ABSTRACTS OF

EUSAAT 2012

(14th Annual Congress of EUSAAT)

and

Linz 2012

(17th European Congress on Alternatives to Animal Testing)

Contents

Introduction to the Abstracts of EUSAAT 2012 (14th Annual Congress of EUSAAT) and Linz 2012 (17th European Congress on Alternatives to Animal Testing)

Dear friends and colleagues,

On behalf of EUSAAT, the European Society for Alternatives to Animal Testing, I welcome you to the 'EUSAAT 2012–Linz 2012' congress, which is actually the 14th annual congress of EUSAAT and the 17th congress on alternatives in Linz.

During the past two decades the 'Linz Congress' has emerged as one of the major international scientific events in the field of the Three Rs. EUSAAT 2012 is hosting presentations, discussions and exchange of new ideas for the benefit of alternative methods to animal experiments. The Scientific Committee has invested its efforts to address the most important subjects related to the Three Rs that are of interest to scientists in Europe.

The EUSAAT 2012 congress is focusing on new approaches and methodologies — for example, disease models, non-animal tools for basic biomedical research, '-omics' techniques and advanced 3D models, are all subjects of the sessions and poster presentations.

EUSAAT 2012 is providing a forum for reviewing the latest developments in the European chemicals and cosmetics policies, in the context of the use of alternative methods. In 2012, the most important topic is the implementation of the new *Directive 2010/63/EU* on the protection of animals used for scientific purposes, since it will affect all European scientists working in the life sciences in academia, industry and government institutions.

A very hot topic is addressed in the 'Round Table' discussion, where members of the Scientific Committee will discuss the 'pros and cons' of the new EU directive for speeding up the validation and regulatory acceptance of new Three R methods.

It is quite important that the number of sponsors of the EUSAAT congresses has increased over the years, since, without their continuous support, we would not be able to maintain the high scientific standard and to keep the congress fee low. Both elements are equally important, in order to attract young scientists from all over Europe and beyond. Therefore, the organisers and Scientific Committee want to thank all of the sponsors of EUSAAT 2012 on behalf of the participants.

Finally, you may notice that for, the first time, the abstracts of an EUSAAT conference on the Three Rs are published by *ATLA*. We are most grateful that, at very short notice, the *ATLA* Editorial Office was able to edit and process the EUSAAT 2012 abstracts in a professional manner.

On behalf of the Organising Committee
Horst Spielmann
President, EUSAAT

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Recognising the Vision of Toxicity in the 21st Century By Implementing Stem Cell Derived Cardiomyocytes and Real-time Monitoring in Cardiotoxicity Testing

Y. Abassi, X. Xu and X. Zhang
ACEA Biosciences, San Diego, CA, USA

The vast majority of the drugs withdrawn from the market due to association with Torsades de Pointes (TdP) arrhythmia appear to interfere with the Ikr repolarisation current mediated through the hERG potassium channel. Consequently, the ICH S7B guidelines recommend that all new chemical entities should be subjected to the hERG repolarisation assay, typically using cell lines that recombinantly express hERG protein. However, in the last decade it has become evident that not all hERG channel inhibitors result in TdP and not all compounds that induce QT prolongation and TdP necessarily inhibit hERG. In order to better understand and assess the different kinds of drug liabilities associated with hERG channel inhibition and modulation, we have used a panel of drugs and compounds which (i) directly bind and inhibit hERG channel function (overt inhibitors); (ii) inhibit hERG, as well as other channels, and therefore compensate for the Ikr block (covert inhibitors) and (iii) interfere with the trafficking of hERG channel protein to the plasma membrane (trafficking inhibitors). We have assessed the activity of these compounds by using human induced pluripotent stem cell-derived cardiomyocytes (iCells), together with a system that can measure the beating activity of the spontaneously beating cardiomyocytes. Our data clearly show that overt hERG channel inhibitors disrupt the periodicity of beating of iCell cardiomyocytes, leading to plateau oscillations and a signature waveform that is typical of this class of compounds. Covert hERG channel inhibitors at physiological concentrations do not appear to affect cardiac function and therefore appear to be safe. hERG trafficking inhibitors display a time-dependent effect on the periodicity of beating that manifests several hours after compound dosing. In summary, the results clearly show that dynamic monitoring of iPS-derived cardiomyocyte beating can be used in a predictive way to assess various types of hERG channel modulators and provide additional information to electrophysiological methods. Furthermore, the use of human iPS-derived cardiomyocytes in this assay has the potential to reduce animal testing for cardiotoxicity assays.

Responsibility in Animal Experimentation: Assistance for Harm–Benefit Analysis in the Context of the EU Directive

N. Alzmann
Messerli Research Institute, University of Veterinary Medicine, Vienna, Medical University of Vienna, University of Vienna, Vienna, Austria

Harmonisation within the EU and, at the same time, the strengthening of the protection of animals still used in scientific procedures, were the grounds for and the objectives of the proposal for a directive on the protection of animals used for scientific purposes. This was presented by the European Commission on 5th November, 2008. One of the reasons why the former EU *Directive 86/609/EEC* (from 1986) needed to be revised was the lack of an obligation to evaluate a proposal from an ethical point of view. In the 2008 proposal, it was accentuated that a "comprehensive ethical evaluation of projects using animals […] forms the core of the project authorisation" (Recital 37), and that "an independent ethical evaluation should be carried out as part of the authorisation process" (Recital 38). *Directive 2010/63/EU*, adopted in September 2010, demands that a harm–benefit analysis should be undertaken, which should take into account ethical considerations (Article 38 (2), d). Animal protection and ethical considerations are not only a demand of the new directive. Scientists looking for good quality research data also strive for the ethical justification of their experiments. In

order to gain ethical justification, the scientist is in need of feasible methodology. However, the parties involved (i.e. those who file an application for animal experimentation, committee members, members of the authorising bodies, as well as animal protection appointees) are usually specialised only in the sciences. The following presentation will identify major shortcomings of the outlined process: a) the legal framework of many member states has so far not been specific — even the adopted EU directive does not offer an adequate support for the involved parties to evaluate scientific proposals in ethical terms; b) a desideratum of expertise has been observed. Researchers, as well as persons involved in the approval procedure of proposed animal experiments, are usually not adequately trained in ethics. At this point, it is not personal moral intuition and feelings that are demanded, but rather a systematic argumentation and a well-defined decision making process. Our project at the Messerli Research Institute focuses on this desideratum. A guideline to reflect the scientist's moral responsibility will be developed, as well as a feasible methodology to support harm–benefit analysis. Continuing a PhD research study, this project is built on the comparative analysis of a number of criteria catalogues for the assessment of the ethical justification of proposed animal experiments. The existing catalogues that have been developed elsewhere, are intended to overcome some of the shortcomings, to provide a basis for objective, transparent evaluation and, finally, to ensure the fair evaluation of project applications and a coherent standard of protection for experimental animals. An appropriate catalogue has to include all aspects that are ethically-relevant. A first draft of an improved catalogue will be presented.

A Combined *In Vitro* Assay Taking Into Account Binding and Enzymatic Activity, Allows Sensitive Detection of Active Tetanus Neurotoxin in Vaccines

H. Behrensdorf-Nicol, U. Bonifas, L. Boehl, B. Krämer and K. Weisser
Paul-Ehrlich-Institut, Langen, Germany

Tetanus toxoids (i.e. formaldehyde-inactivated preparations of tetanus neurotoxin) are used for the production of human and veterinary vaccines against tetanus. In order to exclude the presence of residual active toxin or a reactivation of the detoxified material during storage, toxicity tests in guinea pigs are prescribed by the European Pharmacopoeia for each produced bulk of tetanus toxoid. Our aim is to replace these mandatory animal tests for "absence of toxin and irreversibility of the toxoid", which require thousands of guinea pigs annually, by a suitable *in vitro* method. Tetanus neurotoxin (TeNT) consists of two disulphide-linked protein subunits: the heavy chain — which mediates the toxin binding and uptake by neurons, and the light chain — which cleaves the neuronal protein, synaptobrevin, and thus causes a spastic paralysis. Most *in vitro* assays for tetanus toxicity described to date have solely been based on the detection of the toxin's specific proteolytic activity. Such methods, however, are prone to false-positive results caused, for example, by interference of free TeNT light chains (which are proteolytically active, but non-toxic due to lack of a receptor binding domain). In order to better reflect the *in vivo* situation, we have developed a combined assay which also takes into account the toxin's receptor binding capability. In this assay, only TeNT molecules that display both receptor binding and synaptobrevin cleaving features on separate, disulphide-linked subunits, will generate a signal. Comprehensive optimisation studies have allowed us to markedly improve the sensitivity of this combined assay. A key improvement has been the addition of the osmolyte TMAO (trimethylamine-*N*-oxide) to the cleavage reaction, which strongly increases the toxin's enzymatic activity, and thus leads to an enhancement of the specific assay signals. The optimised combined assay is capable of detecting pure TeNT with very high sensitivity (below 0.1ng/ml), which is equivalent to, or even better than, the detection limit of the animal test. The combined assay allows the detection of TeNT, not only in pure buffer solutions, but also in spiked toxoid samples. The toxoid batches of some vaccine manufacturers induced high background signals, which might limit the applicability of the method for these batches. For the toxoids of the remaining relevant manufacturers, how-

ever, the method could represent a suitable alternative to the prescribed animal tests. An internal validation study has been performed, in order to define the applicability of this method for detecting toxicity in tetanus toxoids. The results of this study are presented.

Characterisation of Human Cornea Constructs for the Rapid Screening of Preservatives and Evaluation of Their Use in Permeability Assays to Reduce Animal Testing

G. Guzman,[1] S. Reichl,[2] U. Bock[1] and E. Haltner,[1]
[1]Across Barriers GmbH, Saarbrücken, Germany; [2]Institut für Pharmazeutische Technologie, Technische Universität Carolo-Wilhelmina zu Braunschweig, Braunschweig, Germany

Ocular diseases have dramatically increased in the last years. For instance, in the United States alone, 2.2 million people had glaucoma in 2010, and it has been predicted that this number will increase by 36%, by 2020. A large number of animals must be involved to investigate the usefulness of the drugs, or the effects of the excipients used. For this reason among others, *in vitro* cornea cell culture models have been developed. As a consequence, the aim of the present investigation was to assess the compatibility of the preservatives that are most commonly used, such as benzalkonium chloride (BAC), methyl paraben (MP), and thiomerosal (Thio). The second objective was to evaluate the impact of the compatible concentrations during the diffusion transport of the hydrophilic molecular marker sodium fluorescein (Na-FLU), the prostaglandin analogue bimatoprost (Bi), and the beta-blocker Timolol (Ti), through the pre-validated human cornea constructs (HCC). Pre-validated human cornea constructs, based on immortalised human cornea epithelial (HCE-T) and human cornea keratocytes cell lines, were cultured in serum-free KGM medium at 37°C and 5% CO_2. Different concentrations of the preservatives were dissolved in KRB buffer at pH 7.4 and added to the apical side of the HCC. Compatibility testing of the preservatives and the permeability studies of the substances were all performed at 37°C. Evaluation of the transepithelial electrical resistance (TEER) values suggests that BAC between 0.005% to 0.1%, MP between 0.05% to 0.1%, and Thio between 0.0005% and 0.02%, resulted in the modification of the TEER values to less than 400ohm.cm^2. These values indicate potential disruption/change of the epithelial barrier of the cells. The compatible concentrations of the preservatives were at 0.0005%, 0.001% and 0.001% for BAC, 0.001% for MP, and 0.00005% for Thio, because the TEER values of the cells remained at approximately 400ohm.cm^2 during the course of the investigation. No significant influence on diffusion transport was apparent for Na-FLU + BAC at 0.0005%, or for Na-FLU + MP at 0.001%, when they were compared to the P_{app} of Na-FLU alone. Significant difference, however, was apparent for Na-FLU + Thio at 0.00005%. Additionally, the non-compatible concentration of BAC (0.005%) significantly increased the P_{app} of Na-FLU alone. On the one hand, the statistical analysis of the intrinsic P_{app} of Bi indicates significant modification after being combined with BAC at 0.0005%, even when the P_{app} remained in the same order of magnitude. On the other hand, the diffusion transport of Bi in the presence of MP at 0.001%, and Thio at 0.00005%, was not significantly modified. We conclude that our characterisation of the *in vitro* human cornea model leads to the possibility of evaluating its potential for a larger test screening of new or modified ophthalmic preparations, in order to reduce animal experiments. Furthermore, the modification of TEER values demonstrated that the model can differentiate between compatible and non-compatible concentrations of the preservatives.

From Neuronal Activity to Brain Dynamics: Novel Insights into Epilepsy from Human *In Vitro* Studies

M. Cunningham and A. Simon
Newcastle University, Newcastle upon Tyne, UK

The use of human neocortical tissue for *in vitro* brain slice electrophysiological studies permits unprecedented access to the cellular and synaptic elements that determine the ability of neocortical microcircuits to generate organised electrical activity. Such human neocortical tissue is, in the majority of cases, removed during elective neurosurgery for the treatment of drug-refractory epilepsy. It is therefore presumed that this tissue contains the epileptogenic focus. Previous studies have failed to observe spontaneous epileptiform activity, and have reverted to artificially manipulating the recording conditions to bring about this behaviour. Work from this laboratory has focused on the use of electrophysiological, anatomical and computational modelling approaches to study spontaneous epileptiform behaviour in epileptic human brain slices. This presentation will highlight recent work concerning the mechanistic features of electrophysiological signatures previously only observed with *in vivo* recordings in epileptic human patients. One such pattern, termed 'very fast oscillation' (VFO; > 80Hz), is characterised by brief high frequency events usually associated with an inter-ictal sharp wave spikes. However, persistent runs of VFO can precede an ictal event. By using an *in vitro* approach, we have been able to characterise the mechanistic features of these events. Following on from these initial studies, we have also described a novel form of VFO in epilepsy patient electrocorticographic (ECoG) and brain slice recordings, that can occur prior to ictal events, in which the frequency increases steadily from ~30–40Hz to > 120Hz, over a period of seconds. We dub these events 'glissandi' and describe a possible model for them.

When is Animal Testing a Last Resort Under REACH?

A. Currie
People for the Ethical Treatment of Animals (PETA) Foundation, London, UK

The REACH Regulation contains many mechanisms to reduce numbers of animals used to fulfil information requirements, including the Annex XI provision that animal tests can be waived by using weight-of-evidence arguments. These arguments can include, *inter alia*, non-animal methods yet to be validated or OECD approved. Although the legal text contains explicit requirements for animal tests, it also requires that vertebrate testing is conducted only when all other options have been exhausted. In the context of Annex XI and other measures, this 'last resort' obligation means that information requirements for any endpoint can potentially be met without the use of animal tests and, where that is the case, animal methods in fact must not be used. The effectiveness of these provisions in ensuring the use of non-animal methods wherever possible, requires both commitment from registrants and effective monitoring in dossier evaluation. Contrary to the 'last resort' requirement, however, official publications and other sources of information show that avoidable testing has been conducted. Examples include instances in which specific REACH provisions have apparently been breached and obsolete animal tests have apparently been employed. In the case of weight-of-evidence arguments, expert judgment may be required, but possible examples of non-compliance in this context are also identified. Despite this evidence of breaches of the 'last resort' requirement, there has been a lack of action to address non-compliance. The reasons for this, including legal issues, policy decisions by the regulator, and areas of scientific uncertainty, are examined. As possible breaches of *Directive 2010/63* may also arise from the improper use of animal tests for REACH, measures are needed to inform and empower relevant national authorities to take action. Steps to ensure that the general requirement to avoid animal testing is fully complied with are identified.

Investigating the Interaction of Pyrogenic Contaminations with Medical Devices by Using a Human Specific *In Vitro* Assay

S. Fennrich,[1] K. Stang,[1] M. Post,[1] S. Stoppelkamp,[1] I. Burgener[2] and H-P. Wendel[1]
1University Hospital Tübingen, Clinical Research Laboratory, Clinic of Thoracic, Cardiac and Vascular Surgery, Tübingen, Germany; 2Clinic for Small Animals, University of Leipzig, Leipzig, Germany

Any medical device which will come into contact with blood needs to be tested for its haemocompatibility as well as pyrogenic activity, since contamination and surface contact can start pro-inflammatory reactions and the activation of cascades within haemostasis. The ISO 10993-4 regulates quality assessments for haemocompatibility and the interactions of medical devices with blood, whereby endotoxins interfere with haemostasis but are not specifically determined. Recently, a novel detection method for pyrogenic activity has been implemented into the European Pharmacopoeia (6.7, Chapter 2.6.30: Monocyte-activation test) as an *in vitro* substitute for the rabbit pyrogen test and supplement to the *Limulus* assay. While the *Limulus* assay detects endotoxins only, the novel PyroDetect detects all pyrogens, i.e. endotoxins and non-endotoxins such as Gram-positive and fungal pyrogens. Here, we demonstrate the suitability of this test to detect pyrogenic activity in various medical devices and raw materials. Slides made of clinical grade steel were tested for their haemocompatibility according to standard procedures in our GLP-certified laboratory, in compliance with ISO 10993-4. The monocyte activation test (MAT) was then used to detect pyrogenic activity on the steel slides and other medical devices (stents and stent systems). Critical steps were performed under sterile conditions in a laminar air flow hood. Briefly, the devices were transferred into pyrogen-free, 6-well plates and incubated with increasing concentrations of endotoxin (WHO standard, *E.coli* O113:H10:K) and freshly-drawn human whole blood. After overnight static incubation, the blood cells were pelleted and the supernatant fluid tested for its interleukin-1β content. Initially, clinical-grade steel, used as a raw material for many medical devices, was tested for its haemocompatibility and all tests showed no specific activation. The same material was then assessed in the MAT, where again no interference by the material was detected. Further devices were tested, directly in contact with the human whole blood as the sensor, and yielded good and specific recovery of pyrogens on bare metal stents and stent systems. The MAT proved to be ideal for detecting pyrogenic contaminants on medical devices. With its human-specific setup and detection of all pyrogens, it exceeds the spectrum of the *Limulus* assay, which detects only endotoxins. In addition, when using the MAT, the material to be tested is in direct contact with the 'sensor blood'. When using the *Limulus* assay, the contaminants need to be washed off the surface with an unknown recovery level. Therefore, we propose the implementation of the MAT into the ISO, in order to cover the current gap in detection of the whole spectrum of pyrogens.

In Vitro Assessment of Developmental Neurotoxicity by Using 3D cultures: Species Can Make a Difference

E. Fritsche
Leibniz Research Institute of Environmental Medicine, Düsseldorf, Germany

Neurospheres are three-dimensional (3D) cell culture models consisting of neural progenitor cells (NPCs), which proliferate in culture and can migrate and differentiate into neurons and glial cells, thus mimicking basic processes of brain development *in vitro*. Thereby, the neurosphere system is able to distinguish between positive and negative developmental neurotoxicity (DNT) test compounds. Comparison of effects in human *versus* rat neurospheres reveals species-specificities. Besides compound screening, we investigated the mechanisms of action of selected chemicals. For example, polybrominated diphenyl ethers (PBDEs) inhibit human neural differentiation by inhibiting cellular thyroid hormone signalling, while the mouse is less sensitive. Moreover, polycyclic aromatic hydrocarbons selectively inhibit mouse

neural migration by Arylhydrocarbon receptor (AhR) activation, while human neurospheres remain unaffected. AhR inhibition only inhibits mouse NPC proliferation. The lack of effects on human cells is due to an absence of AhR expression. Moreover, the Nrf2 pathway, which is necessary for NPC antioxidative defence, functions very effectively in proliferating NPC. This approach opens the possibility of causally connecting adverse outcome pathways (AOPs) with cellular functions in 3D *in vitro* systems. Our data show that neurospheres can be used for DNT testing in a medium-throughput way. Moreover, they are useful for identifying AOP-related chemical modes-of-action. In this regard, species comparisons are of great value for hazard assessment, as humans might be more, less or equally sensitive than their rodent counterparts.

Immortalisation of Urine-derived Renal Proximal Tubular Epithelial Cells: A Non-invasive Cell Source for Relevant and Standardisable Kidney Toxicity Testing and for Generation of Induced Pluripotent Stem Cells

J. Grillari,[1] R. Grillari-Voglauer,[1] L. Fliedl,[2] P. Jennings[3] and M. Esteban[4]
[1]Vienna Institute of BioTechnology (VIBT–BOKU), Vienna, Austria & Evercyte GmbH, Vienna, Austria; [2]Vienna Institute of BioTechnology (VIBT–BOKU), Vienna, Austria; [3]Division of Physiology, Innsbruck Medical University, Austria; [4]Key Laboratory of Regenerative Biology, Chinese Academy of Sciences, South China Institute for Stem Cell Biology and Regenerative Medicine & Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Guangzhou, China

Nephrotoxicity of chemicals or therapeutics can be caused by damage to all parts of the kidney, including the glomeruli and the tubuli. As many biomarkers of acute and chronic nephropathy depend on enzymes and proteins found in urine that derive from the proximal tubuli, proximal tubular injury seems to be an event of prime importance. Therefore, establishing human proximal tubular epithelial cell cultures is necessary for nephrotoxicity testing and for the screening of kidney-protective substances that can, for example, be co-administered during the chemotherapeutic treatment of cancer patients to diminish kidney injury. So far, the short replicative life span and the limited access to human renal proximal tubular epithelial cells (RPTECs) have limited their use in the field of *in vitro* toxicology. We recently established the telomerase-immortalised RPTEC/TERT1 cell line from kidney tissue that retains all functional characteristics of primary cells tested so far. Now we present that RPTECs can be isolated and immortalised from human urine. This non-invasive source of cells gives unlimited access to cells from any consenting individual, irrespective of health or disease status, gender, or age. The cells retain morphology, functionality and gene expression patterns similar to tissue-derived RPTECs, including the formation of domes indicative of intact transport and tight junctions, responsiveness to parathyroid hormone or formation of solitary ciliae. The cells also express gamma glutamyltransferase (GGT), an enzyme involved in cisplatin toxicity. Furthermore, these cells also represent a non-invasive source of cells for generation of induced pluripotent stem cells, as we were able to reprogram them to an embryonic stem cell-like state and then differentiate them to hepatocyte-like cells. Taken together, we present a novel non-invasive source of human, primary-like cells that represent a relevant and standardisable model system for toxicity studies.

Advanced Microfluidic-based *In Vitro* Models for Lung Research

O. Guenat
University of Bern, Bern, Switzerland

Current *in vitro* models do not prevent the occurrence of high attrition rates for new drug candidates in therapeutic areas, mainly because they are poor representatives of human metabolism. Similarly, data obtained with animal models often cannot be extrapolated to humans because they weakly predict adverse reactions in humans. The drug discovery R&D projects related to the respiratory system are no exception, with an extremely low probability of success. To tackle this problem, the ARTORG Lung Regeneration Technologies Lab focuses of the development of advanced *in vitro* models based on microfluidics that better reproduce the *in vivo* conditions of the lung. Those conditions are particularly complex (an air–liquid interface, respiratory movements, shear stresses induced by liquids over the epithelial layer and at the endothelium, etc), and are the reasons why accurate *in vitro* alveolar models do not exist to date. In sharp contrast to standard Petri dishes, in which cells are cultured in a static environment and are submerged in a large volume of physiological medium, microfluidic cell-based assays allow the mimicking of the shear stress generated by the blood perfusion, and the accurate delivery and removal of nutrients and waste products, respectively. Furthermore, microtechnology allows fabricating extremely thin stretchable membranes that can reproduce the mechanical stress induced by the respiratory movements. We will first report about a microfluidic wound-healing assay created using a focused flow of trypsin allowing for accurate modelling of epithelial microinjuries that are the trademarks of pulmonary fibrosis. Significant differences between standard *in vitro* techniques and microfluidic assays demonstrate that hepatic growth factor is a potent agent to accelerate alveolar epithelial regeneration, the key element affected in pulmonary fibrosis. In a second example, a microfluidic *in vitro* model equipped with a 10μm-thick polymeric and stretchable membrane, on which alveolar epithelial cells are cultured to confluence, is presented. In preliminary experiments, the epithelial layer was cyclically stretched over a 30-minute period, without any viability changes. Finally, a microfluidic chemoresistive platform using 3D cell culture with a continuous perfusion, which is a more appropriate model for the study of cell–cell interactions occurring in cancer, will briefly be discussed. Spheroids of malignant pleural mesothelioma, a lethal type of lung cancer with high chemotherapeutic resistance, were trapped in a microfluidic chip and exposed to different concentrations of drugs. Such models are deemed to be extremely important for the understanding of the pathophysiology of pulmonary diseases, as well as for *in vitro* toxicology studies. Furthermore, such models may also be implemented in personalised medicine assays.

