaluation of neurotoxic risks to humans (25, 26). One of the aims was to develop 3-D research models for determining metabolic activation in early phases of the development of new drugs. In order to model the blood–brain barrier (BBB), 3-D models were developed by cultivating various barrier cells (retinal pigment epithelial cells, pig brain capillary endothelial cells or endothelial cell lines) on filter inserts, with the glial cells under the barrier cells and the target neurons opposite the barrier, on the bottom of the outer well of the double-well system (27). The barrier properties were evaluated in studies of the specific transporters and the tightness of the barrier cell layer. At present, the research to optimise the human cell line-based BBB model is continuing.

Hanna Tähti acted as the chairman of the Finnish Society of Toxicology (1996–1998), and was the first chairman (2003–2007) of the Finnish Consensus Platform for Alternatives (FINCOPA), which was established 2003 in Tampere. She has been a EUROTOX executive board member and chief editor of the EUROTOX proceedings (1997–2003), and an advisory board member of the ACuteTox project, and was a member of the EU expert group on alternative (non-animal) methods for cosmetic testing in 2010.

2005: Hasso Seibert and Michael Gülden (Germany) — for work on factors that influence effective concentrations of chemical compounds *in vitro* by defining algorithms for the calculation of free chemical concentrations at the target organ

Michael Gülden was born on 26 October, 1950, in Mittelbollenbach in Germany, and Hasso Seibert on 7 February, 1950, in Einst. Höfe in Germany. They both studied biology at the University of Kiel, Germany, and received their PhD qualifications in 1981 (MG, in Physiology) and 1983 (HS, in Zoophysiology). They are senior researchers and lecturers in toxicology at the Institute for Toxicology and Pharmacology for Natural Scientists, University Hospital Schleswig-Holstein, in Kiel, Germany.

Michael Gülden and Hasso Seibert came together in 1985, to work on a project on alternatives to animals in acute toxicity testing, financed by the German Federal Ministry of Research and Technology. Acute toxicity can be caused by various modes of action at various targets in the body. Therefore, with their colleagues, they developed and standardised a battery of *in vitro* test systems, by using differentiated mammalian cells (bovine spermatozoa, primary cultured rat liver, and skeletal muscle cells), an undifferentiated cell line (Balb/c 3T3), and co-cultures of rat liver cells and Balb/c 3T3 cells. Multiple endpoints of chemical action were assessed: a) to enable the measurement and discrimination of general cytotoxicity, metabolism-mediated cytotoxicity, cell-specific cytotoxicity and interference with cell-specific functions; b) to reveal modes of toxic action; and c) to develop *in vitro* toxicity profiles. The test systems were characterised in terms of their responsiveness toward various modes of toxic action. In order that they could evaluate the relevance and the predictive value of the test systems, they took part in the MEIC project. Based on these investigations, an *in vitro* testing strategy for the classification and labelling of chemicals according to their acute toxicity was proposed in 1994 (28).

For toxicological hazard assessment, *in vitro* effective concentrations have to be related to equivalent *in vivo* doses. For that purpose, the biokinetics of the compounds, both *in vivo* as well as *in vitro*, have to be taken into account. Focusing on

in vitro biokinetics, Michael Gülden and Hasso Seibert found that cellular and extracellular binding can significantly affect the bioavailability of chemicals *in vitro*. Nominal concentration–effect relationships were shown not to be suitable for use as measures of toxic potency *in vitro*, as they depend on the composition of the *in vitro* test sytem. Taking into account serum protein binding and partitioning into lipids, they developed an equilibrium distribution model for chemicals *in vitro*, and defined algorithms to calculate free chemical concentrations corresponding to nominal effective concentrations. On the basis of these investigations, they postulated that quantitative *in vitro–in vivo* extrapolation should estimate external or internal doses/concentrations, resulting in the free concentration determined to be effective *in vitro* (the 'equivalent exposure concept'). This approach was followed: a) to predict toxic human serum concentrations from *in vitro* cytotoxicity data (29); and b) to considerably improve *in vitro* cytotoxicity testing for acute toxicity assessment in fish (30). The continuation of these investigations indicates that the proper *in vitro* dose measurements to be used for quantitative *in vitro* comparison and *in vitro–in vivo* extrapolation depend on the fate of the chemical in the *in vitro* test system, and may be represented by the free concentration, the area under the concentration–time curve (AUC), or the cell dose.

At present, Michael Gülden and Hasso Seibert are continuing their research in different areas of cell toxicology, including possible applications in hazard and risk assessment. Hasso Seibert was a member of various international expert committees, e.g. the OECD Validation Management Group, Non-animal Assays for Endocrine Disruptors, and the ECVAM Scientific Advisory Committee (ESAC). Furthermore, he was Vice-President of ESTIV and Chairman of the SSCT.

2006: Cecilia Clemedson (Sweden) — for work on the MEIC, EDIT and ACuteTox projects, together with her invaluable efforts for the SSCT

Cecilia Clemedson was born on 30 November, 1960, in Stockholm, Sweden. She received her PhD in neurotoxicology from Stockholm University, in 1992. She then worked as a researcher in the Department of Pharmaceutical Biosciences, Division of Toxicology, Uppsala University, Sweden. Between 1992 and 1999, she was the Scientific Secretary of the MEIC programme, which involved 97 laboratories, and worked on both the *in vitro–in vitro* and *in vitro–in vivo* evaluations. She started the CCTox Consulting Company, which initiated the Nordic Information Centre for Alternative Methods (NICA). From 2001 to 2009 she was a scientific director of the Expertrådet AB, Sollentuna, Sweden. Since 2009, she has been the managing director of AdvocoTox consulting AB, Stocksund, Sweden — a consulting company offering services such as risk and safety assessment of chemical products. From 2007, she has been the President of the Swedish Fund for Research without Animal Experiments; she is also secretary and treasurer of Swecopa, the Swedish platform for Three Rs alternatives. She has also been a member of the executive board of the European Society of Toxicology *In Vitro* (ESTIV) and the Scandinavian Society for Cell Toxicology (SSCT). Dr Clemedson was the Scientific Coordinator of the ACuteTox project from October 2005 to 2010, and the Coordinator of the ForInViTox project from September 2007 to 2009.

Cecilia Clemedson was Björn Ekwall's collaborator for many years on the MEIC and EDIT projects. She was mainly involved in the validation of *in vitro* cytotoxicity methods for use in the estimation of acute systemic toxicity in humans. She was also involved in the collection of human blood concentration data for the MEMO database. She published extensively on *in vitro* neurotoxicology and on the prevalidation of *in vitro* methods for toxicity testing, e.g. she is a co-author of the majority of the MEIC publications in *ATLA* (6–12).

Later, in 2005, EDIT was merged with other European activities within the field, to form the ACuteTox project (2005–2010), which was an integrated project within EU Framework Programme (FP) 6. The goal of ACuteTox was to develop and prevalidate a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes. Thirty-five partners from industry, academia, governmental institutes and SMEs worked for five years to develop the most powerful *in vitro* test strategy (31). The aim of ACuteTox was to improve *in vitro–in vivo* correlation to a level sufficient enough to ensure a valid prediction of acute toxicity. Outliers obtained in earlier correlation studies have been evaluated in order to introduce further parameters, such as adsorption–distribution–metabolism–excretion (ADME) and organ specificity, which might improve the correlation. This would allow the integration of alerts and correctors in the prediction algorithm, which, together with robust implementation of medium-throughput approaches, would permit the establishment of a new testing strategy to provide a better prediction of toxicity in humans.

The data generated in the project were stored in a novel internet-based database (AcutoxBase) developed within the project, and were used to assess the within-laboratory variability, the preliminary predictive capacity and, in some cases, also the between-laboratory variability of each *in*

vitro assay (32). Based on statistical analysis, eight test methods were assessed as being promising for inclusion in the testing strategy, and therefore were selected for participation in the prevalidation study (www.acutetox.eu).

Although the ACuteTox project is now finished, the validation of the *in vitro–in vivo* data collected in this project continues at ECVAM and also in other laboratories. The Swedish Fund for Research without Animal Experiments recently granted C. Clemedson *et al.* the funding for a project entitled, *Extended Statistical Analysis of* In Vitro *Data from the ACuteTox Project by Using Human Toxicity Data.*

2007: Rodger Curren (USA) — for work on the development, optimisation, validation and acceptance of alternative (non-animal) testing and research methods

Rodger Curren was born on 10 June, 1946, in Marion, OH, USA. He received his PhD in bacterial genetics in 1975, and followed this with post-doctoral studies on human cell mutagenesis and chemical carcinogenesis at the Michigan Cancer Foundation and Michigan State University, MI. He then went to work for Microbiological Associates, Rockville, MD (now BioReliance), eventually leading the genetic toxicology division and starting an *in vitro* toxicology group. Subsequently, the *in vitro* toxicology activities became his main focus and, in 1997, he helped found a non-profit organisation, the Institute for *In Vitro* Sciences (IIVS). IIVS focuses not only on optimising and conducting *in vitro* methods, but also on providing training for toxicologists internationally.

In his BEMF lecture, Dr Curren stressed that it is almost always a struggle to change existing paradigms, especially those that have been established internally through our education and professional experience. This has been the challenge that *in vitro* toxicology has faced, and one that Björn Ekwall tried to overcome. Slowly, industry has seen that animal testing is not always a sure way to assess product safety, and even more slowly, the regulatory community is attempting to break from the legislative mandates that require a check-box style of traditional assays. Björn Ekwall came to know this well, as he frequently met with a sceptical reception for his new ideas — ideas which stretched the imagination of complacent thinkers. His message of 'outside the box' only slowly took hold, as a few individuals became at least curious enough to test his assumptions, and found empirical evidence that at first surprised them and then eventually moved them to conversion.