A Possible Roadmap for Animal-free Systemic Toxicity Testing

N. Hasiwa,[1] M. Leist,[1] M. Daneshian[1] and T. Hartung[2]
[1]University of Konstanz, Konstanz, Germany; [2]Johns Hopkins University, Baltimore, MA, USA

Animal testing for the safety assessment of cosmetics is about to be banned in 2013. This has enhanced the development of alternative testing methods but, despite the huge efforts, the areas of toxicokinetics, repeated dose toxicity, carcinogenicity, skin sensitisation, and reproductive toxicity are still presenting further scientific challenges. Therefore the Centre for Alternatives to Animal Testing (CAAT–Europe) and the Trans-Atlantic Think Tank of Toxicology (t4) organised an expert workshop to take a larger perspective regarding the respective areas. A forward-looking strategy was discussed, taking into account latest scientific innovations, to develop a roadmap for systemic toxicity testing for cosmetics and other substances. Each area was intensively reviewed and the following recommendations were achieved: Exist-

ing testing approaches should be evaluated, applying an evidence-based approach to detect useless tests and strive towards their abolition. Key events should be identified, which could be tackled specifically, and information-rich single tests should be developed. The same approach could be used to identify useful tests that provide the information leading to the classification of a compound, and their optimisation should be boosted. The area of *in silico* approaches should be promoted further, and integrated into a larger context. The idea of Tox21c was embraced and the identification of Pathways of Toxicity (PoT) was considered the way forward. All these efforts should lead to Integrated Testing Strategies (ITS), which are scientifically sound and valid, to perform risk assessment toward an enhanced consumer safety without the use of animals.

A 3D-Cardiovascular Construct to Mimic the Properties of Native Heart Tissue for Safety and Efficacy Testing of Chemical Substances

T. Heinonen, H. Vuorenpää, L. Ikonen, J.R. Sarkanen, O. Huttala, K. Kujala, K. Aalto-Setälä and T. Ylikomi
University of Tampere, Tampere, Finland

The assessment of cardiac effects of compounds is one of the most critical areas in chemical safety testing, as well as in efficacy testing during drug development. Cardiotoxic substances can cause arrhythmias and heart failure, which may be fatal. As no relevant human cell-based heart tissue model exists, the testing is, at present, performed by using animal models. However, the results from animal models are not necessarily transferable to human situations. The cellular microenvironment is critical in biologically-relevant responses to drugs or other chemicals. Three-dimensional models have the ability to capture cell–cell and cell–matrix interactions that are lacking in traditional 2D *in vitro* models. Also, the communication between parenchymal and stromal cells makes 3D models ideal for replacement of equivalent animal testing. Although the general model for 3D applications relies on the use of exogenous scaffolds or extracellular matrix-based materials, scaffolds may elicit adverse host responses and interfere with cell–cell interactions, as well as cell assembly. Myocardial tissue consists of cells at high density — this is necessary for the generation of synchronous beating through gap junctions. The use of scaffolds in 3D cardiac models has been found to be associated with reduced cell–cell interactions, as well as inflammatory reactions. The cardiac tissue has a dense vascular network. Therefore, the *in vitro* cardiac models should also include vascular-like structures. In this study, we developed a natural-like, xeno-free *in vitro* cardiovascular construct, devoid of added biomaterial. Our initial aim is to show successful performance of the construct (i.e. proof of concept) with rat neonatal cardiomyocytes, after which the rat neonatal cardiomyocytes will be replaced by human embryonic stem cell-derived cardiomyocytes. A fully human cell-based functional cardiovascular construct is the final goal. In the construct, cardiomyocytes are seeded on top of a supporting and interacting platform of a vascular-like network. The intense formation of the vascular-like network formed by human adipose stromal cells (hASCs) and human umbilical vein endothelial cells (HUVECs), serves as a natural scaffold. Results show that hASCs and HUVECs self-assemble into a multilayered, 3D network, expressing a complete basement membrane and vessels supporting cells with contractile properties. It was shown that rat neonatal cardiomyocytes aligned with the vascular-like network and formed a synchronously beating construct with elongated functionality for at least up to 2 weeks. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (βFGF) were found to be critical for vascular-like network formation. A fully human cell-based functional 3D cardiovascular construct is under development. The developed cardiovascular construct with 3D properties will be an excellent model for assessment of the pharmacological and toxicological effects of compounds in a natural-like heart tissue environment. In addition, this approach provides a first step toward techniques applicable in cardiac tissue engineering.

The Consistency Approach in Lot Release Testing of Vaccines

C. Hendriksen,[1] B. Metz,[2] G. Kersten[2] and J. van der Gun[1]
[1]Netherlands Vaccine Institute, Bilthoven, The Netherlands; [2]National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Vaccine lot release testing is characterised by extensive use of laboratory animals, particularly to demonstrate product safety and potency. Successes have been achieved in replacing existing animal models by Three R methods ranging from cell-based methods and serological approaches, to the implementation of humane endpoints to remove lethality as the test parameter. However, progress is tedious, time-consuming and costly. A new paradigm in lot release testing of established vaccines (e.g. Tetanus and Diphtheria toxoid) is the consistency approach. This approach starts from the idea that subsequent lots of vaccine produced can be compared to an earlier (reference) lot (clinical, historical batch) with a thoroughly tested and well-defined profile of safety and efficacy/potency. The concept of consistency for lot release has come within reach, due to improvements in production and control — i.e. the vaccine starting material is better characterised (quality by design); production processes have been optimised and standardised; a tight protocol for in-process testing has been implemented; quality monitoring systems such as GMP and QA are now state-of-the-art, and pharmacovigilance is used for post-marketing surveillance; and, last but not least, new physicochemical and immunochemical techniques have become available. Consistency testing may lead to a significant reduction in animal use, since a narrow set of animal tests performed on each final lot, with questionable power to predict vaccine behaviour in the target populations, may be replaced by a battery of meaningful physicochemical, immunochemical, and eventual *in vitro* functional tests with enhanced capacity to measure equivalence to batches of proven safety and efficacy. The paradigm of consistency is an interesting strategy for vaccine manufacturers, in that it might also allow for a reduction in testing costs and a shortening of the testing period. The concept of consistency testing was adopted in 2011 by the European Partnership on Alternative Approaches to animal testing (EPAA), as a promising strategy to animal reduction. EPAA is a public–private partnership between the European Commission and industry. This presentation will provide an introduction of the consistency approach and discuss its advantages and limitations. The main focus of the presentation, however, will be on the aims, objectives and activities of EPAA's Vaccine Project on the Three Rs and consistency testing.

The Reconstructed Epidermis EST1000 Allows Sub-classification of Corrosive Chemicals *In Vitro*

J. Hoffmann,[1] T. Klein,[2] D. Fuchs,[2] O. Engelking[1] and H. Fuchs[1]
[1]CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany; [2]Advanced CellSystems GmbH, Troisdorf, Germany

The reconstructed human epidermis EST1000 (CellSystems, Germany) is used for *in vitro* skin corrosion, skin irritation, skin sensitisation and skin phototoxicity, as well as in a wide range of research and efficacy applications. The OECD Test Guideline 431 (TG 431) describes the test method for *in vitro* skin corrosion tests for reconstructed human epidermis, in order to classify substances as corrosive and non-corrosive. EST1000 has proven to reliably discriminate between these two classes of chemicals. The introduction of international packaging standards for the labelling of dangerous chemicals according to the United Nations Globally Harmonised System (UN-GHS) requires further classification of corrosive chemicals into the subcategories 'very dangerous' (class 1A), 'medium danger' (class 1B) or 'minor danger' (class 1C). The aim of this study was to prove that EST1000 is suitable for the sub-classification of corrosive substances. A set of more than 40 chemicals was tested with EST1000 following the standard skin corrosion protocol (as defined in OECD TG 431). These chemicals

included liquids and solids. We used a comparable number of non-corrosive, 1A and 1B/C chemicals. For sub-classification, the prediction model of EST1000 was refined in order to distinguish between 1A and 1B/C chemicals: non-corrosive = ≥ 50% viability after 3-minute exposure AND ≥ 15% viability after 60-minute exposure; corrosive (class 1A) = < 50% viability after 3-minute exposure; corrosive (class 1B/C) = ≥ 50% viability after 3-minute exposure AND < 15% viability after 60-minute exposure. We found 93.3% sensitivity and 92.9% overall specificity. The accuracy for corrosives and non-corrosives was 93.75%. The sensitivity for class 1A chemicals was 86%. No over prediction of non-corrosives as class 1A chemicals was observed. This study showed that EST1000 is a suitable test system for sub-classification of corrosive chemicals according to UN-GHS.

The Predict-IV project: Integrating Omic Data and Pharmacokinetic Modelling

P. Jennings
Innsbruck Medical University, Innsbruck, Austria

The overall aim of Predict-IV is to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase, by an intelligent combination of non animal-based test systems, integrated omics and pharmacokinetic modelling. This is achieved by harnessing technological advances in transcriptomics (TCX), metabolomics (MTX) and proteomics (PTX) and the utilisation of established *in vitro* methods serving as models of the kidney, liver and CNS. Here, I present data generated in the human renal proximal tubule cell line (RPTEC/TERT1), which was treated with the immunosuppressive nephrotoxic compound, Cyclosporine A (CsA), for up to 14 days. The analysis of each omic stream, and their integration together with pharmacokinetic modelling, provides deep insight into the molecular perturbations brought about by high concentrations of CsA. Moreover, primary pharmacology could be separated from toxicology. This experiment proves the concept that high-content, multi-omic data collected from simple *in vitro* systems provide much more useful information than classical cell death assays. We believe such a strategy has the potential to create a new paradigm in safety pharmacology.

Modern Approaches to Assessment of the Photo-toxicity (Photo-irritation) Potential of Substances and Formulations by Using Tiered Testing Strategy Combining Analytical and *In Vitro* Test Systems

H. Kandarova,[1] D. Jirova,[2] K. Kejlova[2] and M. Liebsch[3]
[1]MatTek In Vitro Life Science Laboratories, s.r.o., Bratislava, Slovakia; [2]National Institute of Public Health, Praha, Czech Republic; [3]Federal Institute for Risk Assessment, Berlin, Germany

The goal of photo-safety testing is to detect the adverse effects of substances and products in the presence of light. This type of testing is relevant for products that enter the skin via dermal penetration or systemic circulation. Photo-toxicity testing is required only for substances that sufficiently absorb UV-light in the wavelength range 290–700nm and where local and/or systemic exposure is foreseen. Studies on the extent of photo-stability, structure–activity relationships (SARs), and the generation of reactive oxygen species (ROS) after irradiation by UV and visible light, can provide additional indicators that substance or formulation may trigger photo-toxicity. However, the biological effect and bioavailability also need to be examined. For

photo-toxicity testing, a thoroughly validated *in vitro* method, the 3T3 Neutral Red uptake photo-toxicity assay (3T3 NRU PT assay), is validated and accepted internationally. This assay provides indication that a substance might cause photo-toxicity/photo-allergy or other photo-induced effect. While highly sensitive, this assay does not provide information on the bioavailability/accumulation of the substance in the skin or eyes. Therefore, more-advanced *in vitro* 3D tissue constructs are suggested as second tier tests, to exclude possible false positives from 3T3 NRU PT assays. Protocols have been developed for both systemic and dermal exposures. These advanced 3D models can be also used for the assessment of photo-potency and non-phototoxic doses of a phototoxin, whereby a product (e.g. a pharmaceutical) can be still used in human therapy after careful considerations of other possible side effects. The use of the testing strategy described above may significantly minimise risk of photo-toxicity effects, while still permitting the use of substances that would be excluded from further use by a single test. This testing strategy is applicable to pharmaceuticals, as well as consumer products/cosmetics, and helps to distinguish the possible hazards from the real risks.

Micro-Raman Spectroscopy — An Alternative Tool for the Non-invasive Analysis of Cellular States and Identification

S. Koch,[1] K. Schuetze,[1] H. Walles,[2] M.L. Hansmann,[3] M. Baquie[4] and K.H. Krause[5]
[1]CellTool GmbH, Tutzing, Germany; [2]University Würzburg, Würzburg, Germany; [3]University of Frankfurt, Frankfurt am Main, Germany; [4]Neurix SA, Geneva, Switzerland; [5]Universite de Geneve, Geneva, Switzerland

Animal experiments are, to date, important aspects of drug development and testing. Raman spectroscopy may become an alternative method to identify cell–agent interactions and cellular states, as indicators for the effects of chemicals or active agents. The technique is contact-free and non-destructive, and therefore an ideal tool for preservative quality control, e.g. of tissue-engineered products, but also very useful as a cell-based biosensor for cellular differentiation and cell reactions to chemicals. In this work, we describe the detection of different cancer cell types from neuronal tissue and from lymphoma cell lines, as well as the determination of viability states of cells as a possible indicator for cell–agent interaction. Tissue-engineered neuronal models consist of neuronal cells and neuronal stem cells, seeded on a semipermeable membrane. After several days of culturing, the tissue was fixed and cut into 10µm slices. The embedding medium was removed and the slides were rehydrated for Raman measurements. A second model, with glioma spheres, was generated and treated in the same way. Lymphoma cell lines were derived from B- and T-cell Hodgkin lymphoma and compared to a non-Hodgkin lymphoma cell line. For cell viability analysis, we combined a temperature induction of apoptosis and necrosis in the cell lines SAOS-2 and SW-1353 with flow cytometry and fluorescence labelling for apoptosis and necrosis. The results were compared to the standard fluorescence-based technologies. Analysis of Raman spectra was performed by Principal Component Analysis (PCA) and a Support Vector Machine (SVM) was trained to classify the unknown cells within the mixed population. Spectral analysis of neuronal cells and glioma cells revealed significant differences in the PCA. Spectral differences can be assigned to changes in protein and DNA content of the cells. With the aid of the SVM, a model could be generated for the classification of unknown spectra in the mixed neuronal and glioma cell model. Hodgkin and non-Hodgkin lymphoma can be discriminated by using Raman spectroscopy to determine the biochemical fingerprint. Viability states can be identified as viable, early apoptosis (as indicated by Annexin V-positive cells), necrosis and late apoptosis (double-positive staining). Raman spectroscopy is a new tool for cell analysis. The technology provides valuable information about various types of cell and tissue. The purely laser light-based method is reliable and efficient for cell and tissue characterisation, especially when standard methods lack the ability for safe identification. In stem cell research and tissue engineering, Raman spectroscopy may become a powerful supportive technology.

Transposition of *Directive 2010/63/EU*: Missed opportunities in Germany

R. Kolar and I. Ruhdel
Animal Welfare Academy/German Animal Welfare Federation, Neubiberg, Germany

Before November 2012, EU Member States must transpose *Directive 2010/63/EU* on the Protection of Animals Used for Scientific Purposes into national law. To this effect, the German government published its first proposals in January 2012. Instead of bringing forward a new, clearly arranged self-contained law on the use of animals for scientific purposes, the Government chose to implement the Directive by changing the existing animal welfare act and by creating a new legal ordinance specifically regulating animal experimentation. In large parts of the new German rules, the German Government closely followed the provisions laid down in the Directive. In addition, the Government maintained some provisions from the existing act that are stricter than those of the Directive: experiments on animals for the development or testing of weapons, ammunition and related equipment, as well as for the development of tobacco products and detergents, will be prohibited. Also, the so-called ethical committees that advise authorities regarding the licensing of animal experimentation applications will be maintained. Disappointingly, however, the new rules fail to make use of any of the instances where the Directive offers some scope in increasing the protection of laboratory animals. The present draft neither contains an unconditional ban on the use of great apes, nor on experiments that involve severe pain, suffering, or distress that are likely to be long-lasting. Even worse, there are points where the draft rules do not comply with the new Directive. Experiments subjecting vertebrates to lasting or repeated severe pain or suffering are explicitly allowed, if the results are expected to be of outstanding importance for the fundamental needs of humans or animals, including the solution of scientific problems. Only experiments causing permanent pain or suffering are prohibited, but even there, possibilities for exceptions exist. Also, one of the main issues of the EU-Directive is the promotion of alternative approaches with the ultimate aim of ending animal experiments. Germany has not transposed respective measures. The transposition process in Germany is not yet complete. The German Animal Welfare Federation not only has lobbied hard to achieve the best possible protection of laboratory animals during the revision of the EU-Directive, but is determined to continue its efforts. From our standpoint, where the Directive allows a degree of freedom in national implementation, this has to be used for the benefit of the animals. This means that experiments on great apes and procedures involving severe suffering have to be banned without exceptions. Also, all experiments must undergo a strict project authorisation process. Projects summaries of all projects and their retrospective assessment should be published. Presently foreseen exceptions, like continuing a simplified procedure, e.g. for regulatory required animal tests, should discontinue. Finally, the promotion of the Three Rs has to be enshrined in the new legal text.

Putting the Parts Together: Combining *In Vitro* Methods to Test for Skin Sensitising Potential

S.N. Kolle,[1] C. Bauch,[1] T. Ramirez Hernandez,[1] T. Eltze,[2] E. Fabian,[1] A. Mehling,[3] W. Teubner,[4] B. van Ravenzwaay[1] and R. Landsiedel[1]
[1]BASF SE, Ludwigshafen; [2]BASF Jobmarkt GmbH, Ludwigshafen, Germany; [3]BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany; [4]BASF Schweiz AG, Basel, Switzerland

Allergic contact dermatitis is a common skin disease that is elicited by repeated skin contact with an allergen. In the regulatory context, currently only data from animal experiments are acceptable to assess the skin sensitising potential of substances. Animal welfare and the EU Cosmetic Directive/Regulation call for the implementation of animal-free alternatives for

safety assessments. The mechanisms that trigger skin sensitisation are complex, and various steps are involved. Therefore, a single *in vitro* method may not be able to accurately assess this endpoint. Non-animal methods are being developed and validated and can be used as testing strategies that ensure a reliable prediction of skin sensitisation potential. In this study, the predictivity of three *in vitro* assays, one *in chemico* and one *in silico* method, addressing three different steps in the development of skin sensitisation, was assessed by using 54 test substances of known sensitising potential. The predictivity of single tests and combinations of these assays were compared. These data were used to develop an *in vitro* testing scheme and prediction model for the detection of skin sensitisers based on the protein reactivity and dendritic cell activation.

Predicting Eye Irritation of Agrochemical Formulations According to Different Classification Schemes by *In Vitro* Methods

S.N. Kolle,[1] A. Buechse,[1] T. Knieriem,[1] W. Mayer,[1] M.C. Rey Moreno,[1] A. van Cott,[2] B. van Ravenzwaay[1] and R. Landsiedel[1]
[1]BASF SE, Ludwigshafen, Germany; [2]BASF Corporation, Research Triangle Park, NC, USA

The bovine corneal opacity and permeability (BCOP) test has been adopted by OECD for the identification of ocular corrosive and severe irritants (GHS category 1) for single component substances and multi-component formulations. Further, human reconstructed tissue models have been suggested for incorporation into a tiered test strategy to ultimately replace the Draize rabbit eye irritation test (OECD Test Guideline [TG] 405), and we have previously shown the suitability of the EpiOcular Eye Irritation Test (EIT) to be used for the prediction of ocular non-irritants (GHS no category). The purpose of this study was to evaluate whether the BCOP, including corneal histology and the EIT, could be used to predict eye irritancy of agrochemical formulations according to different classification schemes including UN GHS, EPA and Brazilian systems. We have compared data on opacity, permeability and corneal histology in the BCOP assay and relative tissue viability in the EIT, for 50 agrochemical formulations, with available *in vivo* eye irritation data. Use of the OECD TG evaluation scheme for opacity and permeability in the BCOP did not prove predictive with respect to severe eye irritation potential for the 50 agrochemical formulations assessed here, while corneal histology grades and the EpiOcular tissue viabilities were useful predictors of eye irritancy potencies. Further, we describe here the statistical evaluation based on the experimental *in vitro* data to predict eye irritancy for the different classification schemes.

Understanding the Lengthy Process to Replace the Draize Test

M. Kooijman,[1] D. van der Zwan,[1] P. van Meer,[2] E. Moors,[1] H. Schellekens[2] and M. Hekkert[1]
[1]Innovation Studies, Copernicus Institute of Sustainable Development, Utrecht University, Utrecht, The Netherlands; [2]Utrecht Institute of Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands

The urgency of the transition to replace animal tests in the safety assessment of cosmetics, pharmaceuticals and chemicals was triggered by societal resistance to animal testing and the scientific dispute concerning the value of animal testing. Since the 1980s, the European Union (EU) has developed policies to stimulate the development of innovative methods to replace animal studies. However, for regulatory testing, these policies have not been very effective, since only a few regulatory safety tests in animals (among which the Draize test, skin sentisation test) have been (partly) replaced by innovative methods. The few 'successful' replacement processes were laborious and took decades. In order to understand why transitions toward the replacement of animal tests in the regulatory safety assessment take so much time, these transitions need to be systematically studied, taking into account all stakeholders

involved in the development and acceptance of innovative methods. We used a framework, called the combined Technological Innovation System–Multi Level Perspective (TIS–MLP) framework, to systematically study transitions, and thereby elucidate the mechanisms that complicate them. We focused on the lengthy transition toward innovative methods for the Draize test. Eye irritation testing can be considered a pioneer in the development and validation of innovative methods to replace animal tests. Several innovative methods have been developed since the early 1980s, and major multi-laboratory validation studies were undertaken as early as the 1990s. It took until 2004 before a thorough review was carried out to advance the validation of innovative methods. Finally, two tests were approved by the OECD in 2009, to partially replace the Draize test — the Bovine Corneal Opacity and Permeability assay (BCOP) and the Isolated Chicken Eye (ICE) test. Both of these tests were already published in 1985, well over 20 years before regulatory acceptance. This study elucidates why this transition was so lengthy. Based on the combined TIS–MLP analysis it can be concluded that, despite the EU policy to stimulate the development of innovative methods and societal resistance, there was initially a lack of resources to further develop innovative methods. The EU ban on the use of animal tests for cosmetics was the key to solving this issue. Without this pressure on manufacturers, there was a lack of incentives for them to invest in innovative methods. In a later stage, the innovation process was impeded due to a lack of guidance with respect to the validation process and unrealistic validation endpoints. In none of the six validation studies, were innovative methods approved. Years of successful use of the Draize test made it the 'gold standard' in the validation studies. The performance of the innovative methods was judged in relation to the Draize test, not taking into account the actual flaws of that test. Due to the pressure of the EU ban on animal tests for cosmetics, it was considered necessary to change the validation strategy. Finally, only a review of the six validation studies finally convinced the OECD to accept the ICE and BCOP in 2009.

Immortalisation of Primary Human Alveolar Epithelial Cells: Development of a New *In Vitro* Model of the Air–Blood Barrier

A. Kühn,[1] D. Wirth,[2] T. May,[2] H. Huwer,[3] C.M. Lehr[4] and N. Daum[4]
[1]Helmholtz-Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany; [2]Gene Regulation and Differentiation (RDIF), Helmholtz-Centre for Infection Research (HZI), Braunschweig, Germany; [3]SHG Kliniken, Klinik für Herz- und Thoraxchirurgie im Herzzentrum Völklingen, Völklingen, Germany; [4]Drug Delivery (DDEL), Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Saarbrücken, Germany

The aim of this project is the establishment of a human *in vitro* cell model of the air–blood barrier, with prolonged or infinite life span simultaneously retaining the characteristics and barrier properties of primary alveolar type I (ATI) cells. Up to now, there is no cell line available that reflects these crucial features. Primary human alveolar epithelial cells (hAEpC) are an appropriate tool to mimic the air–blood barrier *in vitro*. However, their limited availability, as well as their time- and cost intensive isolation procedures, in addition to ethical concerns, limit their availability and applicability. According to the Three Rs principles (Replacement, Reduction, Refinement) for minimising the use of animal testing, the development of a novel *in vitro* model of the air–blood barrier is therefore of utmost importance. The model is based on the immortalisation of hAEpC via lentiviral vectors, in combination with a set of more than 33 genes with immortalising capability. After transfection and subsequent passaging, promising cell lines undergo characterisation regarding their epithelial origin, the expression of lung cell-specific markers and their barrier properties. The lines are analysed by morphological studies, immunofluorescence staining techniques, real-time PCR and transepithelial electrical resistance (TEER) measurement. Furthermore, the culture conditions are optimised and transport studies with model substances are carried out. Instead of using only classical transformation genes like hTERT or SV40LTAg, so-called 'mild' proliferators were used to prevent de-differentiation of the transformed cells. Thereby, seven human cell lines with prolonged lifespan could be generated by the lentiviral transfection approach and are currently under investigation regarding their cellular identity and retention of *in vivo*-like characteristics. Three cell lines showed

expression of tight junctions in fluorescence microscopy studies, of which one cell line developed the requested barrier properties (TEER values > 500Ω.cm^2). The generated immortal alveolar cell line with barrier properties reflecting the *in vivo* situation of the air–blood barrier will serve as an *in vitro* model for various applications. This system will allow standardised toxicity and transport studies for newly developed compounds and delivery systems. Additionally, it will help to elucidate infection pathways across the respiratory tract, in the context of aerosol-transmitted infectious diseases (e.g. swine flu, tuberculosis, etc), and will facilitate the discovery of drug compounds capable of treating these diseases.