Rodger Curren emphasised that we must continue to move forward with an open mind. For example, he brought up the significant restrictions on animal testing that were to be imposed "as early as 2009" by the 7th Amendment to the Cosmetics Directive. This is a potential problem for toxicologists responsible for genotoxicity assessments, since it is common to retest positive *in vitro* responses in an animal micronucleus or chromosome aberration test. His laboratory imagined that this might actually be an opportunity to investigate an improved approach — one that introduces human biology as well as the complexity of a tissue response. They used the already commercially available 3-D human skin constructs (33) as a suitable substrate to conduct micronucleus studies (34). Subsequent studies have shown that this novel approach is very promising. Thus, by keeping an open, inquiring mind, we are often able to find even better ways forward.

Rodger Curren has been chairman of the Genetic Toxicology Association, president of the *In Vitro* Speciality Section of the Society of Toxicology (SOT), has served on the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) advisory committee, and currently serves on the ECVAM Science Advisory Committee. He has received the Russell and Burch award, as well as the Alternatives Research and Development Foundation (ARDF) award, for service to alternatives research.

2008: Erik Walum (Sweden) — for work in the field of *in vitro* neurotoxicology, the description of mechanisms for chemically-induced cellular injuries and the development of test methods

Erik Walum, born on 24 August, 1945, received his PhD in animal physiology from the University of Göteborg, Sweden, in 1976, and became an associate professor in animal physiology in 1979, associate professor in neurochemistry in 1984, and professor in neurotoxicology in 1995, at Stockholm University, Sweden. At present, he is the CEO of Glucox Biotech AB, in Stockholm.

Erik Walum's interest in the development of cellular models for the study of neurotoxicology started with his employment at the National Defence Research Institute (FOA) in 1976. The National Defence, at that time the biggest employer in Sweden, wanted to improve working conditions, and saw the development of cell-based testing methods for environmental pollutants as an important step in that direction. With additional support from the Swedish Working Environment Foundation, Professor Walum started to use cultured neuronal cell lines to develop test methods and study mechanisms of toxicity of problem compounds. This work continued when he took up a research position at Stockholm University in 1979.

As a result of his early work, Professor Walum was able to define some of the inherent problems with *in vitro* systems in toxicology testing. Among these were the non-physiological conditions experienced by traditionally cultured cells, the special toxicokinetic properties of a cultured test system, and the shortcomings of continuous cell lines. These problems were discussed, and solutions were presented, in his book, *Understanding Cell Toxicology: Principles and Practice* (35), in 1990.

The problems of *in vitro* to *in vivo* extrapolation and the development of concepts for risk prediction became the focus of Erik Walum's academic work during the late 1980s and early 1990s, and he developed ideas for the use of cellular methods for the identification of neurotoxic chemicals and the estimation of neurotoxicological risk (36).

In 1994, Erik Walum moved to the pharmaceutical industry, but maintained a part-time position at the university for more than 10 years and continued to lecture in neurochemistry and neurotoxicology. As head of cell and molecular biology at Pharmacia, and later at Biovitrum, he was a strong advocate of the use of *in vitro* methods for target validation and early drug characterisation and selection. In his BEMF prize lecture, presented at the 15th International Conference of ESTIV, in Djurönäset, Sweden, in 2008, he described a project aimed at the development of a compound for neuroprotection in the retina:

"Glaucoma is a group of diseases involving loss of retinal ganglion cells in a characteristic pattern of optic neuropathy. Although not fully elucidated, the mechanism of neuronal degeneration is believed to be related to restricted blood flow and impairment of nerve cell energy metabolism, possibly with the contribution of elevated levels of neurotransmitter glutamate. Raised intraocular pressure is a significant risk factor for developing glaucoma, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogues represent effective treatments by increasing aqueous humour outflow and thereby reducing the pressure.

When SH-SY5Y cells are exposed to glutamate, intracellular calcium increases and the membrane potential decreases rapidly. Later, a time- and concentration-dependent increase in c-Jun expression is evident, followed by pronounced neuronal cell death.

During the project, the question of whether $PGF_{2\alpha}$ may be neuroprotective arose. SH-SY5Y cells expressing a recombinant FP receptor were therefore exposed to increasing concentrations of glutamate in serum-free medium. The glutamate caused a decrease in neuroblastoma cell survival, which was fully reversed by the presence of 1μM PGF_{2a}. In non-transfected SH-SY5Y cells, the prostaglandin lacked protective effects. A simple and well-characterised cell culture system thus gave useful information on an interesting and, for human health, relevant question, using *in vitro* neuropathy as end-point."