Microsensors for Monitoring the Functions of Liver Cells in a Modular-based Microfluidic Polymer System

J.B. Larsen,[1] S. Prill,[2] M. Jäger,[2] L. Barbe[1] and S. Generelli[1]
[1]CSEM SA, Landquart, Switzerland; [2]Fraunhofer IBMT, Potsdam-Golm, Germany

The European project, HEMIBIO, aims to develop a hepatic 3D microbioreactor, reproducing the complexity of the liver for preclinical long-term toxicity testing. One of the major challenges in building a 3D-liver bioreactor is the lack of data on the complex environment present inside the bioreactor where the cells live, aggregate and differentiate. Most often, physiological samples are aliquoted to determine hepatic function and metabolism, which is a time-consuming and often destructive process, particularly when the sample volumes are small. In these circumstances, microsensors connected to the cell chamber by an automated microfluidic system can play a vital role, by monitoring cell culture conditions. This, in turn, allows the cell culture to be viable and stable over a longer period of time, thus enabling long-term toxicity testing. A modular approach for the sensors has been selected, which allows sensors to be changed, according to the toxic insults, and also allows faulty sensors to be replaced. Independent modules for cell culture and sensing will be connected through a microfluidic switchboard. The sensing module will contain an array of differently functionalised microsensors, which will integrate alanine-aminotransferase (ALT), urea, NH_4^+, lactate and pH electrochemical microsensors. ALT, a specific marker of hepatocyte injury, and urea, which is related to the urea cycle, have been chosen as highly specific markers of liver cell metabolism. The NH_4^+ sensing will be complementary to urea and serve to distinguish between parenchymal and non-parenchymal metabolism. The activity of ALT is determined indirectly, by measuring the level of glutamate. pH, lactate and oxygen sensors are aimed at monitoring the general status of the cell culture. Oxygen content will be measured by phosphorescence from a sensor integrated into the bioreactor structure, in order to register the oxygen content in close proximity to the cells. All microsensors will be interrogated at specific times and will provide crucial information related to the current cell conditions. The modular concept will not only allow replacement of faulty sensors during cell cultivation if needed, but also will facilitate the implementation of additional sensors that are able to discriminate specific patterns of liver injury, such as cholestasis and steatosis. The developed sensors will be validated against commercial references and assay kits, to ensure proper calibration and reliable readout of the sensors. Oxygen, glucose and ALT sensors have demonstrated a linear response, with a limit of detection at approximately 2% O_2 in cell culture medium, 2mM glucose in used cell culture medium, and 5mM glutamate in buffer solution, respectively. The pH and NH_4^+ sensors show a logarithmic response, with a dynamic range and sensitivity of about pH 4–8 and –20mV/dec and pK 5–1 and –59mV/dec, respectively. Experiments are currently ongoing in order to achieve better sensitivity and a lower limit of detection for the amperometric glucose and ALT sensors. Improving sensor performance will enable faster identification of changes in cell performance. The development of new long-term reliable sensors will allow repeated time point measurements or potentially real-time measurements, and provide insights into the kinetics of toxic insults.

Pre-validation of the PCLS *Ex Vivo* Model for the Prediction of Respiratory Toxicology

L. Lauenstein,[1] A. Hess,[2] S. Vogel,[2] X. Schneider,[3] C. Martin,[3] R. Pirow,[4] M. Liebsch,[4] R. Landsiedel,[2] A. Braun[1] and K. Sewald[1]
[1]Fraunhofer ITEM, Hannover, Germany; [2]BASF SE, Ludwigshafen, Germany; [3]RWTH, Aachen, Germany; [4]Federal Institute for Risk Assessment, Berlin, Germany

For acute inhalation toxicity studies, animals inhale chemicals at certain doses. In particular, at the beginning, it is difficult to estimate non-toxic doses for *in vivo* inhalation studies. In the context of REACH and the principles of Three Rs, there is an increasing public demand for alternative methods. The goal of this BMBF-funded project is the standardisation and prevalidation of precision-cut lung slices (PCLS) as a suitable *ex vivo* alternative approach to replace pre-studies for inhalation toxicology. The project is conducted in three independent laboratories (Fraunhofer ITEM, BASF SE, RWTH Aachen), together with BfR providing support in validation and biostatistics. In all participating laboratories, PCLS were freshly prepared and exposed to five increasing concentrations of industrial chemicals in serum-free DMEM under standard submerged cell culture conditions for 1 hour. After 23 hours of post-incubation with serum-free DMEM, chemical-induced toxicity was assessed by the release of lactate dehydrogenase by using the LDH assay, and by determination of metabolic enzyme activity by using the WST-1 assay. In addition, PCLS protein content and pro-inflammatory cytokine IL-α were measured with the BCA assay and ELISA, respectively. For all endpoints, a sigmoid dose-response model was fitted to the data and EC50 values were calculated. In addition, based on the variability obtained in positive and negative controls and test samples, test acceptance criteria were established for each endpoint. This study shows the results of the first six tested substances out of 20. In all laboratories, concentration-dependent toxicity could be shown for aniline, glutaraldehyde, Triton X-100 and paracetamol, but not for lactose and methyl methacrylate with the WST-1 and LDH assays. EC50 values obtained for the WST-1, LDH and BCA data were very similar in all participating laboratories. No increase in IL-1α level was observed for these chemicals. We conclude that local respiratory toxicology, including irritation and inflammation induced by chemicals, could be tested with comparable results in the PCLS model without *in vivo* experiments in three independent laboratories. The standardisation of the PCLS method was successful and the reproducibility of the results is very promising after the testing of the first six substances.

Use of Normal Human Three-dimensional (NHu-3D) Tissue Models (EpiDerm, EpiAirway) for Nanotoxicology Applications

S. Letasiova,[1] P. Hayden,[2] A. Armento,[2] V. Karetsky,[2] S. Ayehunie,[2] Y. Kaluzhny[2] and M. Klausner[2]
[1]MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia; [2]MatTek Corporation, Ashland, MA, USA

Nanomaterials are increasingly utilised in numerous commercial applications where dermal contact, inhalation, or oral ingestion is likely. However, their toxicological properties are largely unknown. Potential adverse effects of nanoparticle exposure include allergenicity, cytotoxicity, and genotoxicity. Nanomaterials may enter the body by interacting with, and eventually crossing, epithelial barriers, including the skin, airway, and intestinal epithelium. Once inside the body, additional interactions with internal organs such as the heart, liver, brain, kidney, and others, are possible. Therefore, there is an urgent need for animal alternative tissue models that can be utilised for the toxicological evaluation of nanoparticle materials. This lecture summarises results from experiments with *in vitro* NHu-3D skin (EpiDerm, EpiDerm-FT) and airway (EpiAirway) models and well characterised

nanomaterials. By using confocal microscopy, we observed the penetration of fluorescently-labelled polystyrene nano-beads into NHu-3D tissue models, EpiAirway and EpiDerm; significantly higher penetration of 49nm *versus* 100nm particles was observed. In addition, comet assay genotoxicity experiments showed dose-dependent increases in percentage tail DNA after treatment of EpiDerm tissues with: a) single wall carbon nanotubes (1–4nm), Fullerenes C60 (average particle size = 120nm), ultra-fine titanium oxide (3–10nm), and crystalline silica (average particle size = 450nm). These studies and others already in the literature demonstrate that *in vitro* NHu-3D models are useful tools for the study of nanoparticle interactions and potential toxicologic effects on epithelial tissues.

Development and Validation of Alternative Approaches According to Article 47 of *Directive 2010/63/EU*: Validation at Stake?

M. Liebsch, M. Oelgeschläger and A. Luch
Federal Institute for Risk Assessment, Berlin, Germany

It took 15 years from the first validation concepts (Amden I) to international consensus on the OECD Guidance Document No. 34 (GD 34) on the principles and procedures of validation and acceptance of new toxicological test methods in 2005. Interestingly, during the process of harmonisation at the OECD level, finally everyone agreed that these principles and procedures are relevant for all new methods, including new animal tests, though they were originally only defined to assure that the validity of new alternatives is assessed with a high level of scrutiny. GD 34 is based on real experiences with prospective and retrospective validation studies and, since reflecting fundamental principles applied in science, it is used as a comprehensive template also for other areas. Six years after publication of GD 34, the world has changed. The EU REACH chemicals legislation and the 7th Amendment of the Cosmetics *Directive 76/768/EEC* are calling for avoidance of animal testing, in the latter case even for a total ban. Moreover, publication of the concept of 'Tox21' by the US Research Council has led to research funding programmes with a drastic increase of *in vitro* testing intended to be used in the regulatory context. Finally, new toxicological concerns are calling for new tests for regulatory use that will require validation. This pressure has speeded up formal consolidation processes for method acceptance, both at the EU and OECD level — shortened commenting periods and consolidations in written procedure. However, is this only streamlining a far-too-slow process, or is the process of thoroughly evaluating the validity of new assays at stake? Our answer is "Yes, scrutiny is at stake". For the 'Tox 21' approach, we do not yet have an answer as to how validity may be assessed in the end. For the 'classical' approach (validation of single methods), we do have procedures that have been successfully proven to work. However, although *Directive 2010/63/EU* on the protection of animals used for scientific procedures requests, in Article 47, Commission and Member States to share the burden of validation, in EU Framework Programmes, only R&D of alternatives are being funded. The 2011 call for validation laboratories from ECVAM/EURL clearly states that, apart from help in management and chemical distribution, not much support will be given by the European Commission. How about the Member States? In Germany, we do have five programmes for funding alternatives at the federal and local state level, and one by a foundation, all of them supporting R&D exclusively. Only the largest programme also funds assessments up to the level of prevalidation. Even if a German laboratory applied for national funding to participate in an EU co-ordinated validation study, national funding procedures and timelines would not allow this technically to happen. Shall we really leave validation up to those who might have a conflict of interest and therefore work for free? Has the saying "He who pays the piper calls the tune" become less true? We make a passionate plea for spending a few percent of R&D funding for well-designed and well-managed validation studies!

New Devices for Model Biological Barriers in Nanotoxicology, Nanomedicine and Inhalation Studies

M. Liley,[1] C. Jud,[2] F. Leonard,[3] E. Collnot,[3] S. Ahmed,[1] B. Rothen[2] and S. Angeloni[1]
[1]CSEM SA, Neuchatel, Switzerland; [2]Adolphe Merkle Institute, University of Fribourg, Marly, Switzerland; [3]Helmholtz Institute for Pharmaceutical Research Saarland, Germany

Recent developments in *in vitro* models of biological barriers have resulted in a number of new and highly relevant model biological barriers (BBs) that are either commercially available (e.g. Mucilair, Episkin), or have been described in the literature. However, new devices are needed in order to exploit these models to their full potential. We are exploring the use of ultrathin microporous cell supports for *in vitro* BB models, especially in the fields of nanotoxicology and nanomedicine. Studies of the transport or translocation of species across model BBs is highly problematic for these fields: very slow transport, or even no transport, of nanomaterials across BBs is commonly observed. However, this has frequently been found to be due to the polymeric microporous support, which may block or slow transport — even in the absence of cells! We have therefore tested novel ceramic (silicon nitride) supports made by using silicon microfabrication techniques. With a thickness of 0.5 microns, these supports are at least 20-times thinner than commonly used commercial supports, and allow much faster transport of nanoparticles than commercially available microporous inserts. We have shown good cell growth and differentiation to give tight epithelial layers on these supports. Not only simple epithelial layer growth, but also complex co-culture models have been successful, with more physiological geometries than can be obtained by using polymeric culture supports. Different devices have been developed for use with the ultrathin ceramic supports. A special holder allows them to be used like a commercial insert, while a small bioreactor means that they can be incorporated into a fluidics system. Studies of nanoparticle transport across, and distribution within, these models are now underway. Improved tools for transepithelial electrical resistance (TEER) measurements are also necessary. TEER measurements can be used both as a toxicological endpoint, and also for quality control of *in vitro* model BBs. TEER measurements are routinely carried out for models in a liquid/liquid environment. However, to date this has not been possible for lung models at the liquid/air interface. An approach to this problem, together with initial results, will be presented.

The Bionas Discovery™ 2500 System — An Alternative Replacement Method for the Cytosensor Microphysiometry to Identify Eye Irritants

C. Lohaus, A. Kob, R. Ehret and S. Frech
Bionas GmbH, Rostock, Germany

Previously, in a retrospective analysis, the ECVAM scientific advisory committee chose the Cytosensor Microphysiometry (CM) method as one of several replacement methods for the Draize eye irritation test. The CM system was developed in the early 1990s, and provides an online measurement capable of detecting the acidification of medium caused by cellular activity. As the instrument is not longer manufactured, other technologies that use similar principles of measurement may be regarded as replacement alternatives. In the current study, which is based on the validated INVITTOX protocol 130 for the CM system (a modified version of protocol 102), the CM method was adapted to the Bionas Discovery™ 2500 (B2500) system. This sensor-based multiparametric *in vitro* technology works on a similar principle. The system monitors physiological parameters of living cells in a label-free and non-invasive assay (acidification, respiration, cell impedance/adhesion). The detection of extracellular acidification is one parameter used to determine acute rates of cellular energy metabolism, and pro-

vides essential information on cellular changes on response to external stimuli. By using different chemicals, which were tested with the CM system and categorised as eye irritants, the method was adopted for the B2500 system. In addition, parallel studies with the CM method and the B2500 system were performed and MRD50 (metabolic rate decrement) values were determined. Finally, the measured MRD50 values were correlated with the historical data provided by other studies (e.g. COLIPA). The results obtained revealed comparable results between the two systems on the one hand, and showed that the MRC50 values were comparable to the historical CM data from other studies on the other hand. The MRD50 values obtained with the B2500 system in this proof-of-principle study were highly similar and had similar accuracy to the validated CM system. Furthermore, the multiparametric system provided two additional physiological parameters, namely respiratory activity and cellular impedance, gaining deeper insight into the metabolic and cellular activity of the cells after external stimulation. Therefore, the multiparametric Bionas Discovery™ 2500 system may be regarded as an alternative replacement method in the near future.

Working Together Toward Successful Transposition

S. Louhimies
The European Commission, Directorate-General for the Environment, Brussels, Belgium

Around 12 million animals are used on a yearly basis in scientific procedures in the EU today. The majority of these are used in the field of pharmaceuticals in applied research, as well as during product development, production and testing. Current scientific knowledge does not yet allow us to replace the use of animals for scientific purposes, which is our ultimate goal. Therefore, the EU has recently strengthened the legislation in this field. A new Directive on the protection of animals used for scientific purposes, replacing *Directive 86/609/EEC*, came into force in November 2010. Member States of the EU have until November 2012 to transpose the requirements of the Directive into their respective national legislation, and it will take full effect on 1 January 2013. The main innovations of the revised Directive include a requirement for a systematic project (ethical) evaluation and a project authorisation. The scope of the Directive is enlarged to include under its protection specific invertebrate species and fetuses of mammalian species in their last trimester of development, as well as animals used for the purposes of basic research, education and training. It sets minimum standards for the housing and care of the animals and puts far greater emphasis on improved transparency and enforcement through regular, risk-based inspections. The principles of the Three Rs — the replacement, reduction and refinement of the use of animals in experiments — is the corner stone of the new Directive. The Three Rs are embedded throughout the text and must be taken fully into account during all aspects of animal use and care. The requirement to use a non-animal method instead of one using animals, when such method is recognised by the EU legislation, is clear and explicit in the legal text. Finally, more resources are foreseen for the development, validation and application of alternative methods. To ensure that the aims set for the new Directive are achieved, there needs to be a clear understanding of the legal requirements by all those affected by it. A number of topics were identified following the adoption of the Directive which would benefit from further discussion among experts to facilitate uniform transposition and application of the new Directive. These topics include, *inter alia,* the development of a statistical reporting format for the use of animals; how the creation, breeding and use of genetically altered animals are to be considered under the new Directive; the development of a framework for mutually recognisable education and training system, as well as the development of a severity assessment framework. Significant progress has been made and a number of results are already available for the benefit of a wider audience. The fruits of the revised Directive can only be harvested following a close co-operation between all those affected by the legislation, together with a committed approach to its complete and correct application. The EU can claim to have the highest standards of experimental animal welfare in the world, whilst promoting high quality, competitive science and research in Europe.

A Triple Culture Model to Study Inhalation Toxicology

C. Pohl
Universitätsmedizin Mainz, Mainz, Germany

Particles and toxins can enter the body by passing through biological barriers. The air–blood barrier formed in the respiratory tract is a main portal for inhaled substances. Bronchial epithelial cells represent not only the first line of defence, but also one of the first barriers encountered by inhaled substances. Two of the essential elements of the bronchial protection are coordinated ciliary movement and mucus lining fluid. To form a functional barrier, tight junctions (TJ) and adherens junctions (AJ) have to be generated and maintained between adjacent cells. In order to come closer to the *in vivo* situation, we cultured primary normal human bronchial epithelial cells (HBEC) with lung fibroblasts as a bilayer on a Transwell filter plate, maintained at an air–liquid interface. Barrier properties and morphological phenotype were compared over 28 days. Mucus production and cilia formation developed within 21 days. Additionally, these cilia showed a beat frequency of about 16 to 20Hz. The differentiated state of the bronchial cells was maintained for up to three months, correlating with average transepithelial resistance values of between 600–800Ω.cm^2 and prolonged ciliary beating. Approximately 600–800 dendritic cells (DCs) per mm^2 are located in the bronchial tract and are responsible for the initial immune response in the lung. Therefore, we stimulated monocytes with various cytokines and other relevant stimuli (IL-4, GM-CSF, TNF-α, Ionomycin) for 7 days to induce differentiation, before they were added to the bronchial model. The DCs incorporated into the bronchial epithelium without modifying the barrier properties of the model. In patients with altered mucosal permeability, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which contribute to the proinflammatory cascade, are both elevated. To mimic an affected barrier function, both bronchial cell types in co-culture were exposed to the proinflammatory cytokines TNF-α and IFN-γ, in concentrations found in cystic fibrosis patients. In the untreated triple culture, cell–cell contact proteins, like ZO-1 and occludin, were located between adjacent cells, whereas cells stimulated with the cytokines showed discontinuities in the expression pattern in the epithelium. DCs seem to be of importance in the model. It appears that they migrate to the damaged cell–cell contacts and try to compensate for the damage. Moreover, significant differences in the release of proinflammatory markers, including IL-6 and IL-10, could be shown between cultures with and without added DCs. In summary, as dendritic cells play an essential role in the pathomechanisms of lung injury, we incorporated them into a triple culture model of the upper respiratory tract. Our data indicate that they exert protective effects in our stimulated model *in vitro*. Current work is studying their possible modulatory role in the prevention of damage by inhaled particles.

The Use of the SHE Cell Transformation Assay in Hazard and Risk Assessment for Industrial Chemicals under REACH

A. Poth
Harlan Cytotest Cell Research GmbH, Rossdorf, Germany

The prediction and assessment of carcinogenicity of chemical compounds is an essential step in their development. The 2-year bioassay is the standard method for carcinogen detection, and this assay is time- and resource-intensive. Many short-term tests, especially genotoxicity tests, have been developed to aid the identification of potential carcinogens. However, the end-point of these systems is genotoxicity, and the concordance between rodent bioassays is only about 60%. A battery of short-term genotoxicity tests cannot improve the overall concordance. *In vitro* cell transformation tests with SHE, Balb/c-3T3 and Bhas 42 cells, simulate the process of animal two-stage carcinogenesis. These tests are suited for the *in vitro* detection of the carcinogenic potential of test compounds in safety and risk assessment. Results from cell

transformation assays can provide information that, in combination with data from other testing methods, is useful for identifying the carcinogenic potential of chemical compounds. The SHE cell transformation assay results were used in combination with other information such as genotoxicity data, structure–activity analysis, *in vivo* toxicity data and pharmacokinetic/toxicokinetic information, to facilitate a relatively comprehensive assessment of the carcinogenic potential of a chemical. The SHE cell transformation data were used in order to gain additional information when the biological significance of the bioassay results was uncertain, for clarification of the meaning of positive results from genotoxicity assays in a weight-of-evidence assessment, for compounds classes where genotoxicity data have only limited predictive capacity, for investigation of compounds with structural alerts for carcinogenicity, to demonstrate differences or similarities across a chemical category, and for investigation of the tumour-promoting activity of chemical compounds. The presentation will provide some examples where the SHE cell transformation assay was used in the hazard and risk assessment of chemical compounds.

A Testing Strategy for the Identification of Mammalian, Systemic Endocrine Disruptors, with a Particular Focus on Steroids

T. Ramirez Hernandez, S.N. Kolle, H. Kamp, R. Buesen, B. Flick, V. Strauss and B. van Ravenzwaay
BASF SE, Ludwigshafen, Germany

Most endocrine disruptors interact with hormone receptors or steroid biosynthesis and metabolism, thereby modifying the physiological function of endogenous hormones. Here, we present an alternative testing paradigm for the detection of endocrine modes-of-action, that replaces and reduces animal testing through refinement. Receptor-mediated endocrine effects were assessed by using yeast-based receptor-mediated transcriptional activation YES/YAS assays, and effects on steroid hormone biosynthesis were assessed by using the human cell line H295R in the steroidogenesis assay. In our testing paradigm, we propose to complement the *in vitro* assays with a single *in vivo* repeated dose study, in which plasma samples are analysed for their metabolome profile in addition to classical parameters such as histopathology. The combination of these methods not only contributes to refinement and reduction of animal testing, but also has significantly increased the efficient allocation of resources and allows for a sound assessment of the endocrine disruption potential of compounds. Thus, this proposal constitutes a potentially attractive alternative to EPA's Endocrine Disruptor Screening Programme to identify mammalian, systemic endocrine modes-of-action. Data on 14 reference substances, for which the *in vitro* YES/YAS and steroidogenesis assays and the *in vivo* metabolome analysis were performed to assess their putative endocrine modes-of-action, are presented here.

Requirement for a Comprehensive EU Strategy to Decrease Animal Testing

K. Reid
EWLA/Eurogroup for Animals, Brussels, Belgium

We are concerned that new legislative proposals continue to promote animal testing. Therefore as an animal welfare NGO, we believe that there is a need for a comprehensive EU strategy to be set up, to ensure that the number of animals used will decrease and ultimately be fully replaced by alternatives. As part of such a strategy, any impact assessment prepared by the Commission should consider the effect on the number of animals used, as a result of intro-

ducing a new policy. This would avoid the situation where new policies, such as on cloning for food, genetically-modified food and feed testing, and nanotechnology, would increase animal use.

'Human-on-a-chip' Concepts to Implement *In Vitro* Repeated Dose Substance Toxicity Testing Predictive of Human Exposure

M. Rosowski and U. Marx
TU Berlin — Institute of Technology, Germany

According to a recently published 'roadmap', systemic toxicity testing forms the cornerstone for the safety-evaluation of substances. Pressures to change from traditional animal models to novel technologies arise from the limited prediction of human health effects and animal welfare considerations. This change requires human organ models combined with the use of new technologies in the field of '-omics' and systems biology, as well as respective evaluation strategies. *In vitro* organ emulation needs an appropriate model for each organ system, i.e. what makes a heart a heart, a liver a liver, etc. In this context, it is important to consider combining such organs into systems. Immunological and inflammatory reactions need to be incorporated. A multi-organ-chip technology has been established, based on a self-contained smartphone-sized chip format. A micropump has been successfully implemented into the microcirculation system for long-term operation under dynamic perfusion conditions. Performance of the technology has been proven by 28-day chip-based bioreactor runs of single perfusion circuits combining human 3D liver tissues and human foreskin. Data on metabolism, viability and endpoint histology will be presented. The inclusion of organ equivalents for intestine, kidney and bone-marrow will extend the multi-organ-chip use to ADMET testing.

Considerations About How the Reproductive Toxicity Has Been Managed in the REACH Registration Dossiers. What Will Happen in the Future?