From the initial preparations for the MEIC programme, and during its active phase, Erik Walum served as the Chairman of the MEIC committee (1985–1999). He was a member of the Swedish National Board for Laboratory Animals (CFN; 1986–1994), and the Chairman of its Alternatives Research Committee (1988–1994). He joined the ECVAM Scientific Advisory Committee (ESAC) in 1996, and served for nine years.

2009: Annalaura Stammati (Italy) — for *in vitro* studies on the toxicity of synthetic and natural compounds, leading to the development of strategies for prediction of biokinetics in humans

Annalaura Stammati was born in Montalcino, Siena, Italy, in 1944, and graduated in Biology from the University 'La Sapienza', Rome, in 1967, with an experimental thesis on the effects of glucides on the diet. In 1971, she became a master in Food Science and, in 1994, she attended a post-graduate specialisation course on Bioethics at the same university.

In 1967, she joined the Veterinary Laboratory in the Virology Unit and, in 1975, she became a member of the permanent staff of the Istituto Superiore di Sanità (ISS) in Rome, where she first worked in the Veterinary Department on the identification and characterisation of pathogenic viruses. In 1976, she joined, first, the Toxicology Laboratory, and then the Comparative Toxicology and Ecotoxicology Laboratory, where she co-ordinated, as senior researcher, a group addressing the applications of cell culture in toxicology. This work involved the study of the toxicity of synthetic and natural compounds by using different cell lines, and covered the screening for toxic molecules, as well as the investigation of their mechanisms of action (37). From 2004 to 2009, she was director of the Toxicity Mechanisms Unit in the ISS Department of Environment and Primary Prevention. In recent years, the research has been focused on compounds that are toxic for the gastro-intestinal tract, and also investigating their absorption, by using *in vitro* intestinal models. This non-animal-based approach, integrating toxicodynamics and biokinetics, should lead to the development of a strategy for measuring the real exposure of cells to chemicals and to the prediction of biokinetics in humans (38–40).

Annalaura Stammati has been involved in a number of national and international expert committees, including membership of the ESAC from 2002 to 2009. She was also an expert in the OECD

Test Guidelines Programme (1998–2009), and in the Italian delegation in the Committee of Experts on Cosmetic Products of the Council of Europe in Strasbourg (2005–2007). She also founded IPAM, the Italian Platform on Alternative Methods, in 2003.

Annalaura Stammati was one of the closest co-workers of Dr Björn Ekwall, having participated in the MEIC project from the very beginning, and she was a co-author of several MEIC publications (e.g. 6, 7, 9). The basic principles of the MEIC project have been taken up by the ACuteTox project, with the collaboration of her group.

Annalaura Stammati has been retired from the ISS since June 2009, but she is still active as Past-President of IPAM and CellTox (the Italian Association for *In Vitro* Toxicology), so she continues to contribute to the promotion of alternative methods.

2010: Richard Clothier (UK) — for significant work in the field of *in vitro* toxicology, the development, implementation and validation of alternative toxicity tests, and for substantial contributions to the FRAME Research Programme

Richard Clothier was born in Kent, UK, in 1944. He graduated in Zoology and Botany from the University of London in 1967, and also became a qualified teacher. He moved to East Anglia, where he taught in schools and undertook an external PhD at the University of East Anglia, Norwich, which he completed in 1972. He took up a research position at the University of East Anglia in 1973, then moved to the University of Nottingham as a Research Assistant, in 1975. Later, he became a lecturer, reader and, finally, an associate professor in the School of Biomedical Sciences at the university, where he taught Anatomy and Histology and was the departmental safety officer. He retired in 2005.

Richard Clothier is a Trustee of FRAME (Fund for the Replacement of Animals in Medical Experiments) and, before he retired, was the Director of the FRAME Alternatives Laboratory (FAL) situated in the Medical School at the University of Nottingham, UK. He initially worked on tumour induction in amphibians. In these studies, it proved very difficult to induce tumours in the South African Clawed toad (i.e. they were highly resistant to cancer; 41). This led to a detailed evaluation of the animal's immune system, in collaboration with Professor Laurens Rubens at Reed College, Portland, OR, USA. This further study revealed some of the mechanisms whereby these animals resisted tumour formation (42). One aspect of this research included *in vitro* tissue culture, initially with amphibian tissues, then later focused on *in vitro* culture and toxicity testing with mammalian cells. This work was conducted in partnership with Michael Balls, his collaborator since 1967. When Professor Balls moved to Italy in 1993, to establish ECVAM, Dr Clothier took over the directorship of the FAL. Under his directorship, the FAL's research focused on the development of *in vitro* alternative assays for toxicity (43). During this period, the FAL also participated in the evaluation, via national and international validation studies, of the relevance, applicability and reliability of the *in vitro* approach for acute toxicity prediction in humans. The FAL has been involved with FRAME's practical studies since 1983 (44), the EC/HO and COLIPA projects on eye irritancy, the EU/COLIPA studies on phototoxicity (45), and the MEIC, SDA, ECVAM/ICCVAM and EU ACuteTox studies on acute toxicity *in vitro*.

The FAL was involved in the development of FRAME Kenacid Blue assay, the Neutral Red Release assay, the Fluorescein Leakage assay, innervated corneal epithelial models and bronchial models for the prediction of squamous metaplasia, and a human skin model for schistosoma infection.

2011: Päivi Myllynen (Finland) — for significant work in the field of reproductive toxicity, and for development of the placental perfusion method for the evaluation of the toxic effects of chemicals in humans

Päivi Myllynen was born on 5 November, 1974, in Suomussalmi, Finland. She graduated from the University of Oulu Medical School, Oulu, Finland, in 1999, and completed her PhD in the Department of Pharmacology and Toxicology at the same university, in 2003. Currently, she has a position as a senior lecturer in the Department of Pharmacology and Toxicology, Institute of Biomedicine, at the university.

One of the main methodologies used in Päivi Myllynen's laboratory is human placental perfusion, in which a delivered-term human placenta is kept alive for a few hours by using artificial circulation. Human placentas are usually disposed of after delivery, and the use of placental tissue in research does not affect the management of the delivery or the newborn in any way.

Päivi Myllynen became familiar with placental perfusion methodology during her PhD project, when she studied the placental transfer of antiepileptic medications. Since then, her focus has been mostly on the placental transfer of environmental contaminants and food-borne carcinogens. Recently, she was involved in the EU ReProTect

project as part of the research group of Professor Kirsi Vähäkangas. In this project, a dual recirculating human placental perfusion methodology was prevalidated by independent research groups, following the modular approach developed by ECVAM for the validation of *in vitro* test systems (46).

The placenta is not merely a passive barrier, but an organ which actively transfers compounds between the maternal and fetal circulations. Placental function may therefore be a source of person-to-person variation in fetal exposure. In the placenta, one of the major functions for some of the maternal-facing efflux transporters, such as ABCB1 and ABCG2, is the protection of the fetus against exogenous insults. It is known that transporter expression varies, e.g. due to genetic polymorphisms. Dr Myllynen and her co-workers showed recently, by using *ex vivo* perfused human placentas, that the placental transfer of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP; an abundant food- borne carcinogen) correlates with the ABCG2 expression level in the placenta (47). On the other hand, the placental transfer of another food-borne carcinogen, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), is not affected by ABCG2 (48). Although the fetoprotective function of ABCG2, and variation in ABCG2 expression in the placental tissue, have also been established in several other studies, Dr Myllynen's results are the first to show that ABCG2 expression may result in person-to-person variation in placental transfer in humans.

Päivi Myllynen has recently established a research group that will continue the study of placental transfer processes. Understanding the mechanisms leading to variation in transplacental transfer is essential, because it may be one of the factors which determine the consequences of maternal exposure to xenobiotics. Dr Myllynen's aim is to characterise the factors which cause person-to-person variation in fetal exposure to food-borne carcinogens and environmental contaminants, and the significance of this variation for the child's health, by focusing on transporter proteins. Her hypothesis is that placental function, especially with regard to the transporter proteins, modifies fetal exposure to food carcinogens and environmental contaminants. In the current project, she is going to combine a toxicokinetic study that uses full-term human placental tissue from a traditional birth cohort, with questionnaires and sample collection at delivery. In addition, cell culture methods will be used for mechanistic studies.

Conclusions

The work of Björn Ekwall, and the investigations he initiated, have proven to be of great significance for the further development of *in vitro* methods in toxicology. His ideas have inspired wide circles of scientists — academic fellows and regulatory toxicologists — in their efforts to replace animal testing with more-relevant and scientifically-sound alternative methods. Today's achievements in this field would not be possible without EU-supported projects based on the ideas of Björn Ekwall. As a result, there was an immense motivation to honour his memory with a prize bearing his name, the Björn Ekwall Memorial Award. All the award-winners presented here have made important contributions in the field of cell toxicology, e.g. by developing new *in vitro* tests or by mechanistic or validation studies. The Björn Ekwall Memorial Foundation will continue to reward distinguished scientists in the field of *in vitro* toxicology, and thereby continue to contribute toward the acceptance of non-animal methods in toxicology.

Dr Björn Ekwall (1940–2000)

Acknowledgements

The Björn Ekwall Memorial Award has been made possible by the generous financial support of: Scandinavian Society for Cell Toxicology (Scandinavia); Swedish Fund for Research without Animal Experiments (Sweden); People for Ethical Treatment of Animals (USA); Djurens Rätt, Karlskrona section (Sweden); Djurens Rätt, Norrrtälje section (Sweden); Mrs Ethel Thurston (USA); Hans and Margareta Beerman (Sweden); Djurskyddet Sverige (Sweden); Italian Platform on Alternative Methods (Italy); Djurens Vänners Riksorganisation, Malmö section (Sweden); New England Anti-vivisection Society (NEAVS; USA); American Fund for Alternatives to Animal Experiments (AFAAR; USA).