C. Rovida
University of Konstanz, Konstanz, Germany

Before the whole REACH adventure began, it was well known that reproductive toxicity was among the less studied endpoints in the characterisation of chemical substances. REACH now requires that screening tests on reproductive toxicity must be performed on all substances manufactured or imported in quantities above 10 tonnes per annum (Annex VIII). When this threshold exceeds 100 tonnes per annum (Annex IX), full two-generation reproductive toxicity must be investigated in detail. In this case, the method of choice should be the OECD Test Guideline (TG) 416. The first REACH deadline (30th November, 2010) was mainly for substances manufactured or imported in quantities above 1000 tonnes per annum and substances classified as CMR, i.e. carcinogenic, mutagenic and toxic for reproduction category 1 for CLP in quantities above 1 tonne per annum. Another provision of REACH states that all studies on vertebrate animals that must be performed in compliance with Annexes IX and X, must receive formal authorisation following the submission of a testing proposal. By analysing 400 dossiers randomly picked from the submitted dossiers that are available in the European Chemicals Agency (ECHA) public database, it was found that no *in vitro* methods have been used in this area and only few *in silico* approaches have been applied. On the other hand, the percentage of existing data is much higher than expected, and both waiving and read-across strategies have been probably over-exploited. Another important point relates to existing studies, which count for about 40% — a proportion

very much higher than estimates. This value must be considered together with the percentage of dossiers containing one or more testing proposals. Summing up both current and previous proposals, there are 343 proposals for new tests on either reproductive or developmental toxicity, or both. Compared to 3599 registered substances, they represent only 9.5% of the total. Unfortunately, the fact that only 9.5% of the dossiers had a testing proposal in the area of reproductive toxicity does not mean that 90.5% of the studies in that area are available. By analysing the data in more detail, a trend in the application of the screening test for reproductive toxicity (OECD TG 422) is evident, demonstrating that many submitters preferred to do the screening test instead of the full study as it is requested by REACH. This approach has many advantages: the study can be performed without the preventive authorisation of ECHA, it is much less expensive and time consuming, and it can provide a very useful clue about the toxicity of the substance. However, it must be stated that this approach alone is not compliant with REACH request. A good compromise that ECHA may accept immediately is substituting the request for a full study, such as the OECD 422, with the new Extended One-generation Reproductive Toxicity Study (EOGRTS; OECD TG 443). At the present time, there is an ongoing discussion within the Member State Committee at ECHA, as some Member States are proposing the application of the EOGRTS instead of the OECD Test Guideline (TG) 416 two-generation study for two substances.

Meeting the Deadline 2013 — The Animal Welfare Perspective

I. Ruhdel
Deutscher Tierschutzbund/German Animal Welfare Federation, Neubiberg, Germany

Due to the pressure by European animal welfare organisations and the public, a Europe-wide marketing ban on cosmetics containing animal tested ingredients was announced in 1993, as part of the European Cosmetics Directive. Because of the clear political decision to end animal testing for cosmetics, the efforts to develop and validate animal-free testing methods have been intensified in recent years, so that in the meantime tests on animals could be replaced in some areas of safety evaluation. The number of animals used for the safety testing of cosmetics has receded drastically in the EU. But now, 18 years after the first announcement of the marketing ban, the last step — which should take effect in 2013 — is put into question. Experts, who documented the availability and development of animal-free testing on behalf of the EU-Commission, have drawn the conclusion that it will still take decades until all animal-based tests for chemicals can be replaced. Based on this, the EU-Commission could either postpone the ban or provide exceptions to it. From the point of view of animal welfare, a further postponement cannot be justified. Many high-quality animal-free testing methods are available. With these, a testing strategy specifically for cosmetic ingredients could be developed, with special regard to their application for humans. The consumer will not be put in jeopardy due to the marketing ban, nor will industry be impeded to produce further innovative products. The compliance of the marketing ban also has an important signalling effect concerning the reliability of political decisions in the European Union. Therefore, the marketing ban for cosmetics must become effective in March 2013, no matter whether animal-free testing methods are available or not.

The Russian 'Human-on-a-chip' Programme for Animal-free Substance Evaluation

D. Sakharov and A. Tonevitsky
Institute of Pathology and Pathophysiology, Moscow, Russia

Substantial reforms of law and regulations on medicines in Russia over the last ten years have created an unprecedented pipeline of new substances for clinical trials in Russia. As a result, the existing animal tests for preclinical evaluation of such substances have been challenged with regard to their predictive power. Similar to the EU and US, it has been recognised that the existing set of mandatory preclinical tests are inadequate to reliably predict pathways of toxicity or modes-of-action of new drug substances, prior to human exposure. Therefore, researchers at the Russian Academy of Sciences and Academy of Medical Sciences have performed a worldwide survey of the best alternative methodologies available. The human-on-a-chip concepts have been found to be the most viable way to replace animal testing while increasing predictivity. A development programme to establish a Russian human-on-a-chip platform has been initiated. The aims of the programme, current development status and the next steps forward, will be presented.

Using the Go3R Search Engine to Collect Toxicological Data and Three Rs-relevant Information for REACH Registration Dossiers

U.G. Sauer,[1] T. Wächter,[2] M.R. Alvers,[3] B. Grune,[4] L. Hareng,[5] B. Huhse,[4] R. Landsiedel,[5] A. Langsch,[5] B. Wareing[5] and M. Schroeder[6]
[1]Scientific Consultancy — Animal Welfare, Neubiberg, Germany; [2]TU Dresden & Transinsight GmbH, Dresden, Germany; [3]Transinsight GmbH, Dresden, Germany; [4]Federal Institute for Risk Assessment, Berlin, Germany; [5]BASF SE, Ludwigshafen, Germany; [6]TU Dresden, Dresden, Germany

The European REACH *Regulation No. 1907/2006* on the Registration, Evaluation and Authorisation of Chemicals obliges companies to register all chemicals manufactured or imported in quantities above 1 tonne per year. Since detailed information on the intrinsic properties of chemical substances has to be provided in the registration dossiers, comprehensive collection of all existing relevant data serves to avoid animal testing. Also, when new data have to be compiled to obtain hazard information, testing on vertebrates may only be undertaken as a last resort. Hence, meeting the challenges of the REACH legislation further requires the continuous updating of the information on newly-developed test methods that replace, reduce and refine animal testing. The knowledge-based search engine Go3R (www.Go3R.org) has been designed to assist scientists from industry and regulatory authorities in accomplishing both tasks. Analysing the 22 million documents of the PubMed and Toxicology Data Network (TOXNET) databases, Go3R makes use of expert knowledge on Three Rs methods and regulatory toxicology, laid down in a network of concepts, terms and synonyms, to 'recognise' the contents of documents. Search results are automatically sorted into a table of contents, which is displayed alongside the list of documents retrieved. By clicking onto different sections of the table of contents, the user can quickly filter the set of documents by topics of interest. Documents containing hazard information are automatically assigned to the 'IUCLID5 toxicological information' section of the table of contents that follows the endpoint-specific IUCLID5 categorisation required for REACH registration dossiers. To enable Go3R to sort documents by the toxicological endpoint being investigated, complex endpoint-specific search queries were compiled and integrated into the search engine. Dependent upon the endpoint, up to 100% of relevant documents containing either *in vitro* or *in vivo* hazard information were correctly sorted into the respective IUCLID5 category. Since Go3R can be customised to search in different databases, the IUCLID5 categorisation is also applicable to the screening and sorting of unpublished in-house data, and it can also be used to group substances. The 'Three Rs specific searches' section of the table of contents recognises

Three Rs-relevant documents and sorts these by Three Rs-specific information. The table of contents provides an overview on available test methods that replace, reduce or refine animal testing — sorted by area of use, types of cells or tissues used in *in vitro* systems, *in vitro* endpoints and *in vitro* endpoint detection methods. Since important information on Three Rs alternatives is published not only in scientific journals, but also on dedicated internet sites, Go3R further allows searching the entire world-wide-web employing a Google-based search tool with prior-ranking of information from Three Rs relevant websites. This option supports the retrieval of information on the status of validation and regulatory acceptance of specific test methods or their Standard Operating Procedure. The presentation will provide examples on how to use Go3R to retrieve substance-specific toxicological data and Three Rs relevant documents and will discuss the ongoing evaluation of the Go3R search engine.

Toward the Replacement of *In Vivo* Repeated Dose Systemic Toxicity: The SEURAT-1 Research Initiative

M. Schwarz
University of Tübingen, Tübingen, Germany

The aim of the SEURAT-1 Research Initiative, which started in January 2011, is the development of a long-term research strategy for research and development work leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals. The overall goal is to establish animal-free Innovative Toxicity Testing (ITT) methods, enabling robust safety assessment that will be more predictive than existing testing procedures. In order to achieve this, a cluster of projects has been organised, consisting of five research projects, supported by a 'data handling and servicing project' and a 'coordination and support project'. SEURAT-1 combines the expertise in cell culturing for the preparation of stable human cell lines, with the establishment of sophisticated experimental systems such as organ simulating devices, and links the experimental work with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration. So far, research in SEURAT-1 led to the development of quality control standards that can be applied in routine pluripotent stem cell-based toxicity testing. Pluripotent stem cells were engineered allowing the introduction of high-resolution fluorescent markers as reporters for the activation of, for example, transcription factors. Furthermore, different miniaturised microfluidic bioreactor prototypes, equipped with a set of microsensors to control culture conditions as well as to monitor toxicological effects on tissues grown in these bioreactors, were generated. In addition, the first toxicity tests have been carried out in more-conventional, less-sophisticated test systems, and '-omics' profiles, as well as structural changes, were monitored in order to identify biomarkers indicative for repeated dose systemic toxicity. Integrative and predictive computational systems biology tools were applied to integrate information obtained from iterative cycles of model predictions and experimental validations by *in vitro* experiments, in order to eventually predict the possible toxicity of test compounds *in vivo*. Furthermore, a process-based model able to simulate the dynamics of a chemical compound in cell-based assays has been developed as a basis for *in vitro–in vivo* extrapolations. All in all, the SEURAT-1 research strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health. This has triggered the selection of standard reference chemicals to be used in the research projects, which was made based on the clear association of a molecule with a particular mode-of-action and underlying mechanisms or effects. As the identification and full description of modes-of-action in the field of repeated dose systemic toxicity is still an open task, SEURAT-1, as a whole, follows a case study approach, i.e. a few well-described cases will be selected and toxic effects will be reproduced by both experimental approaches to identify appropriate readouts ('biomarkers'), and *in silico* approaches to increase the predictive power of the respective computer models. The final output of the SEURAT-1 Research Initiative will be to deliver a proof-of-concept showing if and how the developed scientific tools and the know-how can be combined in test systems to create innovative decision support for human safety assessment.

Three R Innovations and Best Practices in European Pesticide Regulation

T. Seidle
Humane Society International, Toronto, Canada

A paradigm shift is under way in the field of toxicology, toward a more robust understanding of the fundamental 'pathways of toxicity' in humans and other species and the development of more-efficient, species-relevant, and animal-free experimental and computational tools for risk assessment. The vision of '21st century toxicology' has achieved widespread support as a long-term objective, but this should not exclude near-term efforts toward the replacement, reduction or refinement (Three Rs) of animal use where possible. The recent revision of Europe's biocidal product (non-food pesticide) regulation offers a prime example of the substantial Three Rs progress that can be achieved within a product sector today without any additional research and development or validation effort, all the while maintaining a high level of human health and environmental protection. Adoption of the new biocide regulation followed the normal EU legislative procedure, beginning with a proposal by the European Commission, which was subsequently amended by the European Parliament and Member States through two rounds of political negotiations. The revised data requirements reflect dozens of technical amendments developed by Humane Society International and its partners, with solid support from the regulated industry, European Commission, key MEPs and Member State authorities. The new regulation not only incorporates all approved and applicable OECD Three R test methods and guidance, but takes substantial steps to move away from redundant multi-route (e.g. oral and dermal and inhalation) and multi-species (e.g. rodent and non-rodent) studies in a number of endpoint areas. Approved calculation approaches are substituted in place of redundant animal testing for finished products, and companies are encouraged to submit mechanistic *in vitro*, -omic and modelling data, in parallel with traditional apical endpoint studies to build confidence in the emerging '21st century' tools and approaches. Under optimal conditions, the new data requirements for biocides could enable an animal use reduction of 40 percent or more, making it a model for 'Three R best practices' in the pesticide sector globally, as well as for other sectors (e.g. REACH chemicals).

Cell and Tissue Culture Models for the Assessment of Cytotoxicity and Inflammation Induced by Inhalable Carbon Black Nanoparticles

O. Shpigelman,[1] J. Kopf,[1] M. Ströbele,[2] J. Hohlfeld,[1] A. Braun,[1] H. Fehrenbach,[3] T. Hansen[1] and K. Sewald[4]
[1]Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover, Germany; [2]Karlsruhe Institute of Technology, Karlsruhe, Germany; [3]Karlsruhe Institute of Technology, Borstel, Germany

Nanoparticles are widely used in the industry due to their size and unique properties. Nevertheless, a comprehensive safety evaluation of nanomaterials remains a challenge in nanomaterial toxicology. Biological effects of the nanoparticles may lead, for example, to inflammation and toxicity mainly in the lung tissue following pulmonary exposure. To understand the mechanisms of the effects of nanomaterials on human lung, a BMBF joint project (NanoCare) for the prediction of human toxicological effects, with five co-operation partners using *in vivo*, *in vitro* and *ex vivo* models of the lung, was funded and undertaken. The aim of the project was to assess a multi-stage procedure for the screening of carbon black nanomaterials for the impact on different lung regions. Precision-cut lung slices (PCLS) and human cell lines are relevant acute exposure models for the evaluation of

nanomaterials. By using Printex® 90 and acetylene soot particles, this work provides a comparison of lung *in vitro* (three different cell lines) and *ex vivo* models (precision cut lung slices). Cytokine response data of organotypic lung cultures of mice, rats, and humans after exposure to carbon black nanoparticles was compared with data from *in vitro* experiments in cell lines. Three different human cell lines (16HBE14o-, Calu-3, A549) and PCLS (murine, rat and human) were exposed to different concentrations of Printex® 90 and acetylene soot particles, for 24 hours. The size distribution of stable particle suspension in DMEM medium was determined by dynamic light scattering. Viability of PCLS was assessed by LIVE/DEAD® viability staining and determination of metabolic activity by using the WST-1 assay. Pro-inflammatory immune responses connected to particle exposure were quantified by using ELISA. The cytokines interleukin (IL)-1α and IL-1β, tumour necrosis factor (TNF)-α and the chemokine IL-8, were measured. The concentration-dependent effect of carbon black particles on the lung tissue and different cell lines could be shown after a 24-hour exposure. Printex® 90 nanoparticles were nearly non-toxic at concentrations between 50µg/ml and 0.5µg/ml in the cell culture and in the PCLS of all three species. Compared to these results, acetylene soot showed a toxic effect at 50µg/ml in two cell lines and the PCLS by using the vitality assay. These results will be verified by LIVE/DEAD® viability staining for PCLS after 24 hours, by using confocal microscopy. The results of two different dispersants (5% FCS and 0.5% BSA) added to the culture medium were compared for Printex® 90. The inflammatory response was assessed by measuring pro-inflammatory cytokine content (IL-1α and TNF-α) in the supernatants and tissue lysate. We conclude that the comparison of *in vitro* and *ex vivo* data provides a promising test system to reflect the acute effects of nanomaterials on lungs. Furthermore, it can be used to establish a test for finding subtoxic concentrations for humans and to gain better understanding of inflammatory mechanisms.

The EU FP7 Project AXLR8: Drafting a Roadmap to Innovative Toxicity Testing in the 21st Century ('Tox21') in Europe and the USA

H. Spielmann,[1] T. Seidle,[2] V. Kral,[1] G. Schoeters[3] and M. Schäfer-Korting[1]
[1]Freie Universität Berlin, Berlin, Germany; [2]Humane Society International, Toronto, Canada; [3]VITO-CARDAM, Mol, Belgium

AXLR8 is a co-ordination and support action funded by the European Commission (FP7) within the funding framework on *Alternative Testing Strategies: Replacing, reducing and refining use of animals in research*. AXLR8 is particularly aimed at accelerating a transition in Europe toward a more sophisticated approach to chemical and product safety assessment with the common goals of improved health and environmental protection, positioning the EU on the leading-edge of a rapidly developing global research area, and working toward replacement of animal testing. An essential element of the AXLR8 project is the organisation of annual workshops to provide a scientific platform for high-level information exchange and critical discourse among coordinators of EU-funded projects and independent European and international scientists on progress achieved in developing alternative testing strategies, as well as challenges, needs, and priorities for future EU research. The second annual AXLR8 workshop (AXLR8-2) was held in 2011, with a focus on developing a 'roadmap to innovative toxicity testing'. It was the general view that limitations intrinsic to conventional high-dose *in vivo* studies limit their relevance and utility as tools for modern safety assessments for protecting and improving human health (e.g. in relation to nanomaterials, endocrine disruptors, and environmental chemicals), and that the way forward requires a shift toward a pathway-based paradigm for safety assessment. In particular, the assessment of a substance's toxic 'mode-of-action' was considered by the AXLR8 Scientific Panel to be a cornerstone of '21st century' safety assessment. To provide the DG Research & Innovation (DG R&I) of the EU Commission with a vision and strategy for

funding in the field of toxicology in the 'Horizon 2020' programme, which will start in 2014, the third AXLR8 workshop (AXLR8-3) in 2012 discussed a "roadmap to next-generation safety testing under 'Horizon 2020'". The recommendations of the AXLR8-3 workshop will be presented, and will be published later this year in the 'AXLR8 Progress Report 2012 on Alternative Testing Strategies'.

An Inflamed and Non-inflamed Triple Culture Model of the Intestinal Mucosa to Assess Cytotoxicity of Engineered Nanoparticles

J. Susewind,[1] C.M. Lehr[1] and E. Collnot[2]
[1]Saarland University, Saarbrücken, Germany; [2]Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany

Recently, we established a 3D co-culture model of the inflamed intestinal mucosa, combining Caco-2 enterocytes with primary human blood-derived dendritic cells and macrophages. In permeable cell culture inserts, intestinal epithelial cells are grown to confluence on a collagen layer in which the immunocompetent cells are embedded. The model can be inflamed upon addition of cytokine interleukin (IL)-1β to the apical side and then reflects the pathophysiological changes observed *in vivo*, such as decreased epithelial barrier properties and increased release of the pro-inflammatory marker, IL-8. Screening different anti-inflammatory formulations in the system provided mechanistic insight and allowed identification of the most potent candidates for further *in vivo* testing. In this study, the 3D model was utilised for the first time to assess the toxicity of engineered nanoparticles. Slightly adapting the model, PMA-stimulated THP-1 cells were used as macrophages and Mutz-3 were employed as dendritic-like cells. Various engineered nanomaterials differing in size, composition and surface coating were evaluated in the adapted co-culture system by using numerous toxicity endpoints such as LDH release, mitochondrial activity and pro-inflammatory effects. Results were compared to findings in Caco-2 monoculture experiments. Significant differences were observed between testing in a Caco-2 single culture and in the co-culture set-up, both in the non-inflamed and inflamed state. The co-culture model was more resilient to possible cytotoxic stimuli. LC50 values for toxic nanoparticles such as NM300 Ag particles were about 2–4 fold lower in the LDH assay or the Alamar blue assay in the co-culture set-up, as compared to Caco-2 cells alone. The difference cannot be ascribed to a mere change in cell numbers. Instead, nanoparticles are preferentially taken up by immunocompetent cells, reducing the burden on enterocytes but changing the *in vitro* response pattern: an increased release of pro-inflammatory cytokines, such as IL-8 and TNF-α, is observed in the 3D co-culture. The effect is potentiated if the barrier is stressed by previous inflammatory damage, as simulated by the addition of IL-1β. Thus, the novel 3D co-culture model presents a valuable tool for advanced mechanistic and toxicity studies, better reflecting the physiology of the intestinal mucosa in both the inflamed and non-inflamed state.

The First REACH Deadline: Experience With the Testing Proposals System to Reduce Animal Testing

K. Taylor
BUAV/ECEAE, London, UK

In order to reduce animal testing under the new EU REACH legislation, companies must submit a proposal to do certain tests on animals. This proposal is then published for public comments, evaluated by the European Chemicals Agency (ECHA) and finally agreed by its Member State Committee (MSC). Only a proportion of the animal tests that are required under REACH go through this system. These are the so-called 'higher-tier animal tests' such as 90-day repeated dose tests, reproductive toxicity tests on rats, and developmental toxicity tests on rats and rabbits. According to ECHA's Evaluation Report, a total of 715 testing proposals were received from the first registration deadline of December 2010 for chemicals produced in quantities of more than 1,000 tonnes per year. We estimate that if all of these proposals were agreed, then this would consume nearly one million animals alone. The European Coalition to End Animal Experiments appears to have taken the lead on providing comments on these testing proposals as they are published. Between August 2008 and December 2011, we submitted comments on 260 proposals, i.e. 37% of the 699 published by the ECHA. We estimate that our comments have been approximately 50% of those received by the ECHA. In this presentation, I will summarise the endpoints for which testing proposals have been made, and the type of comments we have provided. I will also share the successes and problems that we have experienced with commenting. It has proved difficult to find existing data on public databases, so the various forms our comments have taken has ranged from proposing read-across on substances already known to the company, providing data on similar substances to enable read-across, and suggesting QSAR models and *in vitro* methods that could be used in a weight-of-evidence argument. We have also suggested testing strategies to reduce testing, when more than one animal test has been proposed. To date, the ECHA website has only published 12 final decisions (11 were agreed that they needed to be done, one was withdrawn by the company). In fact, due to the extremely narrow approach taken by ECHA, there have been no reported rejections of testing proposals to date. Companies tend to withdraw their proposals earlier on in the process. We are aware of five instances where this has been as a direct consequence of our comments. We have also been successful in helping the Agency to now adopt, as standard, several approaches of which they were at first critical — such as asking for tests to be done sequentially or conditionally rejecting proposals until the results of other tests are known. The ECHA have also frozen decisions on two-generation reproductive toxicity tests until the Commission makes a decision. There remain several problems with the quality of decisions by ECHA and its MSC, and we hope that some will be considered by the Board of Appeal.

Potential of Pertussis Toxin-sensitive Genes in Human Dendritic Cells to Evaluate Safety of Pertussis Vaccines

S. Vaessen,[1] J. Pennings,[2] M. Bruysters,[2] S. Verkoeijen,[1] R. Vandebriel,[2] C. Krul[1] and A. Akkermans[2]
[1]University of Applied Sciences Utrecht, Utrecht, The Netherlands; [2]National Institute for Public Health and the Environment, Bilthoven, The Netherlands

The histamine-sensitisation test (HIST) is the current standard test to ensure the absence of active pertussis toxin (PTx) in pertussis vaccines. Working toward a mechanism-based *in vitro* alternative for the HIST, we performed microarray analysis of six relevant human cell types after exposure to PTx. Monocyte-derived dendritic cells (MoDCs) were the only cells in which PTx induced differential expression of genes. A second experiment, with MoDCs from four

donors, confirmed the observed induction of genes by PTx and extended the data by showing specificity for PTx in comparison to *E. coli* lipopolysaccharide and *B. pertussis* lipo-oligosaccharide. Statistical analysis, with a false/discovery rate (FDR) of 2% and an up-regulation by a ratio > 4 of PTx against any of the other exposures, indicated six genes that were significantly up-regulated by PTx: IFNG, IL-2, XCL1, CD69, CSF2 and CXCL10. Increased levels of the secreted proteins in the supernatants of PTx-treated MoDCs, corresponded to the up-regulated genes (IFNG, IL-2, XCL1, and CXCL10), confirming the effects of PTx on these genes. Although further evaluation is required, these genes have the potential to control the safety of pertussis vaccines.

The Future of Non-human Primates in the Development of Biopharmaceuticals

P. van Meer,[1] M. Kooijman,[2] J.W. van der Laan,[3] E. Moors[2] and H. Schellekens[1,2]
[1]Utrecht Institute of Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands; [2]Copernicus Institute of Sustainable Development, Innovation Studies, Utrecht University, Utrecht, The Netherlands; [3]Medicines Evaluation Board, Utrecht, The Netherlands

Due to the characteristics unique to monoclonal antibodies (MAbs) and other biotech products, non-human primates (NHP) are often assumed to be the only relevant species to evaluate safety and efficacy of MAbs. However, the scientific value of NHP in MAb development has never been adequately established, and has been debated since it became clear that the non-clinical safety programmes used for small molecule therapeutics would not be appropriate for MAbs. In this retrospective analysis, we studied the value of using NHP to evaluate the safety and efficacy of these products. To do this, we had unique access to the drug registration files of all MAbs marketed in the European Union. Inadequately justifying the use of NHP as a primary non-clinical species and the use of study designs that were considered ineffective led to a needless increase of NHP use. The value of NHP in non-clinical assessment was further limited by immunogenicity. But more importantly, NHP do not stand out as a predictive model because MAbs primarily exert their expected pharmacological effect. Nevertheless, their use continues to increase. There is an urgent need for a reevaluation of the need for routine studies with NHP to develop MAbs.