References

1. Walum, E. (2001). Björn Ekwall MD PhD, 1940–2000: A pioneer in the field of *in vitro* toxicology. *ATLA* **29**, 5–6.
2. Kolman, A. & Walum, E. (2010). Björn Ekwall, an outstanding Swedish cell toxicologist. *Toxicology in Vitro* **24**, 2060–2062.
3. Ekwall, B. (1983). Screening of toxic compounds in mammalian cell cultures. *Annals of the New York Academy of Sciences* **407**, 64–77.
4. Bondesson, I., Ekwall, B., Hellberg, S., Romert, L. & Walum, E. (1989). MEIC — a new international multicenter project to evaluate the relevance to human toxicity of *in vitro* cytotoxicity tests. *Cell Biology & Toxicology* **5**, 331–347.
5. Ekwall, B., Clemedson, C., Ekwall, B., Ring, P. & Romert, L. (1999). EDIT: A new international multicenter programme to develop and evaluate batteries of *in vitro* tests for acute and chronic systemic toxicity. *ATLA* **27**, 339–349.
6. Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocañas, L., Gómez-Lechón, M.J., Gülden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Ohno, T., Persoone, G., Roguet, R., Romert, L., Sawyer, T.W., Seibert, H., Shrivastava, R., Stammati, A., Tanaka, N., Torres-Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F. & Ekwall, B. (1996). MEIC evaluation of acute systemic toxicity. Part I: Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. *ATLA* **24**, 249–272.
7. Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocañas, L., Gómez-Lechón, M.J., Gülden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Malmsten, A., Ohno, T., Persoone, G., Pettersson, R., Roguet, R., Romert, L., Sandberg, M., Sawyer, T.W., Seibert, H., Shrivastava, R., Sjöström, M., Stammati, A., Tanaka, N., Torres-Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F. & Ekwall, B. (1996). MEIC evaluation of acute systemic toxicity. Part II: *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. *ATLA* **24**, 273–311.
8. Clemedson, C., Barile, F.A., Ekwall, B., Gómez-Lechón, M.J., Hall, T., Imai, K., Kahru, A., Logemann, P., Monaco, F., Ohno, T., Segner, H., Sjöström, M., Valentino, M., Walum, E., Wang, X. & Ekwall, B. (1998). MEIC evaluation of acute systemic toxicity. Part III: *In vitro* results from 16 additional methods used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. *ATLA* **26**, 93–129.
9. Clemedson, C., Andersson, M., Aoki, Y., Barile, F.A., Bassi, A.M., Calleja, M.C., Castano, A., Clothier, R.H., Dierickx, P., Ekwall, B., Ferro, M., Fiskesjö, G., Garza-Ocañas, L., Gómez-Lechón, M.J., Gülden, M., Hall, T., Imai, K., Isomaa, B., Kahru, A., Kerszman, G., Kjellstrand, P., Kristen, U., Kunimoto, M., Kärenlampi, S., Lewan, L., Lilius, H., Loukianov, A., Monaco, F., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Segner, H., Seibert, H., Shrivastava, R., Sjöström, M., Stammati, A., Tanaka, N., Thuvander, A., Torres-Alanis, O., Valentino, M., Wakuri, S., Walum, E., Wang, X., Wieslander, A., Zucco, F. & Ekwall, B. (1998). MEIC evaluation of acute systemic toxicity. Part IV: *In vitro* results from 67 toxicity assays used to tests reference chemicals 31–50 and a comparative cytotoxicity analysis. *ATLA* **26**, 131–183.
10. Ekwall, B., Clemedson, C., Crafoord, B., Ekwall, B., Hallander, S., Walum, E. & Bondesson, I. (1998). MEIC evaluation of acute systemic toxicity. Part V: Rodent and human toxicity data for the 50 reference chemicals. *ATLA*, **26**, 571–616.
11. Ekwall, B., Barile, F.A., Castano, A., Clemedson, C., Clothier, R.H., Dierickx, P., Ekwall, B., Ferro, M., Fiskesjö, G., Garza-Ocañas, L., Gómez-Lechón, M.J., Gülden, M., Hall, T., Isomaa, B., Kahru, A., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lewan, L., Loukianov, A., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Stammati, A., Tanaka, N., Valentino, M., Walum, E. & Zucco, F. (1998). MEIC evaluation of acute systemic toxicity. Part VI: Prediction of human toxicity by rodent LD50 values and results from 61 *in vitro* tests. *ATLA* **26**, 617–658.
12. Clemedson, C., Barile, F.A., Chesné, C., Cottin, M., Curren, R., Ekwall, B., Ferro, M., Gómez-Lechón, M.J., Imai, K., Janus, J., Kemp, R.B., Kerszman, G., Kjellstrand, P., Lavrijsen, K., Logemann, P., McFarlane-Abdulla, E., Roguet, R., Segner, H., Thuvander, A., Walum, E. & Ekwall, B. (2000). MEIC evaluation of acute systemic toxicity. Part VII: Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further *in vitro* assays. *ATLA* **28**, 161–200.
13. Ekwall, B., Ekwall, B. & Sjöström, M. (2000). MEIC evaluation of acute systemic toxicity. Part VIII: Multivariate partial least squares evaluation, including the selection of battery of cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 reference chemicals. *ATLA* **28**, 201–234.
14. Scheers, E.M., Ekwall, B. & Dierickx, P.J. (2001). *In vitro* long-term cytotoxicity testing of 27 MEIC chemicals on Hep G2 cells and comparison with acute human toxicity data. *Toxicology in Vitro* **15**, 153–161.
15. De Sousa Vieira, M., De Oliveira, V., Lima, E.M., Kato, M.J. & Valadares, M.C. (2011). *In vitro* basal cytotoxicity assay applied to estimate acute oral systemic toxicity of grandisin and its major metabolite. *Experimental & Toxicologic Pathology* **63**, 505–510.
16. Anon. (2010). *Directive 2010/63/EU* of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (Text with EEA relevance). *Official Journal of the European Union* **L276**, 20.10.2010, 33–79.
17. Anon. (undated). *Executive Summary: The 7th Amendment to the Cosmetics Directive*, 17pp. Available at: http://ec.europa.eu/consumers/sectors/cosmetics/files/doc/antest/(2)_executive_summary_