Inconsistencies in Data Requirements of EU Legislation Involving Tests on Animals

K. Wagner and R. Kolar
Deutscher Tierschutzbund — Akademie für Tierschutz (German Animal Welfare Federation — Animal Welfare Academy), Neubiberg, Germany

Present and future European Union (EU) legislation on the protection of animals used for scientific purposes (*Directives 86/609/EEC* and *2010/63/EU*) requires that alternative methods have to be used instead of animal tests wherever they are available. Unfortunately, this provision is not implemented to its full extent when it comes to risk assessment of chemicals and new products prior to their authorisation and placing on the market in the EU. In this study, the Animal Welfare Academy of the German Animal Welfare Federation screened data requirements of relevant EU law regarding chemicals (REACH), biocides, pesticides and food safety (Novel Food), and found that data requirements as part of the risk assessment do not always reflect the state-of-the-art of science and technology. Most of the data requirements we investigated still include testing on animals for many toxicological endpoints, even though

more than 40 alternative testing methods accepted at the level of the EU or of the Organisation for Economic Co-operation and Development (OECD), are available. This unacceptable state of affairs may be due to a multitude of reasons that range from a shortage both of manpower to implement existing knowledge and of expertise in the field of alternative methods, to unclear and misleading statements on the applicability and state of validation of alternative methods. In conclusion, we strongly suggest a homogeneous EU-wide approach for all areas involving risk assessment of substances, with the aim of better implementing the Three Rs and complying with *Directives 86/609/EEC* and *2010/63/EU*. As a positive side-effect, this would obviously streamline data requirements, save costs on various levels, and enhance product safety for consumers.

Use of a Human Embryonic Stem Cell-based Test System For Neurodevelopmental Toxicity Testing and Epigenetic Profiling

T. Waldmann, N. Stiegler and M. Leist
Doerenkamp-Zbinden Chair of In Vitro Toxicology and Biomedicine, University of Konstanz, Konstanz, Germany

Information about the neurotoxic mechanisms of many chemicals on human development is still sparse. This is mostly due to the lack of effective and reliable test systems. This gap cannot be closed solely on the basis of animal experiments. Most of the current knowledge is based on epidemiological studies. However, such studies are very difficult to interpret because the 'experimental conditions' cannot be controlled. From such studies, it is known that the antiepileptic drug, valproic acid (VPA), and the environmental chemical, methyl mercury (MeHg), have an impact on human neurodevelopment. In humans and rodents, a major target of VPA, which is used as a drug to treat epilepsy, is the process of neural tube closure. In accordance with this, exposure during this developmental period is most detrimental. It may result, for example, in spina bifida, cleft palate, autism-spectrum symptoms or lowered IQ scores, in children exposed to the drug *in utero*. In contrast to VPA, MeHg accumulates in the environment. Most knowledge has been gained from studying environmental pollution (e.g. the mass intoxication in Minamata, Japan). Children that were exposed to MeHg *in utero* showed clear neurological dysfunction, such as sensory defects or mental retardation. Here, we used VPA and MeHg as known compounds that affect human neurodevelopment, to establish an *in vitro* test system based on the differentiation of human embryonic stem cells (hESC). We differentiated hESC to neuroepithelial progenitor cells (NEP), resembling the cells found during the early stages of neural tube closure. The differentiation process was studied by microarray expression profiling, by immunostaining and by RT-qPCR. The microarray studies showed a high reproducibility of differentiation, which is a major pre-requisite for an *in vitro* test system. Expression of marker genes of neural development was chosen as a major endpoint and investigated further by RT-qPCR. In the presence of VPA or related compounds, marker gene expression was changed. There was a relative increase in OCT4 and a reduction in PAX6. In contrast, after treatment with MeHg, we found OCT4 and PAX6 unchanged, but instead, a decrease of FOXG1 was observed. Therefore, changes in marker gene expression show distinct patterns for different compounds. This effect was confirmed by whole genome analysis by using Affymetrix microarray studies. The set of changed genes offers an explanation for potentially disturbed signalling pathways. This system is a step toward modelling chemical-induced neurodevelopmental disturbances on the basis of human stem cells. It can be used to study adverse effects on early human neurodevelopment, and to obtain critical information for the construction on adverse outcome pathways (AOPs).

Esterase Activity in Excised and Reconstructed Human Skin — Biotransformation of Prednicarbate and the Model Dye Fluorescein Diacetate

G. Weindl,[1] F.M. Bätz,[1] W. Klipper,[1] F. Henkler,[2] R. Landsiedel,[3] A. Luch,[2] U. von Fritschen,[4] H.C. Korting[5] and M. Schäfer-Korting[1]
[1]Freie Universität Berlin, Institut für Pharmazie (Pharmakologie und Toxikologie), Berlin, Germany; [2]Bundesinstitut für Risikobewertung (BfR), Abteilung Sicherheit verbrauchernaher Produkte, Berlin, Germany; [3]BASF SE, Experimentelle Toxikologie und Ökologie, Ludwigshafen, Germany; [4]HELIOS Klinikum Emil von Behring, Berlin, Germany; [5]Ludwig-Maximilians-Universität München, Klinik für Dermatologie und Allergologie, München, Germany

Reconstructed human epidermis (RHE) is used in non-animal testing for hazard analysis and reconstructed human skin (RHS) gains increasing interest in preclinical drug development. RHE and RHS have been characterised regarding their barrier function but, as with human skin, knowledge about biotransformation capacity is rather poor. However, this can be highly relevant for the efficacy of topical dermatics, as well as genotoxicity and sensitisation. We compared the esteratic cleavage of the prednisolone diester prednicarbate and the enzyme kinetic parameters (V_{max} and $K_{0.5}$) of the model substrate, fluorescein diacetate (FDA), in commercially available RHS and RHE, and in excised human skin and monolayer cultures of normal and immortalised human keratinocytes and of fibroblasts. Formation of the main metabolite prednisolone, and of fluorescein, ranked as: RHS ~ RHE > excised human skin and keratinocytes > fibroblasts, respectively. Because of the aromatic probe, however, V_{max} of FDA cleavage was not related linearly with prednicarbate metabolism. In conclusion, RHE and RHS may be useful to quantitatively address esterase activity of human skin in drug development and hazard analysis, although an increased activity compared to native human skin has to be taken into account.

Characterisation of Reconstructed Human Skin Following Topical Treatment with Glucocorticoid Creams

G. Weindl, T. Markovič and M. Schäfer-Korting
Freie Universität Berlin, Institut für Pharmazie (Pharmakologie und Toxikologie), Berlin, Germany

Topical glucocorticoids (GCs) represent the most important treatment for inflammatory skin diseases. Besides the desired anti-inflammatory effects, GCs also induce several adverse effects after prolonged therapy, of which skin atrophy is one of the most frequently observed. Previously, we could show that topical application of GC solutions for 7 days strongly reduces the secretion of pro-inflammatory cytokines in reconstructed human skin (RHS). However, GC-induced skin atrophy was not detected by the analysis of epidermal thickness and collagen mRNA expression. The aim of the present study was to further develop the *in vitro* test system based on RHS, for the identification and characterisation of GC-induced skin atrophy. The anti-inflammatory effects and atrophogenic potential of GC-containing creams (prednicarbate, clobetasol 17-propionate) were evaluated on Epiderm-FT and Phenion-FT models. Treatment with GCs, applied every second day for six days, strongly reduced IL-6 release, which correlated well with the different GC classes. Interestingly, the two untreated skin models showed marked differences in the kinetics of IL-6 production. Quantitative gene expression analysis of GC-treated Phenion-FT models showed a more pronounced IL-6 and MMP1 down-regulation in the dermis, as compared to Epiderm-FT. Collagen expression was not affected by GCs in Epiderm-FT. In contrast, a slight reduction of COL1A1 mRNA was detected in the dermis of Phenion-FT models following treatment for up to 6 days with the highly potent clobetasol 17-propionate. When Epiderm-FT models were treated with GCs for 14 days, expression of COL1A1 mRNA was reduced in the dermal compartment. Decreased

collagen type I protein expression was confirmed by immunofluorescence. Of note, the number of epidermal layers was severely reduced in all skin models after 6 or 14 days of treatment with base creams and GC-containing creams, thus the impact of GCs on epidermal thinning could not be evaluated. In summary, GC-induced skin atrophy can be detected by analysis of collagen expression after prolonged treatment with cream formulations. Furthermore, our results indicate that Phenion-FT models appear to be more sensitive to GC treatment, as compared to Epiderm-FT models.

BioChip-based Electrochemical Platform for the Label-free Monitoring of Living Cells

D. Weiss,[1] H. Grothe,[2] B. Wolf[2] and J. Wiest[1]
[1]Cellasys GmbH — R&D, Munich, Germany; [2]Heinz Nixdorf — Lehrstuhl für Medizinische Elektronik, Technische Universität München, Munich, Germany

Measurement of physiological parameters of cultured cells is routinely used by a number of methods to understand inter-functional relationships at the levels of single cells, organs and organisms. Development and supplement of these methods in order to reach the continuous online and real-time registration of metabolic and morphological cell parameters extend the spectrum of their application, e.g. in toxicokinetics, drug development or chemosensitivity analysis. The Intelligent Mobile Lab (IMOLA) delivers metabolic and morphological parameters of living cells that are marker-free, online and in real-time, and represents a key technology for the development of new cell-based methods and therapies. The IMOLA technology was developed in the group of Professor Bernhard Wolf at the Heinz Nixdorf — Lehrstuhl für Medizinische Elektronik of Technische Universität München. The system performs measurements of the extracellular acidification (pH), cellular respiration (pO_2), changes in cell morphology (bioimpedance) and temperature of the cell/tissue probe. The degree of extracellular acidification caused by the products of cell metabolism, in addition to the concentration of dissolved oxygen, act as indicators of cell vitality. Information about cell morphology and proliferation can be derived by means of bioimpedance value analysis. A detailed description of the whole cell monitoring approach and the related signal processing was given previously. Various cell types have been successfully tested with the system. These include cells in suspension, monolayers, tissue-slices and 3D-spheroids. Since the European Community Regulation on chemicals and their safe use (REACH) entered into force on 1st July 2007, the use of dangerous chemical substances is allowed, as long as a chemical safety assessment was established and published by the substance manufacturers. The chemical safety assessment characterises and classifies the adverse effects of chemicals on organisms and on the environment. A study on the influence of different concentrations of mercury on mouse fibroblasts was investigated by means of the IMOLA. The study showed that 5μM HgCl hardly affected the fibroblasts. A significant reduction in extracellular acidification was, however, measured after the addition of 20μM HgCl. This cellular effect seems to be partly reversible because toxin removal leads to the restoration of the acidifying activity of the cells almost up to the untreated level. This study demonstrates that the IMOLA is a suitable method for providing information about interactions between cells and active substances. One of the major advantages of this system is the possibility of performing marker-free long-term investigations, excluding any foreign influences on the cells, and allowing the observation of long-term effects after chronic exposure to toxins. Moreover, it was shown that the IMOLA technology is capable of differentiating metabolic and morphological cellular effects, with respect to different toxin concentrations. This is an essential feature for any technology attempting to replace animal experiments currently used for determining the toxicity of new chemicals.

The Role of Adverse Outcome Pathways in Streamlining Hazard and Risk Assessment

C. Willett
The Humane Society of the United States, Gaithersburg, MA, USA

The 'adverse outcome pathway' (AOP) approach is based on the concept that toxicity results from chemical exposure as a result of molecular interaction with a biomolecule — the initiating event, followed by a description of the sequential progression of events through to the *in vivo* result, or adverse outcome. The AOP concept can be used in the short-term to enhance hazard and risk assessment at multiple levels, for example, by informing chemical category and structure–activity relationships, by increasing the certainty of interpretation of both existing and new information and by facilitating the development of integrated testing strategies that maximise useful information gained from minimal testing. Ultimately, AOPs can be used to identify key events for which non-animal tests can be developed, thereby facilitating mechanism-based, non-animal chemical assessment. This concept has been embraced by the Organisation for Economic Co-operation and Development (OECD), as well as by some regulatory authorities around the world. AOP development is exemplified by three current examples: the AOP for sensitisation developed by the OECD, the AOPs for oestrogen receptor-mediated effects and thyroid hormone-related effects, which were developed by US EPA. Broad application of the AOP concept will facilitate the emergence of an internationally-harmonised predictive toxicological framework.

Development of a First-choice Non-animal Model for Bipolar Disorder Research

R. Williams and A. Frej
Royal Holloway University of London, Egham, UK

Bipolar disorder (or manic depression) is a psychiatric condition that has a devastating effect on individuals and leads to a high socio-economic cost, estimated to be £4.6 billion annually in the UK. The range of pharmacological treatments that are used to control bipolar disorder show variable efficacy in patients, and give rise to a number of adverse side effects (including weight gain, tremors, alopecia, and birth defects). Thus, there is a clear need to develop more-effective and safer treatments for this disorder. Presently, embryo-derived animal cells are the standard model used to investigate both the unknown mechanism of these drugs and to develop improved treatments. In bipolar disorder research, the use of a molecular pharmacology approach to investigate drug mechanisms offers perhaps the best method for understanding the origins of (and means of controlling) the disorder. One breakthrough in this research was the formulation of the inositol depletion theory, which states that bipolar disorder drugs down-regulate inositol based signalling that is over-active in bipolar disorder patients. The exact mechanism of inositol depletion remains unclear. However, we have previously demonstrated, by using primary neurons taken from unborn rat embryos, that this is a common mechanim of action for the three structurally distinct bipolar disorder treatments (lithium, valproic acid and carbamazepine). Current methods to further investigate these drug targets in order to develop better treatments would require the death of thousands of animals. It is therefore imperative to develop better models for this research that do not rely on the use of animals, in order to improve the treatment (and quality of life) of people diagnosed with bipolar disorder. We are now developing a replacement model for animal cells in this research by using the simple biomedical model, *Dictyostelium*. This amoeba is a widely accepted system in a range of research areas, including the development of new therapeutic drugs. In our current studies, we have shown that *Dictyostelium* contains a highly conserved homologue of the INO1 protein, suggested to be involved in inositol depletion caused by val-

proic acid. We have also shown that treatment of *Dictyostelium* with valproic acid causes an increase in the expression of the *INO1* gene, consistent with an inositol depleting effect. We are now creating *Dictyostelium* cells that have deleted or elevated levels of the protein, or contain versions of the fluorescently tagged INO1, expressed from its own promoter. These studies will enable us to understand how the INO1 protein works in a cell and allow us to rapidly screen a large number of novel chemical compounds for the effect of inositol depletion. Our ongoing studies will therefore provide the mechanism by which bipolar disorder treatments (like valproic acid) function within a cell to cause inositol depletion and will ultimately enable the development of more efficacious and safer bipolar disorder treatments without the loss of animal life.

Evaluation of Developmental Toxicants and Signalling Pathways in a Functional Test Based on the Migration of Human Neural Crest Cells

B. Zimmer,[1] G. Lee,[2] N. Stiegler,[1] K. Meganathan,[3] A. Sachinidis,[3] L. Studer[4] and M. Leist[1]
[1]Doerenkamp-Zbinden Chair of In Vitro Toxicology and Biomedicine, University of Konstanz, Konstanz, Germany; [2]Institute for Cell Engineering, Department of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MA, USA; [3]Center of Physiology, Institute of Neurophysiology, University of Cologne, Germany; [4]Developmental Biology Program, Sloan-Kettering Institute, New York, USA

Information on the potential for developmental neurotoxicity (DNT) is scarce for the majority of chemicals. To guarantee high consumer safety, more information is required. However, test capacities for further animal-based testing are limited and associated with ethical problems. Therefore, new animal-free approaches with higher throughput are required. A screening strategy using different assays, based on relevant human cell types combined in a test battery, has therefore been proposed by the EPA and other international key authorities. The feasibility of such test batteries has already been shown in the fields of genetic toxicology and skin sensitisation. Such a test battery does not currently exist for DNT. For this reason, many laboratories, including ours, are developing assays to model neurodevelopment *in vitro* for toxicity screening. Impaired neural crest (NC) function is one of the most prominent causes of the teratogenic effects of chemicals. It is estimated that one third of all congenital birth defects are associated with neural crest cells and their derivatives. Testing of toxicant effects on the NC is highly desirable, and should form an element of a future DNT test battery. We aimed to develop a robust and widely applicable human-relevant NC function assay, allowing sensitive screening of different toxicants, and a definition of toxicity pathways, as well as evaluation of such an assay as part of a larger DNT *in vitro* test battery. We generated NC cells from human embryonic stem cells and, after establishing a migration assay for NC cells (MINC assay), we tested environmental toxicants, as well as inhibitors of physiological signal transduction pathways. Methylmercury (50nM), valproic acid (> 10μM) and lead acetate (1μM) affected the migration of NC cells more potently than the migration of other cell types, including central nervous system progenitor cells. The MINC assay correctly identified the neural crest toxicants, triadimefon and triadimenol (two pesticides). Additionally, it highlighted different sensitivities to various organic and inorganic mercury compounds. Negative control compounds, such as mannitol or acetaminophen, did not alter NC cell migration. By using classic pharmacologic inhibitors and by performing large-scale microarray gene expression profiling, we found several signalling pathways that are relevant for the migration of NC cells in the MINC assay, which could therefore be potential targets for different toxicants. In preliminary experiments, we found that chemicals specifically targeting those pathways indeed have adverse effects on NC cell migration in the MINC assay. The MINC assay faithfully models human NC cell migration, and reveals impairment of this function by developmental toxicants with good sensitivity and specificity. Additionally, the MINC assay can be used to identify important pathways of toxicity (PoT) in the area of NC cell migration. We therefore believe that our MINC assay could play an important role as part of a DNT *in vitro* test battery.

POSTER PRESENTATIONS

Session I: Progress in 3Rs Research: EU FP6 & FP7 Projects

There were no posters submitted to Session I

Session II: Chemicals — REACH and Animal Welfare

Example of a Corrosive Amine Classified as False-negative in an *In Vitro* Skin Irritation Test

D. Lehmeier
BSL BIOSERVICE Scientific Laboratories GmbH, Planegg, Germany

The assessment of skin corrosion and skin irritation has typically involved the use of laboratory animals (OECD Test Guideline [TG] 404). Considering animal welfare concerns, validated *in vitro*/*ex vivo* test methods have been accepted for application within a tiered testing strategy (Annex to OECD TG 404, as revised in 2002). Different three-dimensional reconstructed human skin models have shown to fulfil the ECVAM Performance Standards for *in vitro* skin corrosion testing (OECD TG 431) and *in vitro* skin irritation testing (OECD TG 439). If a test substance is expected to be non-corrosive after assessment of all existing data, an *in vitro* skin irritation test can be performed as first step within a Bottom-Up approach. If the outcome is negative, the test substance is classified as 'non-irritant' in accordance with UN GHS 'No Category'. In this paper, we want to show the risk of misclassification by using a Bottom-Up strategy without confirmation of negative results derived from one single *in vitro* skin irritation test for submission to regulatory agencies. As an example, we present the discrepant results of *in vitro* skin irritation studies performed on two different validated human skin models and the results of skin corrosion tests according to OECD TG 431 and OECD TG 435, with a fatty amine which was expected to have corrosive potential.

Pre-validation of a High-throughput Reporter Gene Assay to Detect Genotoxicity and Oxidative Stress

A.R.M. von Bergh,[1] S.C. van der Linden,[2] B. Lussenburg,[2] L. Jonker,[2] M. Teunis,[1] C.A.M. Krul[1] and B. van der Burg[2]
[1]Innovative Testing in Life Sciences and Chemistry, Research Centre Technology & Innovation, University of Applied Sciences Utrecht, Utrecht, The Netherlands; [2]BioDetection Systems BV, Amsterdam, The Netherlands

Many compounds are known to have multiple toxic effects, and therefore it is important to develop mechanism-based tools. One of the effects of high relevance for both humans and the environment is genotoxicity, as it is related to many adverse health effects, including cancer. While traditional genotoxicity tests mainly focus on mutations, recent test systems use cell lines to detect specific mechanisms of genotoxicity. In this study, we used highly specific luciferase reporter assays that respond to p53 and Nrf2 activation, indicative of genotoxicity and oxidative stress. A third reporter assay serves as a control for cytotoxicity and non-specific luciferase gene expression. The reporter cell lines are based on human cell line U2OS. A total of 83 compounds were tested in two independent laboratories: 35 genotoxic carcinogens (positives), 22 non-genotoxic compounds (negatives) and 25 compounds that give positive results *in vitro* at high concentrations that are non-relevant *in vivo* (false positives). As U2OS is not capable of metabolising genotoxic compounds, S9 was added. The U2OS-p53 luciferase reporter assay detected 19 out of 35 genotoxic compounds as positive. Most of these were mutagenic compounds. Testing with S9 resulted in the positive identification of four more genotoxic compounds. Although some of the current assays produce a large portion of false positives, the U2OS-p53 assay without S9 identified only three compounds incorrectly as positive. Out of the 35 genotoxic compounds, 17 produced a positive response in the Nrf2 assay, while 14 out of 48 non-genotoxic compounds were positive for Nrf2. Therefore, the Nrf2 assay should only be used as follow-up of p53 positives, to identify whether oxidative damage is an indirect mechanism causing the observed genotoxic activity. The reproducibility of the assays is high, as discrepancies between the two laboratories were found in less than 4% (3/83) of

compounds. The assays presented here can be used to assess the genotoxic potential of compounds, as well as oxidative stress. The U2OS-p53 assay is a fast, easy and sensitive method to detect genotoxicity of chemicals, and thus provides a valuable tool for early high-throughput human genotoxicity screening.

Integrated Testing Strategy for Reproductive Toxicity

A. Wolterbeek, A. Beker, S. Bosgra, M. Verwei, J. Westerhout, H. Buist and D. Kroese
TNO, Zeist, The Netherlands

The current system of risk assessment of chemicals is complex, very resource-intensive and time-consuming. This will become very much clearer during the implementation of the REACH regulations. Within these regulations, the requirements for reproductive and developmental toxicology are particularly important, since these studies require most resources. Therefore, there is a great need to modernise the process of hazard and risk assessment, requiring registrants to consider alternative methods for filling the data gaps. Due to the complexity of the reproductive and developmental processes, the use of alternative methods for these endpoints may be problematic. At this moment, there are only a few alternative methods identifying potential reproductive toxic agents with sufficient accuracy, speed and reliability. Simple animal-free *in vitro* models cover only a restricted part of the reproductive cycle. Most models represent underlying processes and dynamics insufficiently, and are therefore of limited use as a stand-alone. The EU project, ChemScreen, aims to fill these gaps and place the tests in a more innovative animal-free integrated testing strategy for reproductive toxicity, which will use combinations of available *in silico* and *in vitro* technologies. A first step in the project is to establish methods for prescreening and prediction of chemicals having specific toxicological properties that do not need further testing for reproductive toxicity according to REACH, i.e. chemicals that need classification as either genotoxic carcinogen or germline mutagen. In this step, methods for prescreening and predicting potential reproductive toxicity by using repeated dose and reproductive toxicity databases and *in silico* methods are also envisaged. A minimal set of medium- and high-throughput *in vitro* test methods to study sensitive parameters will be established as a second step to identify reproductive toxicants. Initially, these methods will be applied and tested for use in a category approach to verify read-across to an *in vivo*-tested agent, while the long-run objective is to develop them into a stand-alone battery. In the final step, all of this information is to be integrated, to allow conclusions on classification and labelling and risk assessment to be made — among others, whether, by applying quantitative *in vitro–in vivo* extrapolation, can the need for (and specifics of) further *in vivo* testing for reproductive toxicity be decided.

Developmental and Neurotoxicity of Organotin Compounds in Zebrafish

A. Wolterbeek,[1] L. te Brake,[1] A. Beker,[1] C. Snel,[2] A. Menke,[2] D. de Groot[1] and D. Kroese[1]
[1]TNO, Zeist, The Netherlands; [2]TNO Triskelion, Zeist, The Netherlands

The zebrafish embryotoxicity test is an alternative test system for the relatively fast and simple screening of the embryotoxic properties of chemicals. Furthermore, zebrafish locomotor activity, and especially the total distance moved as a measure for developmental neurotoxicity, can be easily and rapidly measured in larval zebrafish by using an automated video image analysis system. However, these assays are only useful if they predict the potential (neuro)developmental toxicity of chemicals comparable to observations in *in vivo* developmental toxicity studies. In this study, a group of organotin compounds known to induce general development effects and/or neurodevelopmental effects in studies with rodents, was

evaluated in zebrafish. Various mono- and disubstituted methyltin and butyltin compounds were administered to zebrafish embryos at a range of concentrations (octyltin compounds showed low solubility). At 24, 48, 72 and 96 hours post-fertilisation (hpf), various morphological and physical parameters were scored to assess lethality and/or developmental effects. At 100 hpf, all viable larvae were used to assess locomotor activity by means of a video-tracking system (View-point) and, subsequently, the larvae were processed for microscopic examination and for substance uptake determination. In the embryotoxicity assay, dose-dependent effects were observed on hatching and other abnormalities. In the motor activity assay, the most sensitive parameter was total distance moved, and a dose-related hypoactivity was frequently observed. Furthermore, microscopic examination also revealed histopathological changes in the brain of the larvae. When comparing the results of this study to *in vivo* data, the potency ranking of the compounds for developmental toxicity and neurotoxicity was comparable to their *in vivo* ranking. These results show that the zebrafish model is a promising model for the prediction of toxic potencies and a useful tool to study biological response similarities of structurally related substances.

Local Toxicity and Embryotoxicity of Organophosphate Insecticide (Malathion) Identified by Alternative *In Vitro* Methods

D. Jira,[1] K. Kejlova,[2] S. Janousek,[2] J. Pikula[1] and F. Vitula[1]
[1]University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic; [2]National Institute of Public Health, Praha, Czech Republic

The organophosphate insecticide, Malathion, is reported to be of low toxicity for man. Based on a LD50 value over 2000mg/kg for the rat and the mouse, it is generally not classified as toxic. The toxicity seems to be species-dependent, and is particularly dependent on carboxylesterase activity that breaks down toxic malaoxon generated in the liver by cytochrome P450. Distinct data also suggest inhibition of the thyroid gland hormones, degeneration of ovary follicle cells and increase in the incidence of fetus resorption. Local and systemic toxicity data for birds are rare and/or ambiguous, but a decrease in wild bird densities in areas where malathion was applied is repeatedly reported. The actual intoxication of wild birds may be influenced by the level of exposure from multiple sources, by age and sex of animals, by their state of nutrition and body condition. With the aim of extending knowledge on Malathion toxicity at the cellular and organ levels, we performed a number of experiments with progressive alternative *in vitro* methods that model local and systemic toxicity. The cytotoxicity was assessed in 3T3 fibroblast cultures. Skin and eye irritation potential was determined by using reconstructed skin and eye cornea tissues (EpiDerm™ and EpiOcular™). As no skin and eye cornea irritation potential was demonstrated, the HET-CAM test, which utilises the rich vascular system of the chorioallantoic membrane of chicken embryos in fertilised hen eggs, was used to detect effects on mucosa. The chick embryo model was employed further for an extended study on acute embryotoxicity (mortality and genotoxicity), dependent on the time and place of Malathion intra-embryonal application up to day 8 of chick embryo development. If Malathion was applied into the amnion cavity, then chick embryo mortality was identified at lower doses, at higher incidence and in earlier stages of development, in comparison with application into the air cavity. No genotoxicity was identified by means of the micronucleus test in erythroid MNE I and MNE II cells isolated from the chick embryo chorioallantoic vascular system, following morphological evaluation by optical and fluorescent microscopy. No significant changes in micronucleus and mitosis numbers were detected. The IC50 of 54.2 ± 3.1µg/ml, obtained by using the *in vitro* cytotoxicity test, which was recently validated as suitable to identify non-toxic, i.e. not classified, substances, suggests higher toxic potential of Malathion than is generally declared in literature based on conventional *in vivo* tests on laboratory rodents.

Coupling of a Drug Metabolising System to the BALB/c-3T3 Cell Transformation Assay

M.D. Brauneis, S. Oortgiese and P. Steinberg
University of Veterinary Medicine Hannover, Hannover, Germany

Analysis of the carcinogenic potential of chemicals plays an important role in toxicology. Up to now, the acquisition of such data requires a large number of animal experiments. The optimisation of the BALB/c-3T3 cell transformation assay could, in future prospects, lead to a significant reduction in the number of experimental animals used for carcinogenicity testing. This already well-established and well-suited method for high-throughput screening applications allows a quantitative analysis of the aforementioned carcinogenic potential of different chemicals. The principle of this test system is based on the fact that BALB/c-3T3 cells (murine embryonic fibroblasts) lose contact inhibition upon treatment with carcinogenic compounds. This leads to the development of so-called 'foci', which can be distinguished by characteristic changes in cell growth behaviour. Since foci formation is a direct result of treatment with a transforming compound, the number of foci is directly proportional to the carcinogenic potential of the compound. A major drawback of the 'classic' BALB/c-3T3 cell transformation assay is the examination of chemical compounds, which initially require a metabolic transformation to gain their full genotoxic potential. Hence, without prior metabolic transformation, many chemicals are not detected as being carcinogenic. To overcome this major disadvantage, the BALB/c-3T3 cell transformation assay has been coupled to a drug metabolising system in the present study. In an initial step, the metabolisation of the well-known genotoxic agents, benzo[*a*]pyrene, aflatoxin B1 and *N*-nitrosodimethylamine, by an S9 mix was investigated. All three compounds led to a concentration-dependent increase in the number of foci, whereby it is important to note that this concentration-dependent increase was observed within a non-cytotoxic concentration range. In a next step, the BALB/c-3T3 cell transformation assay will be coupled to further drug metabolising systems. One promising *in vitro* system for metabolic studies is the newly available cell line, HepaRG®, for which different applications will be tested.

Session III: 7th Amendment of EU Cosmetics Directive

Serum-free Long-term Repeated Dose and Acute Toxicity Studies Using the HepaRG Cell Line

S. Klein, D. Mueller and F. Noor
Saarland University, Saarbrücken, Germany

In vitro long-term repeated dose toxicity studies have gained increased importance in the cosmetic as well as the pharmaceutical industry, due to legal, safety and ethical reasons. Primary human hepatocytes (PHHs) are the current 'gold standard' *in vitro* model alternative to *in vivo* animal studies. However, their use is hindered due to their limited availability, short viability and inter-donor genotypic and phenotypic variabilities. The human HepaRG cell line represents a promising alternative for PHHs, not only in toxicological studies, but also for use in bioartificial livers, as has been shown in several studies. In contrast to other hepatic cell lines like HepG2, HepaRG cell metabolism and CYP expression levels are comparable to PHHs. Until now, there has been no available method for the serum-free long-term culture of HepaRG cells. However, serum impedes analytical methods, especially the analysis of the proteome, and might affect other cellular activities due to its inherent compositional complexity. It might also have other effects that have not been fully studied. In addition, the use of serum is ethically questionable. The aim of the study was to culture and maintain HepaRG cells in serum-free conditions for several weeks and to subsequently determine their suitability for long-term repeated dose toxicity studies. Furthermore, acute toxic effects on the viability and metabolism of the HepaRG cells were compared to those of other hepatic cells. With our results, we demonstrate the possibility of serum-free long-term culture of HepaRG cells, and its potential use in long-term toxicological studies. These results will not only have an impact on *in vitro* long-term repeated dose toxicity screening, but will also provide new applications in the '-omics' field of systems toxicology.

Retrospective Analysis of the Ability of Industry, Academia, and Animal Rights Organisations to Meet the Testing and Marketing Bans Established by the 7th Amendment of the Cosmetic Directive

H. Kandarova,[1] P. Hayden,[2] Y. Kaluzhny[2] and M. Klausner[2]
[1]MatTek In Vitro Life Science Laboratories, s.r.o., Bratislava, Slovakia; [2]MatTek Corporation, Ashland, MA, USA

In 2004, a timetable for phasing out animal testing under the framework of the 7th amendment to the Cosmetics Directive (CD) was established. Based on an *ad hoc* group of experts, the Scientific Committee on Cosmetic Products and Non-food Products (SCCNFP) presented opinions regarding whether alternative tests could be developed to meet the cut-off dates provided by the CD. At that point in time, the SCCNFP predicted that non-animal alternatives to the toxicological endpoints, skin irritation, eye irritation, skin adsorption, and UV-induced toxic effects, could be validated to meet the 2009 testing and marketing ban of the CD (attainable group). At the same time, the SCCNFP did not foresee the validation of alternatives to address acute toxicity, skin sensitisation, subacute and subchronic toxicity, genotoxicity, photo-allergy, toxicokinetics and metabolism, carcinogenicity, or reproductive and developmental toxicity prior to the 2009 testing ban, or the 2013 marketing ban, of the CD (non-attainable group). Retrospective analysis of progress in the development of alternatives was performed to determine the accuracy of the SCCNFP predictions. For the attainable group,

OECD guidelines utilising normal human cell-based 3-dimensional (NHu-3D) tissue models for skin irritation and skin absorption have been established, but only pre-validation studies for eye irritation or photogenotoxicity have been completed. For the non-attainable group, no animal alternatives have been validated, but active programmes utilising NHu-3D and other *in vitro* alternatives are ongoing in the areas of skin sensitisation, genotoxicity, photosentisation, and reproductive and developmental toxicity. Based on this analysis, the SCCNFP predictions were partially accurate, while the timetables set forth in the CD were overly ambitious. It is anticipated that validation of alternative assays will continue, but the realistic timetable for validation of these assays will be longer than previously anticipated.

Development of the EpiOcular™ Eye Irritation Test for Hazard Identification and Labelling of Eye Irritating Chemicals in Response to the Requirements of the EU Cosmetics Directive and REACH Legislation

Y. Kaluzhny,[1] H. Kandarova,[2] L. d'Argembeau-Thornton,[1] P. Hayden[1] and M. Klausner[1]
[1]MatTek Corporation, Ashland, MA, USA; [2]MatTek In Vitro Life Science Laboratories, s.r.o., Bratislava, Slovakia

The recently implemented 7th Amendment to the EU Cosmetics Directive and the EU REACH legislation have heightened the need for *in vitro* ocular test methods. To address this need, the EpiOcular™ eye irritation test (EpiOcular-EIT), which utilises the normal (non-transformed) human cell-based EpiOcular tissue model, has been developed. The EpiOcular-EIT prediction model is based on an initial training set of 39 liquid and 21 solid test substances and uses a single exposure period and a single cut-off in tissue viability, as determined by the MTT assay. A chemical is classified as an irritant (GHS Category 1 or 2), if the tissue viability is ≤ 60%, and as a non-irritant (GHS unclassified), if the viability is > 60%. EpiOcular-EIT results for the training set, along with results for an additional 52 substances, which included a range of alcohols, hydrocarbons, amines, esters, and ketones, discriminated between ocular irritants and non-irritants with 98.1% sensitivity, 72.9% specificity, and 84.8% accuracy. To ensure the long-term commercial viability of the assay, EpiOcular tissues produced by using three alternative cell culture inserts were evaluated in the EpiOcular-EIT with 94 chemicals. The assay results obtained with the initial insert and the three alternative inserts were very similar, as judged by correlation coefficients (r^2) that ranged from 0.82 to 0.96. The EpiOcular-EIT was pre-validated in 2007/2008, and is currently involved in a formal, multi-laboratory validation study sponsored by the European Cosmetics Association (COLIPA), under the auspices of the European Centre for the Validation of Alternative Methods (ECVAM). The EpiOcular-EIT, together with EpiOcular's long history of reproducibility and proven utility for ultra-mildness testing, make EpiOcular a useful model for addressing current legislation related to animal use in the testing of potential ocular irritants.

Session IV: *Directive 2010/63/EU* and Other Legal and Ethical Topics

Lack of Ethical Justification for Animal Experiments in Xenotransplantation

U. Gross and R. Kolar
Deutscher Tierschutzbund — Akademie für Tierschutz (German Animal Welfare Federation — Animal Welfare Academy), Neubiberg, Germany

The supply of donated organs for transplantation worldwide far outstrips the need. Legislation may increase the supply of organs, but even then, it is unrealistic to suppose that this need can ever be fully met. Some researchers suggest that a possible solution may be xenotransplantation (XTX), the use of animal organs in humans. Ethical problems associated with this procedure have been discussed elsewhere. However, one issue that has not received due attention is the severity of the animal experiments carried out in xenotransplantation research. The new EU guideline for the protection of animals used in scientific procedures states that animal experiments must be ethically justified. We here argue, with respect to the example of pig-to-primate xenotransplants, that this requirement cannot be met. On the one hand, the severity of XTX experiments must be assessed. Recipient animals, e.g. baboons, are subjected to isolation, handling, surgery, restriction of movement, anticoagulant treatment, and a severe regimen of multiple immunosuppressive modalities, simultaneously including removal of spleen, irradiation, plasmapheresis, and diverse drugs. Death may be caused by surgical complications, coagulation problems, infections, rejection, and graft failure. XTX experiments must be rated as exceptionally severe. On the other hand, can the expected benefits of the project justify the harm to the animals? Survival of transplanted pig hearts in primates only very rarely exceeds 30 days. The combination of intensive immunosuppressive, anticoagulant and other medication is quite impracticable in human patients for any length of time, and it is unrealistic to expect this situation to change, as no revolutionary new immunosuppressives are expected. Some argue that new genetic modifications — to a total of at least seven — in source animals, might decrease the need for immunosuppression. This argument is speculative and does nothing to justify more experiments (which are being planned in some centres) using pigs whose quadruple genetic modifications have already proved insufficient. Further, it is unrealistic to expect the severe and largely ungovernable problems that have occurred in quadruple-GM-pig to baboon transplants, to be the only ones that occur in humans. A new set of complications is to be expected. For all these reasons, clinical xenotransplantation of solid organs is an uncertain prospect and, at best, many say, 25 years in the future. Lastly, doubts are in order as to whether XTX can ever be an ethical treatment option for humans. Realistically, animal organs will always be inferior to human organs. Who decides which patient deserves a human organ, and which has to be content with an animal organ? This question cannot easily be resolved and raises a formidable ethical hurdle to XTX. Alternative treatments of organ malfunction are possible, stem cell therapies or the *in vitro* growing of organs among them. In light of a time horizon of 25 years, progress here is at least as likely as in XTX. We conclude that experiments involving severe, prolonged suffering in primates cannot be justified by doubtful expectations of a therapy that will, at its speculative best, still subject patients to lifelong harsh medication, and may be not realisable at all.

Session V: 3Rs Progress in Other Sectors

A Screening Method to Estimate Dermal Absorption *In Vitro*

E. Fabian,[1] C. Goth,[1] K. Guth,[1] A. Mehling,[2] B. van Ravenzwaay[1] and R. Landsiedel[1]
[1]BASF SE, Ludwigshafen, Germany; [2]BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany

Dermal absorption can be used in the evaluation of the effectiveness of pharmaceutical or cosmetic formulations, but often it is used as a critical parameter in the risk assessment of pesticides or chemicals. Therefore, knowledge of dermal absorption is helpful in formulation development. Skin absorption is routinely measured *in vivo* or *in vitro* following OECD Test Guideline (TG) 427 or 428. However, these tests are complex, time-consuming and expensive. Therefore, a study was developed to allow simple and rapid screening. The method uses dermatomised skin in modified Franz-type diffusion cells. Ten microlitres of test substance preparation are applied to the skin preparation. After 6 hours, the skin is washed and the amount of penetrated substance is quantified. The receptor fluid and the washing solutions are optimised for subsequent analyses by LC-MS. We performed dermal absorption screenings in parallel with our routine guideline studies, and demonstrated a good correlation between the results of both study types. The total recovery found in the screening studies is somewhat lower than in the corresponding guideline studies, but is always in the acceptable range above 80%. The efficacy of the skin washing procedure is less efficacious than under routine conditions, most probably due to the change to an LC-MS-compatible washing solution. Overall, dermal absorption screening is an easy, fast and cost-effective screening method for the estimation of dermal absorption of a wide variety of test substances and formulations.

Bridging the Gap Between Validation and Implementation of Non-animal Vaccine Potency Testing Methods

J. Brown,[1] S. Dozier[2] and A. Currie[3]
[1]PETA, Los Angeles, CA, USA; [2]PETA, Norfolk, VA, USA; [3]People for the Ethical Treatment of Animals (PETA) Foundation, London, UK

In recent years, technologically advanced high-throughput techniques have been developed that replace, reduce or refine animal use in vaccine quality control tests. Following validation, these tests are slowly being accepted for use by international regulatory authorities. Because regulatory acceptance itself has not guaranteed that approved humane methods are adopted by manufacturers, various organisations have sought to foster the preferential use of validated non-animal methods by interfacing with industry and regulatory authorities. We present a paradigmatic approach that seeks to ensure, quicken and confirm implementation of new replacement, refinement or reduction guidance. A systematic analysis of our experience in promoting the transparent implementation of validated non-animal vaccine potency assays has led to the refinement of our paradigmatic process, presented here, by which interested parties can assess the local regulatory acceptance of methods that reduce animal use and integrate them into quality control testing protocols, or ensure the elimination of peripheral barriers to their use, particularly for potency and other tests carried out on production batches.

'3R-Good Information Retrieval Practice' Module for Training of Supervisors (FELASA C)

N. Leich, S. Spätlich, D. Sittner, A. Doerendahl, S. Skolik, B. Grune and D. Butzke
ZEBET at the Federal Institute for Risk Assessment (BfR), Berlin, Germany

Directive 2010/63/EU states that: "The welfare of the animals used in procedures is highly dependent on the quality and professional competence of the personnel supervising procedures, …", and that: "Member States should ensure through authorisation or by other means that staff are adequately educated, trained and competent." (Recital 28) Since ZEBET staff have been engaged in the further education of scientists assigned to FELASA categories B and C for many years, we suggest here a concise module for the straight-forward training of supervisors in the general outline of a '3R-Good Information Retrieval Practice' (3R-IR), providing the basis for transparency and reproducibility in searching for alternatives.

Diversification of Focus: AnimAlt-ZEBET to Retrieve Alternatives in Basic Sciences

S. Spätlich, N. Leich, D. Sittner, A. Doerendahl, S. Skolik, B. Grune and D. Butzke
ZEBET at the Federal Institute for Risk Assessment (BfR), Berlin, Germany

The AnimAlt-ZEBET database now holds about 150 examples of alternative methods. Within the past 2 years, there has been a fundamental diversification of focus, with the objective of providing more alternatives for basic and medical research. Significant new topics thus have been, for example, alternative models for neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease; traumatic brain injury [TBI]) and respiratory diseases (e.g. acute lung injury [ALI]; acute respiratory distress syndrome [ARDS]) and the state-of-the-art of non-invasive techniques in cognitive neurosciences (e.g. MEG, EEG, fMRI, lf-NMR), which are eagerly anticipated to replace invasive primate models in the near future. Here, we depict the conception of the database and highlight some recent entries.

In Vitro Phototoxicity Assay for Systemically Administered Pharmaceuticals Using Reconstructed Human Skin Model EpiDerm

Y. Kaluzhny,[1] L. d'Argembeau-Thornton,[1] M. Kinuthia,[1] H. Kandarova[2] and M. Klausner[1]
[1]MatTek Corporation, Ashland, MA, USA; [2]MatTek In Vitro Life Science Laboratories, s.r.o., Bratislava, Slovakia

Both topically applied and systemically administered medications have the potential to induce photosensitivity. According to current regulatory guidelines, photosafety testing is required for a substantial number of drug development submissions (e.g. due to light absorption in the range of 290–700nm, or because the new compound partitions into the skin or eyes). However, there has been growing concern regarding the performance of the only approved, non-animal *in vitro* phototoxicity assay, 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU-PT), as being overly-sensitive in predicting the *in vivo* photosafety hazard to humans. EpiDerm™, a highly differentiated NHu-3D skin model, is highly reproducible, contains an *in vivo*-like barrier, possesses *in vivo*-like biotransformation capabilities, and has been pre-validated for determining phototoxicity of topically-applied materials. Here, we utilised EpiDerm to develop an *in vitro* assay for screening the phototoxic potential of pharmaceuticals after sys-

temic administration (EPI-sPHO). Test materials are added into the culture medium, allowed to partition into the epidermal skin model, and then exposed to solar radiation. Phototoxic effects are determined by comparing the tissue viability of UV-irradiated *versus* non-irradiated tissues models, as determined by using the MTT assay. A prediction model was established: a material is phototoxic after systemic administration if one or more test concentrations in the presence of irradiation (+UVR) decreases tissue viability by ≥ 30%, when compared to identical concentrations in the absence of irradiation (–UVR); a material is non-phototoxic if the decrease in tissue viability is < 30%. This prediction model resulted in high sensitivity (92.9%) and specificity (95%) for 34 test materials (20 phototoxic and 14 non-phototoxic). The current protocol extends phototoxicity testing with EpiDerm to the risk assessment of systemically administered chemicals and medications.

Session VI: Inhalation and Nanotoxicology

An Improved Co-culture System Mimicking the Cellular Organisation at the Alveolar Barrier to Study the Potential Toxic Effects of Particles

S. Klein,[1] T. Serchi,[1] L. Hoffmann,[1] B. Blömeke[2] and A. Gutleb[1]
[1]Centre de Recherche Public — Gabriel Lippmann, Belvaux, Luxembourg; [2]Department of Environmental Toxicology, University of Trier, Trier, Germany

Exposure to particulate matter (PM) and to engineered nanoparticles (eNPs) has increased in the last century, due to intensified combustion and industrial processes, road traffic, etc. Exposure to small particles of less than 100nm in diameter is linked with an increased risk of respiratory diseases, such as asthma or rhinitis. In addition, it was shown that small particles can cross the alveolar epithelial barrier and affect the underlying cells by inducing oxidative stress. Furthermore, recent data suggest that especially ultrafine particles might be responsible for the detrimental effects of PM. However, to date, more than 1000 customer products contain eNPs, despite the poor knowledge about their toxic potential and health effects. Here, we present a complex co-culture system consisting of four different human cell lines, that mimics the cell response of the alveolar surface *in vitro* after exposure to particles. The system is composed of an alveolar Type-II cell line (A549), differentiated macrophage-like cells (THP-1), mast cells (HMC-1) and an endothelial cell line (EA.hy 926). Since the alveolar surface *in vivo* is also lined by a thin surfactant film, *in vitro* systems to study effects of particles should also consider this additional barrier. Surfactant plays an important role, as it lowers the surface tension, prevents the cells from drying out and facilitates particle displacement *in vivo*. The cells are grown on microporous membranes under submerged conditions until confluence, and then cultured at the air–liquid interface to force the alveolar Type-II cells to release the surfactant. Epithelial cells, macrophages and mast cells are seeded on the luminal side of the membrane. In order to resemble *in vivo* conditions, the endothelial cells are seeded on the outside of the membrane. After differentiation, the macrophage-like THP-1 cells act as efficient sentinels, intercepting particles in the *in vitro* system. The pore size of the membrane plays an important role, since it either prevents or enables communication between cell layers. Small molecules, such as resazurin or sodium fluorescein, seem to be unable to pass through a 0.4μm membrane in any significant amount. The system is used to study the potential of NPs to induce oxidative stress and a potential inflammatory response. To provide a uniform exposure to particles, a state-of-the-art aerosol chamber (Vitrocell) is used to expose the cells at the air–liquid interface. The outcome of these studies will enhance our mechanistical understanding of particle-induced inflammation.

Human 3D Airway Models Compared to Cell Culture Systems and *In Vivo* Acute Inhalation Studies

A. Hess,[1] S. Vogel,[1] S.N. Kolle,[1] U.G. Sauer,[2] B. van Ravenzwaay[1] and R. Landsiedel[1]
[1]BASF SE, Ludwigshafen, Germany; [2]Scientific Consultancy — Animal Welfare, Neubiberg, Germany

Information on acute inhalation toxicity has to be provided for all non-corrosive chemicals manufactured or imported in quantities of 10 tonnes per year or more, if inhalation is the relevant secondary route of exposure, as well as for agrochemicals, regardless of tonnage, if there is likelihood of inhalative exposure. Acute inhalation toxicity is expressed in terms

of LC50 values — the test substance concentration that leads to death of 50 percent of the animals after 4 hours of exposure. With regard to animal distress, toxicity studies are assigned to the highest severity category, and the new EU Directive on the protection of laboratory animals stipulates further implementation of the Three Rs principles of replacing, reducing and refining animal testing. Whereas a reduction and refinement alternative to the traditional *in vivo* acute inhalation toxicity study (OECD Test Guideline 403) was adopted in 2009 (OECD Test Guideline 436), a replacement method is not yet available. To make a contribution toward filling this gap, the usefulness of different *in vitro* test systems in determining single-dose inhalation toxicity was investigated in an in-house validation study. Twenty substances were tested in two 3D *in vitro* human reconstructed epithelial airway models, EpiAirway™ (MatTek, USA) and MucilAir™ (Epithelix, Switzerland), reflecting normal human bronchiole histology, and in an *in vitro* human A549 adenocarcinomic alveolar basal epithelial cell culture system, by using endpoint detection methods applicable to high-throughput screening, such as mitochondrial activity and lactate dehydrogenase release. To compare the respiratory tract-related *in vitro* data to unspecific cytotoxicity, all substances were further tested on 3T3 mouse embryonic fibroblasts. For all test runs, IC50 and IC75 values were calculated. The results from all four *in vitro* assays were compared to available *in vivo* data for the 20 test substances having known modes of toxicological action. *In vitro*/*in vivo* comparisons of the study results showed that the *in vitro* models are only predictive of *in vivo* respiratory toxicity for a subset of substances with specific modes-of-action. Detailed evaluations revealed the diversity of issues that remain to be addressed to enable replacing *in vivo* acute toxicity studies. *In vitro* models require strict definitions of their applicability domains and further test protocol development. *In vivo* respiratory toxicity data to be used for *in vitro*/*in vivo* correlations need to distinguish between different modes-of-action, and their relevance for human health effects should be ensured.