en.pdf (Accessed 10.08.11).
18. European Commission (2007). *REACH in Brief*, 19pp. Brussels, Belgium: Environment Directorate General, European Commission. Available at: http://ec.europa.eu/environment/chemicals/reach/pdf/2007_02_reach_in_brief.pdf (Accessed 10.08.11).
19. National Research Council (2007). *Toxicity testing in the 21st century: A vision and a strategy*, 216pp. Washington, DC, USA: The National Academies Press.
20. Gómez-Lechón, M.J., Castell, J.V. & Donato, M.T. (2008). An update on metabolism studies using human hepatocytes in primary culture. *Expert Opinions on Drug Metabolism & Toxicology* **4**, 837–854.
21. Gómez-Lechón, M.J., Lahoz, A., Gombau, L., Castell, J.V. & Donato, M.T. (2010). *In vitro* evaluation of potential hepatotoxicity induced by new drugs. *Current Pharmacological Design* **16**, 1963–1977.
22. Lahoz, A., Donato, M.T., Castell, J.V. & Gómez-Lechón, M.J. (2008). Strategies to *in vitro* assessment of major human CYP enzyme activities by using liquid chromatography tandem mass spectrometry. *Current Drug Metabolism* **9**, 12–19.
23. Donato, M.T., Hallifax, D., Picazo, L., Castell, J.V., Houston, J.B., Gómez-Lechón, M.J. & Lahoz, A. (2010). Metabolite formation kinetics and intrinsic clearance of phenacetin, tolbutamide, alprazolam and midazolam in adenoviral P450 transfected HepG2 cells and comparison with hepatocytes and *in vivo*. *Drug Metabolism Display* **38**, 1449–1455.
24. Erixon, M., Lindén, T., Kjellstrand, P., Carlsson, O., Ernebrant, M., Forsbäck, G., Wieslander, A. & Jönsson, J.A. (2004). PD fluids contain high concentrations of cytotoxic GDPs directly after sterilization. *Peritoneal Dialysis International* **24**, 392–398.
25. Tähti, H., Nevala, H. & Toimela, T. (2003). Refining *in vitro* neurotoxicity testing — the development of blood–brain barrier models. *ATLA* **31**, 273–276.
26. Toimela, T., Mäenpää, H., Mannerström, M. & Tähti, H. (2004). Development of an *in vitro* blood–brain barrier model — cytotoxicity of mercury and aluminum. *Toxicology & Applied Pharmacology* **195**, 73–82.
27. Nevala, H., Ylikomi, T. & Tähti, H. (2008). Evaluation of the selected barrier properties of retinal pigment epithelial cell line ARPE-19 for an *in vitro* blood–brain barrier model. *Human & Experimental Toxicology* **27**, 741–749.
28. Seibert, H., Gülden, M. & Voss, J.U. (1994). An *in vitro* toxicity testing strategy for the classification and labelling of chemicals according to their potential acute lethal potency. *Toxicology in Vitro* **8**, 847–850.
29. Gülden, M. & Seibert, H. (2003). *In vitro–in vivo* extrapolation: Estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations *in vitro*. *Toxicology* **189**, 211–222.
30. Gülden, M. & Seibert, H. (2005). Impact of bioavailability on the correlation between *in vitro* cytotoxic and *in vivo* acute fish toxic concentrations of chemicals. *Aquatic Toxicology* **72**, 327–337.
31. Clemedson, C., Kolman, A. & Forsby, A. (2007). The integrated acute systemic toxicity project (ACuteTox) for the optimisation and validation of alternative *in vitro* tests. *ATLA* **35**, 33–38.
32. Sjöström, M., Kolman, A., Clemedson, C. & Clothier, R. (2008). Estimation of human blood LC50 values for use in modeling of *in vitro–in vivo* data of the ACuteTox project. *Toxicology in Vitro* **22**, 1405–1411.
33. Spielmann, H., Hoffmann, S., Liebsch, M., Botham, P., Fentem, J.H., Eskes, C., Roguet, R., Cotovio, J., Cole, T., Worth, A., Heylings, J., Jones, P., Robles, C., Kandárová, H., Gamer, A., Remmele, M., Curren, R., Raabe, H., Cockshott, A., Gerner, I. & Zuang, V. (2007). 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. *ATLA* **35**, 559–601.