A Three-dimensional hESC-derived System to Assess Chronic Nanotoxicity to the Developing Nervous System

L. Hoelting,[1] B. Scheinhardt,[1] S. Mecking,[1] V. Tanavde,[2] M. Leist[1] and S. Kadereit[1]
[1]University of Konstanz, Konstanz, Germany; [2]Bioinformatics Institute AStar, Singapore

During development of the nervous system, even small disruptions in differentiation and patterning processes may result in severe impairment of nervous system function. Due to uncertainties in the extrapolation of results based on animal tests to the *in vivo* situation in humans, there is a need for alternative methods to detect developmental neurotoxicity (DNT). Human embryonic stem cell (hESC) technology provides a tool to recapitulate critical aspects of early human neurodevelopment. The rapid development of nanotechnology has led to the wide use of nanoparticles (NP) in various sectors, such as foods, cosmetics, electronics, biotechnology and medicine for drug delivery and diagnostic purposes. However, until now, there has been a lack of data on the long-term accumulation of nanoparticles and their potential risk for human health. A human neural differentiation culture system was established, providing a three-dimensional neurosphere environment in which the balance between neural precursor cell (NPC) proliferation and differentiation shifts toward neuronal differentiation. This system combines many cell types in different stages of differentiation, and allows for a dynamic, three-dimensional communication between cells, thus mimicking the *in vivo* tissue situation. Moreover, exposure can be long-term, thus modelling natural exposure to accumulating NP in the environment. Differentiation was characterised by whole gene expression analysis and qRT-PCR, showing a progressive decrease in neural stem cell markers and concomitant up-regulation of genes involved in nervous system patterning and neuronal precursor differentiation. Toxicity of chronic exposure to inert polyethylene nanoparticles (PE-NPs) was assessed. Fluorophores were incorporated into the PE-NPs to enable tracing in the cells. A rapid incorporation of nanoparticles into the neurospheres was observed after 24 hours of exposure. After 18 days of exposure, our neural differentiation system sensitively

measured toxicity to crucial early neurodevelopmental genes at non-cytotoxic concentrations of chemically inert PE-NPs. In contrast to normal cell lines usually used to assess nanotoxicity, this stem cell-based differentiation system is dynamic, with continuously differentiating cells. Thus, the effects of chronic exposure to nanoparticles on dynamic processes taking place during neural development can be modelled more closely.

Effect of TiO_2 Nanoparticle Size on Possible Skin Penetration *In Vitro*

D. Jirova,[1] K. Kejlova,[1] P. Pikal,[2] L. Kasparova,[1] K. Safarova,[3] L. Kovarikova,[2] H. Bendová [1] and D. Zalabak[4]

[1]National Institute of Public Health, Praha, Czech Republic; [2]Czech Technology Centre for Inorganic Pigments, Přerov, Czech Republic; [3]Regional Centre of Advanced Technologies and Materials, Palacký University, Olomouc, Czech Republic; [4]Centre of the Region Haná for Biotechnological and Agricultural Research Faculty of Science, Palacký University, Olomouc, Czech Republic

The current project, NANOCOVER, supported by the Technological Agency of the Czech Republic, is focused on the development and modification of TiO_2 particles designed for specific applications, e.g. as UV filters, antimicrobial functional textiles or photoactive self-cleaning coating materials. Considering the crucial role of dermal penetration of topically applied substances for systemic toxicity, five newly engineered TiO_2 nanoparticles were subject to *in vitro* skin absorption/penetration studies, in combination with highly sensitive analytical techniques. The nanoparticle size (median diameter d[i,50]), as measured by dynamic light scattering (DLS), ranged from 26 to 200nm. Porcine skin served as the substrate for skin penetration studies *in vitro* combined with histological evaluation. Skin penetration studies were performed according to OECD Test Guideline (TG) 428, by using static Franz cells and a 24-hour application period. The presence of TiO_2 in various skin layers was determined by using inductively-coupled plasma mass spectrometry (ICP-MS). The skin penetration test revealed that none of the tested TiO_2 particles penetrated into the receptor fluid, confirming no risk of systemic exposure via the bloodflow. Penetration of particles with a median diameter of 26nm, and particles above 100nm, exhibited a markedly different pattern. In the case of particles with a median diameter of above 100nm, more than 95% of the recovered TiO_2 remained on the surface of the skin, about 4% of TiO_2 was detected in the stratum corneum/epidermis and less than 1% in the dermis. In the case of neopentylglycol (NPG)-stabilised nanoparticles (particle median diameter of 26nm), only 45% of the recovered TiO_2 stayed on the surface of the skin, while ~45% of TiO_2 infiltrated the stratum corneum/epidermis. Tape stripping proved maximum accumulation in the first four layers (out of 16 strips) of the stratum corneum. About 6% of TiO_2 was identified in the dermis. Although the mass proportion of TiO_2 identified in the dermis in the case of nanoparticles with a diameter of 26nm, is significantly lower when compared to particles with a diameter of over 100nm, the biological risk may be expected to be higher due to a considerably larger biologically-active surface area, depending on the localisation in the tissue. A pilot study utilising scanning electron microscopy (SEM) was performed on cross sections of porcine skin exposed to NPG-stabilised TiO_2 nanoparticles, in comparison with particles with a median diameter of above 100nm. Small specimens (~5 × 5 × 5mm) containing hair follicles were excised by scalpel from the titanium dioxide-treated porcine skin discs. Specimens were subsequently fixed, dehydrated and infiltrated by LR white resin, and embedded in gelatin capsules. Cross-sections of porcine hair follicles were then attached to the SEM holder and characterised by scanning electron microscopy (SEM). The scanning electron microscopy was able to identify nanoparticles (particle median diameter of 26nm) on top of the stratum corneum, but did not confirm presence of nanoparticles in hair follicles or channels. It seems apparent that this method is not suitable for visualisation of these nanoparticles in the large mass of biological material. More robust and relevant optical methods are suggested for the visualisation of nanoparticles.

A Short-term Model for the Induction of Specific Respiratory Hypersensitivity Reactions to Proteins in Rats

C. Stahl
Bayer Pharma AG, Germany

Respiratory sensitisation represents a considerable clinical problem. Low-molecular weight molecules and particles, as well as larger molecules such as proteins, contribute to the development of occupational respiratory lung disease. Today, pre-clinical studies for hazard identification are based on long-lasting and labour-intensive inhalation studies, which vary with respect to experimental design. Regarding the Three Rs concepts, refinement of these methods is, without question, necessary. The goal of our studies is to tap into mechanisms from short-term treatments as a tool for hazard identification of high-molecular weight molecules causing respiratory sensitisation. In our studies, we performed intra-tracheal application to induce specific respiratory hypersensitivity reactions to proteins (ovalbumin [OVA], keyhole limpet haemocyanin [KLH]) in rats, in a 15-day model. Cell counts of eosinophils, a parameter which can easily be determined in bronchoalveolar lavage fluid (BALF), and changes in cytokine pattern, turned out to exhibit potential endpoints. As verified by cross-experiments (OVA ↔ KLH), both proteins provoke a strongly specific secondary immune response after challenge. Especially with regard to the increasing development of biologics, such a screening method will enable reduction of the number of laboratory animals. The general aim is, of course, to further reduce the use of laboratory animals by adequate *ex vivo* or even *in vitro* models as alternatives. Therefore, our attempt is to transfer some of the results gained in the short-term *in vivo* studies concerning specific T-cell activation, to an *ex vivo* model. One of our approaches is the *in vitro* restimulation of T-cells by previously antigen-pulsed macrophages, where we have already obtained initially promising results.

MucilAir *versus* RAW264.7 Cells in Nanotoxicology

F. van Acker,[1] M. Gröllers,[2] A. Reus,[1] Y. Staal[1] and F. Kuper[2]
[1]TNO Triskelion, Zeist, The Netherlands; [2]TNO, Zeist, The Netherlands

Predictive *in vitro* tests are needed to rank nanomaterials according to toxicity and bioavailability, in order to determine priority for subsequent *in vivo* testing. *In vitro* nanomaterials are predominantly studied in A549 cells (representing alveolar epithelial cells) or RAW264.7 cells (representing macrophages). The use of human 3D airway models opens up new possibilities for the testing of nanomaterials. The 3D models consist of fully differentiated human respiratory epithelial cells and allow relevant exposure via air, as they are cultured at an air–liquid interface. To investigate the applicability of human 3D airway models in the safety assessment of nanomaterials, we compared the toxicity of SiO_2 and CeO_2 nanoparticles to MucilAir™ (EpiThelix Sarl) and to RAW264.7 macrophages. MucilAir™ inserts and RAW264.7 cells were exposed to the nanoparticles for 24 hours, via droplet exposure on the tissue surface and via the medium, respectively. Cytotoxicity was measured by LDH assay (in both), by TEER measurement (MucilAir™), or by MTT assay (RAW264.7 cells). Various cytokines were analysed in the culture medium, as a measure of inflammation. Oxidative stress and genotoxicity were evaluated by the determination of HO-1 expression and by comet assay, respectively. In RAW264.7 cells, SiO_2 and CeO_2 were cytotoxic at similar concentrations, but SiO_2 induced only TNF-α, whereas CeO_2 induced only HO-1 expression and caused an increase in percentage tail DNA. In MucilAir™, no significant effects were seen on all endpoints tested, at up to 10-fold higher concentrations. It seems that MucilAir™ is less sensitive toward particle induced toxicity, as compared to cell lines. This may be more realistic, as the interaction of particles with mucus is taken into account and the cells are morphologically similar to human airway epithelium, in contrast to RAW264.7 cells. In the

future, we will further assess the applicability of human 3D airway models by exposure via different routes, and will compare the results with both cell culture and *in vivo* inhalation data. Ultimately, these models may be useful in the safety evaluation of engineered nanomaterials.

An NGO's Role in Nanomaterials Regulation

S. Dozier
PETA, Norfolk, VA, USA

PETA became involved in developing nanomaterial toxicity testing guidance in 2005, when most regulatory agencies were in the early stages of defining nanomaterials. A PETA specialist in nanomaterials participated in these initial meetings both in the United States and Europe, as regulators around the world were grappling with many of the same issues relating to regulating this novel chemical type. The global nanotoxicology community struggled to determine the most effective assessments of nanomaterial toxicity. A top priority was to address concerns relating to the many scientific problems associated with animal-based toxicology in general, and pulmonary toxicity testing specifically. We worked closely with regulators and top toxicologists in the field to steer regulators and industry away from instillation testing, and identified the most promising suite of *in vitro* pulmonary toxicity test methods to replace reliance on rat inhalation tests. We have presented *in vitro* and *ex vivo* barrier testing methods and contributed to international standards that cite scientifically valid, *in vitro* methods. These endpoints include: developmental toxicity and embryotoxicity, the blood–brain barrier, as well as nano-specific concerns (e.g. inflammation and oxidative stress, among others). PETA's seat on national and international standards-making bodies will further contribute to overcoming transatlantic regulatory hurdles that prevent the use of sophisticated non-animal methods in the emergent field of nanotechnology.

Session VII: 3R Goes 3D — Implementation of 3D Methods in Toxicity Testing

Prevalidation of a Bioengineered Cornea Model for Ocular Drug Absorption Studies

M. Zorn-Kruppa,[1] M.R. Hahne,[2] G.M. Grobe,[2] G. Guzman,[3] J.M. Brandner,[1] E. Haltner-Ukomadu[3] and S. Reichl[2]
[1]Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; [2]Technische Universität Carolo Wilhelmina zu Braunschweig, Braunschweig, Germany; [3]Across Barriers GmbH, Saarbrücken, Germany

The steadily growing market for ophthalmic drugs in the past decades has increased the demand for preclinical studies such as transcorneal drug absorption measurements. Due to the lack of alternatives, many experiments are still carried out *in vivo* and *ex vivo* on animals. Bioengineered models of the human cornea could avoid several disadvantages of animal experiments, such as ethical concerns and poor standardisation. However, so far, no validated and generally accepted *in vitro* model is available for drug absorption assessment. In previous studies, we developed a three-dimensional model of the human cornea (Hemicornea, HC), based on immortalised human-derived corneal cells, and we established standard operating procedures (SOPs) for its cultivation and for its use in drug absorption experiments under serum-free conditions. In the present study, the HC model was subjected to a prevalidation approach by using pharmacologically-relevant drugs with varying physicochemical properties. The corneal equivalents were produced independently in three participating laboratories, and the intra- and interlaboratory reproducibility was analysed and compared to *ex vivo* data from rabbit and porcine corneas. Our results showed that the HC model demonstrates a tight barrier, with permeation coefficients for the tested substances which were similar to those generated from *ex vivo* corneas. The methods for HC cultivation and permeation were successfully transferred to the partner labs and generated reproducible results. Intra- and interlaboratory comparison revealed high equivalence of the data. Therefore, the HC model represents a promising *in vitro* alternative to the use of *ex vivo* tissue and offers a well-defined and standardised system for drug absorption studies.

Applicability of *In Vitro* Test Strategies for Skin Irritation to Regulatory Classification Schemes: Substantiating Test Strategies with Data from Routine Studies

S.N. Kolle,[1] K. Sullivan,[2] A. Mehling,[3] B. van Ravenzwaay[1] and R. Landsiedel[1]
[1]BASF SE, Ludwigshafen, Germany; [2]Physicians Committee for Responsible Medicine, Oakland, CA, USA; [3]BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany

Skin corrosion or irritation refers to the production of irreversible or reversible tissue damage to the skin following the application of a test substance, respectively. Traditionally, hazard assessments of chemicals are conducted by using the *in vivo* Draize skin irritation test. Due to animal welfare considerations and regulatory provisions such as the European Cosmetics Regulation, *in vitro* tests with different reconstructed human skin models were developed and have now gained regulatory acceptance (OECD Test Guideline [TG] 431 and OECD TG 439). In the study presented, skin corrosion and/or irritation test protocols with a reconstructed skin model were integrated into testing strategies, to reduce the number of *in vivo* Draize skin irritation tests. The *in vivo* test was performed as required by the regulatory bodies. Over 100

substances have been tested, and include a wide range of different chemical classes. The results were assessed in a regulatory context according to the UN-GHS, CLP, Brazilian and US EPA guidelines.

A 3D Reconstructed Hemi-cornea Model for Predicting the Eye-irritating Potential of Chemicals: Results of an Inter-laboratory Evaluation Study

M. Engelke,[1] M. Bartok,[2] D. Gabel,[2] M. Zorn-Kruppa,[3] K. Reisinger[4] and K. Mewes[2]
[1]University of Bremen, Bremen, Germany; [2]Jacobs University, Bremen, Germany; [3]University Hospital Hamburg-Eppendorf, Hamburg, Germany; [4]Henkel AG & Co. KGaA, Düsseldorf, Germany

Currently, no single *in vitro* test system or respective test battery are able to completely replace the Draize Rabbit Eye Irritation Test. For example, by using validated assays, only severe irritants can be identified. Based on a previously developed 3-dimensional hemi-cornea consisting of both an epithelium and collagen-embedded stromal cells, we intended to establish a test method to reliably distinguish between eye-irritating and non-irritating substances. In an inter-laboratory trial test we aimed to: a) demonstrate the quality controlled production of the hemi-cornea model in two independent laboratories; and b) assess the predictive capacity and intra-laboratory and inter-laboratory reliability of the test system. Both laboratories produced their own hemi-cornea tissues according to the standard operation procedure (SOP). Only tissue models matching the predefined quality criteria based on negative (NC) and positive controls (PC), qualified for subsequent use in the eye irritation study. A set of 20 chemicals was tested under blinded conditions, to assess the performance and limitations of the test system comparing three different prediction models. The test chemicals comprised different chemical classes with different eye-irritating properties according to the Draize Rabbit Eye Irritation Test, covering all three GHS categories. Test chemicals, as well as negative and positive controls, were applied topically onto the surface of the hemi-corneal tissue and the viability of the entire tissue after different exposure intervals was monitored by using the MTT assay. The most suitable prediction model of the test system revealed *in vitro–in vivo* concordance of 80% and 70% in the participating laboratories, respectively, and an inter-laboratory concordance of 80%. Sensitivity of the test was 77% and specificity was between 57% and 86%. Although the results are promising, the test system has to be further optimised in order to enhance the predictive capacity. We conclude that additional physiologically-relevant endpoints in both the epithelium and stroma have to be developed for a satisfactory selectivity of the test system, and hence for the prediction of all GHS classes of eye irritation in one stand-alone test system.

Interferon (IFN) Induction in the 3D Culture

B. Filipic,[1] G. Lidija,[2] C. Avrelija[2] and S. Koren[1]
[1]Institute for Microbiology and Immunology, Medical Faculty, University of Ljubljana, Ljubljana, Slovenia; [2]Faculty of Agriculture and Life Sciences, University of Maribor, Maribor, Slovenia

There is a wealth of evidence that 3D cell cultures better mimic the *in vivo* conditions of a multicellular organism than do 2D cultures, in which cells adhere to glass or plastic surfaces. Often, the responses of cells in a 2D culture to various cues are quite dissimilar from those of cells *in vivo* or in a 3D culture. This is partly because the physiology of a cell is determined, in addition to the genome, by its micro-environment — including the mechanical properties of the extracellular matrix and its physical and chemical anisotropies, which are quite different in 3D and 2D cultures. Therefore, 3D cultures are preferable over 2D cultures for use in a number of fields, such as studies on stem cell differentiation, tissue morphogenesis, cancer biology, cell–virus interactions, and cell-based drug screening and toxicology assays. Here, we report interferon (IFN) induction in a 3D culture of goat intestinal epithelial cells. Cells were cultured on the Alevetex® highly porous, cross-linked polystyrene scaffold, which was sectioned into 200μm-thick membrane discs. These discs provide a suitable 3D structure in which cells can proliferate. Goat cells were seeded at a density of 1.5×10^6 cells in 150μl of medium, per disc. Discs were cultured in modified Eagle's medium with 8% SR-20552. After 7 days of incubation at 37°C in a 5% CO_2 atmosphere, when cell growth was confluent, IFN induction with the Sendai virus (100HA units/10^6 cells) for 24 hours, was performed. In comparison, cells were cultured in the normal 2D plane and IFN was induced with the same amount of Sendai virus. After induction, β-IFN was tested on WISH (human amniotic cell line) cells cultured in modified Eagle's medium with 2% SR-20552 at 37°C in 5% CO_2. The IFN assay was performed in comparison to standard human β-IFN (1000IU/ml), with the VSV (vesicular stomatitis virus) as challenge. It is interesting to note that the level of β-IFN/10^6 cells in the 3D culture was 2–3 times higher than that of cells in 2D culture, where it was aproximately 800IU/10^6 cells. It seems that the 3D micro-environment and cell–cell contacts affect IFN induction and release.

Analysis of the Validated EpiDerm Skin Corrosion Test (EpiDerm SCT) and its Prediction Model for Sub-categorisation of Corrosive Substances According to UN GHS and EU CLP

H. Kandarova,[1] S. Letasiova,[1] P. Hayden,[2] Y. Kaluzhny[2] and M. Klausner[2]
[1]MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia; [2]MatTek Corporation, Ashland, MA, USA

Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the epidermis and into the dermis, following the application of a test material (as defined by the United Nations [UN] Globally Harmonised System of Classification and Labelling of Chemicals [GHS]). OECD has adopted two ECVAM-validated reconstructed human skin models (EpiDerm and EPISKIN) for testing skin corrosion (OECD Test Guideline [TG] 431), and currently is in the process of adopting another two methods (SkinEthic and EST-1000) based on concept of performance standards. However, OECD TG 431 currently does not satisfy international labelling guidelines for transport of dangerous goods, since none of the methods were adopted with a prediction model allowing for sub-categorisation. The UN GHS utilises three corrosion sub-categories (1A: very dangerous, 1B: medium danger, and 1C: minor danger). Labelling a chemical as sub-category 1A has important consequences, including very small volume package limits for air transport, prohibition from passenger aircraft, protective storage conditions, costly containers and low market acceptance. Animal tests are still utilised for assessing the 1A label requirement. An *in vitro* method that discriminates 1A from 1B/1C classes will therefore have a substantial impact on

reducing animal tests for this purpose. The current poster evaluates the ability of the data obtained with the EpiDerm skin corrosion test (SCT), to discriminate between UN GHS 1A, 1B/1C classes and non-corrosives. Data obtained during the ECVAM validation study (Phase I and Phase III), and from additionally tested chemicals, were analysed based on the MTT viability assay and the 3-minute exposure period. For the dataset containing 80 chemicals (with known *in vivo* GHS classifications), the 3-minute endpoint produced sensitivity > 90% for predicting sub-category 1A. It has been demonstrated that the MTT-reducers require special attention and thus additional testing, by using freeze-killed tissues at both the 3-minute and 1-hour endpoints, is necessary. The EpiDerm SCT provides an *in vitro* procedure allowing the identification of non-corrosive and corrosive substances and mixtures. As demonstrated by the results obtained in this study, it also allows a partial sub-classification of corrosives into sub-category 1A, 1B/1C and no category. Adoption of the 3-minute endpoint in the EpiDerm SCT prediction model to identify severely corrosive substances, would lead to significant reduction in animal use for corrosion sub-group package labelling.

Session VIII: Free Communications

Foundation Animalfree Research: Current Activities and Future Goals

S. Schindler
Animalfree Research, Zürich, Switzerland

As in previous years, the foundation Animalfree Research (AfR) has pursued activities in the field of replacement and reduction of animal experiments — on a scientific, public and political level. Nanotoxicology has remained one of our core interests; a presentation on the opportunities and risks of nanomaterials aimed at the general public was held in April 2011 at the University of Zurich. The main topic was, of course, the issues of animal experiments in this field — with regard to animal welfare as well as to science. Other activities comprised, for example, a response to the EFSA statement on the planned required animal testing of nanomaterials in food and feed, which was published in *ATLA*, and the conception of a study that intends to examine the feasibility of integrated testing strategies (ITS) for nanomaterials, starting in the course of this year. In addition, we are proud that one of our major projects will be concluded in 2012; the development of an *in vitro* assay for the testing of residual toxicity of tetanus vaccines has been completed. If it is validated successfully, this alternative method will replace animals in a widely used and potentially highly distressing test. In order to further strengthen the use and implementation of Three R methods in Switzerland, our foundation is planning to launch an information centre on alternative methods. Its activities will include offering advice for researchers, education and information for students and teachers, and the active participation in the legally required courses at the University of Zurich. In a similar vein, we will continue our work in the various organisations and working groups, with the goal of continuously aiding and enforcing the implementation and use of alternatives to animals.

The Isolated Perfused Equine Distal Limb as a New *Ex Vivo* Model for Pharmacokinetic Studies

M. Friebe and M. Kietzmann
Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

Studies investigating the pharmacokinetic properties of intra-articularly administered drugs in horses are crucial because of medication regulations in equine sports. However, *in vivo* studies are impeded by high costs, effort and the potential harm of the physical integrity of the horses involved. Therefore, it was the aim of the present study to establish and validate an *ex vivo* model of an isolated perfused equine distal limb, in order to facilitate pharmacological studies concerning the equine foot. Distal forelimbs of slaughtered horses (n = 11) were perfused with gassed (Carbogen, 95% O_2 and 5% CO_2) bodywarm Tyrode's solution, for up to 8 hours, at a perfusion rate of approximately 65ml/min, based on the reported blood flow in the digital artery *in vivo*. To increase the oncotic pressure within the vessels, sodium carboxymethyl cellulose was added to the perfusion medium (0.15g/L). The venous perfusate was not recirculated (open system). Based on other established models of isolated perfused organs, tissue viability was confirmed by repeated measurements of glucose consumption, lactate production and lactate dehydrogenase activity in the venous perfusate, determination of skin surface temperature and histological examinations of the joint capsule. Before and after perfusion, forelimbs were weighed to quantify oedema formation. Viability parameters did not

show any significant changes over the perfusion period of 8 hours, in 11 separate experiments. Average glucose consumption was 355mg/h, mean lactate production was 252mg/h and LDH activity averaged at 4.5U/h. Mean skin temperature during perfusion was 26.7°C and weight increased by 4.5%. Histological sections of the fetlock joint capsule did not show any changes in the intimal layer of the synovial membrane, while the subintimal connective tissue appeared slightly oedematous. An *ex vivo* model was established to facilitate pharmacological studies concerning the equine distal limb, without the need to use living horses. The model offers several advantages — for example, unique sample sites are available that are hardly accessible *in vivo* and there is the possibility of withdrawing samples in very short time intervals. First applications of the model included the distribution of intra-articularly administered betamethasone into the systemic circulation, as well as the concentration of systemically administered acetylsalicylic acid and salicylic acid into the synovial fluid of the fetlock joint.