34. Curren, R.D., Mun, G.C., Gibson, D.P. & Aardema, M.J. (2006). Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm™). *Mutation Research* **607**, 192–204.
35. Walum, E., Stenberg, K. & Jenssen, D. (1990). *Understanding Cell Toxicology: Principles and Practice*, 206pp. London, UK: Ellis Horwood.
36. Walum, E., Nordin, M., Beckman, M. & Odland, L. (1993). Cellular methods for the identification of neurotoxic chemicals and estimation of neurotoxicological risk. *Toxicology in Vitro* **7**, 321–326.
37. Zucco, F., De Angelis, I., Testai, E. & Stammati, A. (2004). Toxicology investigations with cell culture systems: 20 years after. *Toxicology in Vitro* **18**, 153–163.
38. Rossi, L., De Angelis, I., Pedersen, J.Z., Marchese, E., Stammati, A., Rotilio, G. & Zucco, F. (1996). *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidinone activation by the human intestinal cell line Caco-2 monitored through noninvasive electron spin resonance spectroscopy. *Molecular Pharmacology* **49**, 547–555.
39. Re, G., Badino, P., De Angelis, I., Odore, R., Belloli, C., Stammati, A. & Zucco, F. (2001). Identification and coupling to adenylate cyclase of three different [^{3}H]CGP 12177 binding sites in Caco-2 cell membranes. *Pharmacology Research* **43**, 393–398.
40. Turco, L., Catone, T., Caloni, F., Di Consiglio, E., Testai, E. & Stammati, A. (2011). Caco-2/TC7 cell line characterization for intestinal absorption: How reliable is this *in vitro* model for the prediction of the oral dose fraction absorbed in human? *Toxicology in Vitro* **25**, 13–20.
41. Balls, M. & Clothier, R.H. (1974). Spontaneous tumours in amphibia: A review. *Oncology* **29**, 501–519.
42. Ruben, L.N., James, H.S., Clothier, R.H. & Balls, M. (1984). Genetic limits of thymic immunosuppression of anti-hapten antibody production in *Xenopus laevis*, the South African clawed toad. *Journal of Immunogenetics* **11**, 97–101.
43. Balls, M., Riddell, R.J., Horner, S.A. & Clothier, R.H. (1987). The FRAME approach to the development, validation, and evaluation of *in vitro* alternative methods. In *In Vitro Toxicology. Approaches to Validation. Alternative Methods in Toxicology, Volume 5* (ed. A.M. Goldberg), pp. 45–58. New York, NY, USA: Mary Ann Liebert, Inc.
44. Clothier, R. (2010). The development and evaluation of *in vitro* alternative assays: A personal perspective. *ATLA* **38**, 457–469.
45. Clothier, R.H. (2007). Phototoxicity and acute toxicity studies conducted by the FRAME Alternatives Laboratory: A brief review. *ATLA* **35**, 515–519.
46. Myllynen, P., Mathiesen, L., Weimer, M., Annola,

K., Immonen, E., Karttunen, V., Kummu, M., Mørck, T.J., Nielsen, J.K., Knudsen, L.E. & Vähäkangas, K. (2010). Preliminary interlaboratory comparison of the *ex vivo* dual human placental perfusion system. *Reproductive Toxicology* **30**, 94–102.

47. Myllynen, P., Kummu, M., Kangas, T., Ilves, M., Immonen, E., Rysä, J., Pirilä, R., Lastumäki, A. & Vähäkangas, K.H. (2008). ABCG2/BCRP decreases the transfer of a food-born chemical carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in perfused term human placenta. *Toxicology & Applied Pharmacology* **232**, 210–217.
48. Immonen, E., Kummu, M., Petsalo, A., Pihlaja, T., Mathiesen, L., Nielsen, J.K., Knudsen, L.E., Vähäkangas, K. & Myllynen, P. (2010). Toxicokinetics of the food-toxin IQ in human placental perfusion is not affected by ABCG2 or xenobiotic metabolism. *Placenta* **31**, 641–648.