Human Multipotent Progenitors — A Promising Cell Model for Assessing Developmental Osteotoxicity *In Vitro*

D. Sittner, B. Huhse, A. Luch and A. Seiler
Federal Institute for Risk Assessment, Berlin, Germany

To assess the potential adverse effects of substances on bone development in man, animal experimentation is commonly employed. *In vivo* testing is labour- and cost-intensive and requires a high number of laboratory animals. Up to now, there exists no validated alternative method to assess developmental bone toxicity *in vitro*. The derivation of multipotent progenitors with mesenchymal characteristics from human embryonic stem cells (hES-MPs) constitutes one strategy in regenerative medicine, to work with a cell source that exhibits a low risk of tumour formation after transplantation. These cells display the typical morphology of primary human mesenchymal stem cells and show a similar gene expression profile. In addition, they have a high proliferative activity and the capacity to differentiate into specialised cell types of mesenchymal origin *in vitro* (adipocytes, chondrocytes, osteoblasts). Notably, their experimental use in Germany is exempt from regulatory approval enforced by the German Stem Cell Act. Therefore, hES-MPs appear to be an attractive and promising human-based cell model to screen for potential osteotoxic substances. Crucial stages during osteogenesis involve the proliferation of progenitor cells, followed by their gradual differentiation into functional osteoblasts, the maturation of the extracellular matrix (ECM) and, ultimately, the mineralisation of the ECM. Initial work to study the osteogenic differentiation process of hES-MPs has already been accomplished by another research group. Based on their findings, we further characterised the differentiation process regarding the influence of different inducer cocktails, the delineation of the developmental stages and the expression of lineage-specific protein markers. A diverse range of biochemical and molecular biological methods, e.g. cell viability and proliferation assays, colorimetric assays, cytochemical stainings, flow cytometry and western blotting, were employed to monitor the underlying molecular processes. Additionally, we performed initial experiments to investigate the sensitivity of the differentiating cells toward developmental toxicants. In summary, the hES-MP cell model might prove to be a valuable tool for assessing compound-mediated adversity on human bone development *in vitro*.

Review of Skin Irritation/Corrosion Hazards on the basis of Human Data: A Regulatory Perspective

D. Basketter[1], D. Jirova[2] and H. Kandarova[3]
[1]DABMEB Consultancy Ltd, Sharnbrook, UK; [2]National Institute of Public Health, Prague, Czech Republic; [3]MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia

Regulatory classification of skin irritation has historically been based on rabbit data. However, current toxicology processes are transitioning to *in vitro* alternatives. The *in vitro* assays have to provide sufficient level of sensitivity as well as specificity, to be accepted as replacement methods for the existing *in vivo* assays. This is usually achieved by comparing the *in vitro* results to classifications obtained in animals. A significant drawback of this approach is that neither *in vivo* nor *in vitro* methods are calibrated against human hazard data, and the results obtained in these assays might not correspond to the situation in man. The main objective of this review was to establish an extended database of substances classified according to their human skin irritation hazard, to serve for further development of alternative methods relevant to human health as well as resource for improved regulatory classification. The literature has been reviewed to assemble all the available hazard information on substances tested by using the human 4-hour human patch test, which is the only standardised protocol in humans matching the exposure conditions of the traditional regulatory accepted *in vivo* rabbit skin irritation test. A total of 81 substances tested according to the defined 4-hour human patch test protocol were found and collated into a dataset, together with their existing *in vivo* rabbit classifications published in the literature. While about 50% of the substances in the database are classified as irritating based on the rabbit skin test, when using the 4-hour HPT test less than 20% were identified as acutely irritant to human skin. Based on the presented data, it can be concluded that the rabbit skin irritation test largely over-predicts human responses for the set of evaluated chemicals. Correct classification of the acute skin irritation hazard will only be possible if newly developed *in vitro* toxicology methods are calibrated to produce relevant results to man.

Xenobiotic-metabolising Systems in the Chick Embryo Liver — Development of Simple and Sensitive Assays

W. Hoppe and G. Baron-Ruppert
University of Osnabrück, Osnabrück, Austria

The metabolism of xenobiotics in liver comprises two steps — oxidation by mixed-function oxidases (CYP450) and conjugation with glucuronic acid. Oxidases are also involved in the activation of pharmaceuticals, and thus are required in functional assays. Since few studies have focused on the activity of these enzyme systems in the liver of developing chick embryos, we have developed sensitive assays for the determination of CYP450 and glucuronyltransferase activities, particularly in the first half of embryonic development. CYP450 activities were assayed with 7-ethoxycoumarin (EC) as the substrate and 7-hydroxycoumarin (HC), the product in this reaction, was quantified by fluorescence after separation by a new and simple differential extraction procedure. Activities of glucuronyltransferases were assayed with 4-methylumbelliferone (MU) and the amount of 4-methylumbelliferyl-glucuronide (MU-GlcA) was measured by a sensitive HPLC method. Livers from developing embryos were extracted and fractionated by differential centrifugation. Neither the direct determination of HC by fluorescence in the presence of EC, nor the one-step extraction with chloroform described in the literature, proved to be appropriate methods for assaying CYP450 activities. Therefore, a new two-step extraction procedure was developed and the results presented here show that this procedure is a simple and efficient method, permitting the separation of HC from excess EC and the determination of HC in the low pmol range. By employing this extraction method,

CYP450 activity was determined in liver extracts from d8 and d11 chick embryos. During this period, activity increased by a factor of about five. In d11 extracts, an activity of 2.83pkat (equivalent to 0.17mU) per 0.1mg fresh weight was found. Cell fractionation experiments revealed that CYP450 enzymes were predominantly located in the microsomal fraction. In this cell fraction, the specific activity of CYP450 was about 0.63pkat (0.038mU)/mg protein. Assay conditions for determination of glucuronyltransferase activity were studied with respect to substrate concentrations and the addition of solvents. Under optimised conditions, the formation of MU-GlcA was linear with incubation up to 4 hours and liver extracts corresponding to about 1mg of protein. In contrast to the increase observed for CYP450, the activity of glucuronyltransferases was similar in d8 and d11 extracts: an activity of 1.75pkat per 0.1mg fresh weight was found at d8 and 1.96pkat per 0.1mg fresh weight at d11, corresponding to specific activities of 0.15pkat/mg protein (at d8) and 0.13pkat/mg protein (at d11). In summary, assay systems with standard substrates improved with respect to their sensitivity, efficiency and practicability, have been successfully employed to determine activities of CYP450 and glucuronyltransferases in liver extracts from developing chick embryos. Further studies will now focus on the role of these enzymes in metabolising xenobiotic compounds.

Comparative Distribution of Canalicular and Basolateral Transporters in HepaRG cells, HepG2 cells and Primary Human Hepatocytes

C. Chesne
Biopredic International, Rennes, France

The re-establishment of polarised excretory function and the maintenance of expression and activity of transporters in liver cell models, are critical for the *in vitro* analysis of drug and bile acid excretion, as well as toxicity testing. Previous studies have shown that conventional primary hepatocytes and HepG2 cells do not correctly simulate the *in vivo* situation. In the present work, we have analysed expression, activity and distribution of several canalicular and basolateral transporters in HepaRG hepatocytes, by comparison with HepG2 cells and primary human hepatocytes. As expected, differentiated HepaRG hepatocytes exhibited typical bile canaliculus structures, characterised by F-actin accumulation and restricted by tight junctions positive for Connexin 32. In previous reports, most efflux and influx transporters were found in HepaRG cells and adult hepatocytes at the transcript level. By using immunofluorescence and imaging analysis, MRP2 was localised on the canalicular membrane, while MRP3 and MRP4, as well as the influx transporter NTCP, were found on basolateral membranes. Disposition of the model bile acid, taurocholate, was also investigated by using H^3-taurocholate; both active influx via NTCP activity and canalicular efflux was evidenced. Data obtained with conventional primary hepatocytes, and especially HepG2 cells, showed that these two liver cell models much less closely mimic the *in vivo* situation, and support the view that HepaRG cells represent the most suitable *in vitro* model to investigate drug and bile acid excretion.

SATIS and InVitroJobs: News from Information Portals on Animal-free Science

C. Hohensee,[1] A. Schmidt,[2] A. Schmidt,[3] U. Teichgräber,[3] C. Ledermann,[3] S. Elsner[3] and C. Baumgartl-Simons[3]

[1]InVitroJobs, Federal Association of People for Animal Rights, Aachen, Germany; [2]SATIS, Federal Association of People for Animal Rights, Aachen, Germany; [3]Federal Association of People for Animal Rights, Aachen, Germany

In parallel with international developments in the field of risk assessment of chemicals and cosmetics, where efforts are undertaken to evaluate toxic substances with methods using human specific test procedures, it is necessary that young scientists are able to work on new animal-free methods. With its SATIS and InVitroJobs projects, the Federal Association of People for Animal Rights actively supports this process. The first ethics ranking of German universities shows that currently, high-school graduates and first-year students often have to choose between courses with harmful animal use or to undertake studies outside of the life sciences. Only some institutes exclusively use alternatives to harmful animal use in education and training. On the one hand, this possibility offers freedom for conscientious objection, on the other hand, it shows *in praxis* that education through alternatives is possible. Both requirements are demanded by the new EU-directive, valid from 2013. SATIS is the first Bachelor in Biology without harmful dissection. The biological faculty of the University of Mainz, Germany, has now started a pioneering alternative programme to the obligatory dissection course on animals, after debates with students groups and our organisation. Veterinary faculties use, to some extent, animals from veterinary clinics that have died naturally, or through medical indications. A comparable use is conceivable, but still rarely implemented, in zoological courses, thus the new SATIS guide to so-called 'body donation programmes' for universities. Further hands-on skills can be learned in the upcoming SkillsLabs. More than 25 medical training centres, and the soon-to-open first veterinary SkillsLab at the Universitiy of Hannover, will offer practice on models, simulators and mannekins, which would also be imaginable in biological education. InVitroJobs involves helping students to enter the animal-free employment market after studying. Since ethically-minded and responsible young scientists have recognised the potential of research specifically targeting alternatives to animal testing, with its project InVitroJobs, the Federal Association supports students in their search for thesis assignments and helps qualified scientists in their search for job opportunities. The bi-lingual portal mediates between research groups and graduates. In the three years since its creation, the portal has offered more than 200 research groups from 22 countries, about 400 job opportunities, 125 thesis assignments and nearly 90 internships.

Session IX: 21st Century Non-animal Tools For Basic and Biomedical Research

The Functionality of Uptake Transporters in Primary Hepatocytes of Different Species Can Be Verified by Estrone-3-sulphate

M. Keiser,[1] A. Ullrich,[2] J. Radebold,[1] G. Damm,[3] A. Nüssler,[4] W. Siegmund[5] and D. Runge[2]
[1]Universität Greifswald, Institut für klinische Pharmakologie, Greifswald, Germany; [2]PRIMACYT Cell Culture Technology GmbH, Schwerin, Germany; [3]Charite, Berlin, Germany; [4]Universität Tübingen, Klinik für Unfallchirurgie, Tübingen; [5]Universität Greifswald, Institut für Klinische Pharmakologie, Greifswald, Germany

Primary mammalian hepatocytes are used for several *in vitro* applications like testing of drug metabolism, toxicity and transporter assays. However, little is known about species-specific differences or similarities in the activity of uptake and efflux transporter. Therefore, we started a species-specific characterisation and compared the uptake of different substrates in hepatocytes of species used for drug testing. Human, rat, dog and monkey hepatocytes were incubated in serum-free media. Two to four days after cell isolation, the time- and concentration-dependent uptake of [^{3}H]-estrone-3-sulphate (E3S), bromosulphophthalein (BSP), digoxine (Dig) and taurocholic acid (TA), at 4°C and 37°C, was measured by using liquid scintillation counting. Competition assays were performed with rifampicin. All hepatocytes showed, at 37°C, a time-dependent and saturable increase in E3S and BSP uptake, compared to the uptake at 4°C. By contrast, Dig and TA showed a time-dependent and saturable transport only in the hepatocytes of humanoids and rats, but not in dog. E3S revealed a high affinity to human (K_m = 12.9 ± 10.1µmol/l; V_{max} = 84.2 ± 30.3pmol/mg × min), monkey (K_m = 8.2 ± 2.3µmol/l; V_{max} = 31.3 ± 3.6pmol/mg × min) and dog hepatocytes (K_m = 3.3 ± 2.3µmol/l; V_{max} = 10.0 ± 2.1pmol/mg × min), but not to rat hepatocytes. BSP showed only a high affinity to dog hepatocytes (K_m = 3.3 ± 2.3µmol/l; V_{max} = 10.0 ± 2.1pmol/mg × min), and TA was transported only into the hepatocytes of the monkey (K_m = 17.5 ± 12.5µmol/l; V_{max} = 5.8 ± 2.1pmol/mg × min). Rifampicin clearly inhibited the E3S uptake in monkey and dog hepatocytes, while rat hepatocytes were not influenced. Our data suggest that, from all tested substrates, [^{3}H]-E3S is the most suitable substrate to verify the functionality of uptake transporters in primary hepatocytes of humanoids and dogs, but not of rats. However, none of the tested substrates can be used to verify the functionality of uptake transporters in primary rat hepatocytes. These species-specific differences in hepatocellular uptake-transporter activities may contribute to species-specific differences very often observed when drug metabolism and toxicity are analysed.

Cytotoxic Effects Induced by Sulforaphane in Human Osteosarcoma Cell Line

M. Costa, C. Remédios, P. Pinto, S. Pinho, T. Pedrosa, F. Pinho, H. Oliveira, M. Oliveira and M.C. Santos
CESAM & Laboratory of Biotechnology and Cytomics, Department of Biology, University of Aveiro, Aveiro, Portugal

Osteosarcoma (OS) is the most common bone sarcoma. The disease is most frequently observed in infants and young individuals, being the second cause of death among all types of cancer in this age group. OS is an aggressive malignancy developed from the stem cells that generate the normal bone. Over the last decades, many studies on tumour prevention and

treatment have enabled the discovery of new anticancer agents. Sulforaphane (SFN), an isothiocyanate mainly found in Brassicaceae such as broccoli, has been extensively studied for its putative anticancer properties. SFN may promote inhibition of cancer cell viability and cell cycle arrest, as shown by several studies. However, regarding osteosarcoma cell lines, the effects of SFN are still poorly understood. For this reason, our goal was to study the cytotoxic effects of SFN in the MG-63 cell line, an *in vitro* human osteosarcoma model. *In vitro* cultured MG-63 cell line was exposed to different concentrations (0, 5, 10 and 20μM) of SFN for 24 and 48 hours, and cells were analysed for different parameters. Cell morphology was observed through inverted microscopy and cell viability was determined by using the MTT reduction assay. Cell cycle distribution and the formation of reactive oxygen species (ROS) were monitored by flow cytometry. Total antioxidant activity (TAA) was measured by using an antioxidant assay kit, which is based on the production of a radical cation detected spectrophotometrically at 405nm. Exposure to SFN was associated with disturbed cell morphology and decreased cell viability. Cell viability decrease was dose-dependent for the 48-hour exposure. Intracellular ROS increased with SFN exposure, in a dose-dependent manner. TAA was mostly maintained up to 10μM SFN, with significant reduction at 20μM SFN. The TAA:ROS ratio decreases with increasing SFN concentration, and this decrease is more rapid for 48 hours. SFN exposure was also associated with cell cycle arrest in G2/M phase, in a dose-dependent manner. Moreover, the subpopulation in G0/G1 decreased upon SFN exposure. Our results suggest that SFN is a phytochemical with anticancer potential against human osteosarcoma, since it interferes with several key steps of the carcinogenic process.

Development and Deployment of a Bioinformatic Test for Replacement of the Teratoma Assay

F.J. Müller,[1] J. Loring[2] and A. Schuppert[3]
[1]Zentrum für Integrative Psychiatrie ZIP GmbH, Kiel, Germany; [2]The Scripps Research Institute, La Jolla, CA, USA; [3]Aachen Institute for Advanced Study in Computational Engineering Science, Aachen, Germany

Pluripotent stem cell biology has become one of the key technologies of the 21st century, with anticipated transforming impact for disease modelling, cellular therapies and drug discovery. The recent development of technologies for the ethically unproblematic derivation of so-called 'induced pluripotent stem cells' (iPSC) from human subjects has enormously increased the visibility and adoption of stem cell biology in the biomedical research arena. Currently, iPSC are derived from all patient groups suffering from any type of disease in which genetic or epigenetic factors have been implicated. It is widely assumed that iPSC lines will become as important as DNA as primary substrate for collecting, storing and analysing material from human subjects for genetic studies. As iPSC have the ability to differentiate into every cell type of the human organism, genetic or epigenetic features and cellular responses can be now studied *in vitro* in the relevant cellular systems, such as neurons for the study of neuropsychiatric diseases. However, iPSC require an order of magnitude higher level of expertise in cell culture techniques and molecular and developmental biology for their characterisation and quality control than does the handling of DNA samples from blood or the culture of immortalised cancer lines. Additionally, an anachronistic tumour formation assay in immunodeficient mice that was developed 60 years ago, the so-called 'teratoma assay', is still considered to be the current 'gold standard' for the assessment of pluripotent stem cell features in human pluripotent stem cell lines, and as such is often requested by academic reviewers in high-impact journals. We present a recently developed, open-access bioinformatic alternative to the teratoma assay, termed PluriTest. PluriTest uses genome wide transcriptional profiles obtained through standard microarray analysis of iPSC, and uses a data-driven algorithm to assess pluripotent features in human stem cells. The PluriTest assay requires significantly less time and costs than the teratoma assay, and it can be accessed worldwide through an easy to use online interface at www.pluritest.org. Current PluriTest online usage data illustrate the significant number of iPS cell lines which are generated at the moment, and provides evidence for an urgent need for a widely accepted, animal-free alternative to the teratoma assay.

Development of a Microfluidic Chamber to Study Nerve–Muscle Interaction

E. Matas,[1] C. Jenkins,[1] A. De La Iglesia,[1] X. Tonnellier,[1] S. Marson,[1] M. Lewis[2] and S. Cellek[1]
[1]*Cranfield University, Cranfield, UK;* [2]*University of Bedford, Bedfordshire, UK*

Animals are widely used to study motor nerve degeneration and regeneration. These models usually involve the severing or crushing of one of the motor nerves, and this can be painful and stressful to the animals used. Moreover, the translation of information obtained from such animal models to human disease has been problematic. There has been therefore a need to develop *in vitro* cellular models to study human nerve–muscle interaction which can replace such animal models and will have a better translation to the clinical situation. Although such models have been developed, they have certain limitations such as random axonal growth, random alignment of muscle fibres, limited information on axonal growth factors released by muscle cells and inability to stimulate the neurons without activating the muscle. This project aims to develop an *in vitro* human neuron–skeletal muscle co-culture model by using microfluidic technology, which is expected to replace animal models and address those issues. Although microfluidic technology has been widely used to study neuronal biology, to our knowledge it has not yet been utilised to study the interaction between motor neurons and skeletal muscle cells. A photolithographic technique, with epoxy-based resin SU-8, was used to mould the microfluidic device with poly-dimethylsiloxane (PDMS), a biocompatible, optically transparent polymer. The PDMS device was covalently bonded on a glass slide. In one of the channels of the microfluidic chamber, a human neuroblastoma cell line (SH-SY5Y) was cultured and differentiated by treatment with retinoic acid to model human neurons. In the opposite channel of the chamber, mouse skeletal muscle cells were cultured and differentiated. Both SH-SY5Y and skeletal muscle cells were successfully cultured in the microfluidic chamber. However, some of the SH-SY5Y cells were observed to be migrating through the microgrooves of the microfluidic chamber. In order to solve this issue, we are now in the process of designing a new chamber with a range of microgroove lengths and widths. Successful completion of our study will lead to a better understanding of human motor neuron–skeletal muscle interaction. Such an *in vitro* cellular model will enable scientists to study axonal regeneration toward muscle, axonal transport in the presence of an intact neuromuscular junction, and axonal growth factors released by the muscle cells. It is envisaged that such a model will also accelerate the discovery of novel medicines for diseases such as motor neuron diseases and spinal cord injury. The team believes that such a model can replace several animal models currently being used for research into such diseases.

In Vitro Assessment of Sulforaphane-induced Genomic and Nuclear Damage in an Osteosarcoma Cell Line

C. Remédios, M. Costa, P. Pinto, F. Pinho, T. Pedrosa, S. Pinho, M. Oliveira, H. Oliveira and M.C. Santos
[1]*CESAM & Laboratory of Biotechnology and Cytomics, Department of Biology, University of Aveiro, Aveiro, Portugal*

Over the past years, *in vitro* studies have provided insight into the biological effects of several dietary phytochemicals, reducing the need for testing in animals. Sulforaphane (SFN) is a sulphur-containing compound belonging to the isothiocyanate family, mainly found in cruciferous vegetables. Several *in vitro* studies have shown that SFN exerts an anti-tumoural activity, regulating processes such as apoptosis and cell cycle progression in some tumoural cell lines. Osteosarcoma is an aggressive bone cancer with high incidence in children and adolescents that exhibits high level of resistance to current therapeutic programmes. Therefore, there is an urgent need to discover compounds that may be used efficiently for osteosarcoma therapy.

The effects of SFN have not yet been studied extensively in osteosarcoma. Our aim is to study the possible genotoxic effects of SFN in *in vitro* cultured osteosarcoma cells. As a model for our study we used the human osteosarcoma cell line, MG-63. These cells were exposed to 0, 5, 10, and 20μM SFN, for 24 and 48 hours. After these periods, the genomic/nuclear status was assessed. The SFN genotoxicity was evaluated by using the comet assay, which provided a measurement of the percentage tail DNA. Also, after measurement of the cell's DNA content by flow cytometry, the full peak coefficient of variation (FPCV) of the G1/G0 phase peak of each sample was used as a measure for clastogenic damage. The cytokinesis-blocked micronucleus cytome (CBMC) assay was performed in order to elucidate other aspects related to genomic/nuclear status, such as: i) the nuclear division index (NDI), a measure of the proliferative status that enables the detection of cytostatic effects; ii) nucleoplasmic bridges (NPBs); iii) micronuclei (MN); and iv) apoptosis. Our results showed that SFN increased the number of DNA breaks in a dose-dependent manner for a 48-hour period of exposure, as shown by comet assay, with a significant rise in percentage tail DNA. Moreover, for the same period of exposure, FPCV values were significantly higher for the cultures exposed to SFN when compared to control, suggesting a clastogenic effect of SFN in osteosarcoma cells. Exposure to higher doses of SFN was also associated with a decrease in the NDI for 24 and 48 hours, supporting the cytostatic effect of SFN. The lowest SFN dose was able to induce an increase in the formation of NPBs and MN, after 48 hours of exposure. Furthermore, a time-dependent rise of apoptotic cells was detected. This is the first report on SFN-induced genotoxicity in osteosarcoma. These data suggest that SFN promotes genomic instability at various levels in this osteosarcoma model, probably associated with cytostatic and apoptotic events.

Evaluation of Assay Requirements to Detect Specific Neurotoxicants in a Human Cell-based Test

A. Krug, N. Stiegler and M. Leist
University of Konstanz, Konstanz, Germany

Detecting chemicals affecting embryonal neurodevelopment is a major challenge, as organ-specific toxicity is hard to detect in *in vitro* toxicology. LUHMES human neuronal precursor cells have been suggested as test system to detect neurotoxicants affecting human neurites. A high-content image analysis was used to quantify neurite growth in LUHMES cells that spontaneously extend projections after plating. Labelling methods were all based on a live cell stain, which enabled us to assess cell viability in parallel. Here, we evaluated the assay features that affect robustness, specificity and sensitivity. The use of a general cell death endpoint did not allow specific identification of neurotoxicants. Measurement of neurite growth in developing (d3) LUHMES was more specific than using neurite destruction in mature LUHMES (d6) as an endpoint. Forty-two compounds were tested over a large concentration range for general cytotoxicity and inhibition of neurite growth in d3 LUHMES. The ratio of the EC50 values of these endpoints provided a robust classifier for compounds associated with a developmentally-neurotoxic hazard. A few pesticides showed specific inhibition according to these criteria, as did some microtubule destabilisers. Our test system is also able to detect compounds triggering excessive neurite growth indicating a broad dynamic range. These compounds were associated with the ROCK pathway. Mixtures of different compound classes were used to further define the response dynamics, and both additive and neutralising effects were observed. Based on our findings, we suggest that assays based on cell function (neurite growth), rather than cell survival, have a higher potential to measure specific neurotoxicity.

A Human *In Vitro* Model Using HepaRG and LX-2 Cells

M.R. Lornejad-Schäfer,[1] K.R. Schröder,[1] R. Weiskirchen[2] and C. Schäfer[1]
[1]BioMed-zet Life Science GmbH & zet — Centre for Alternative and Complementary Methods to Animal Testing, Linz, Austria; [2]Institute of Clinical Chemistry and Pathobiochemistry, RWTH University Hospital Aachen, Aachen, Germany

Liver fibrosis is a common consequence of many pathological liver diseases and is accompanied by the activation of collagen-producing cells and excessive deposition of extracellular matrix proteins. Activated hepatic stellate cells (HSC) represent the main cell type responsible for extracellular matrix deposition. Most *in vivo* liver fibrosis models lack human origin, and *in vitro* models are unable to resemble the complexity of human liver fibrosis. To explore the role of HSC on fibrogenesis and the hepatocyte-typical functions of differentiated HepaRG cells, we co-cultured HepaRG cells with the immortalised cell line LX-2 (Prof. S.L. Friedman, MSSM, USA; Prof. R. Weiskirchen, UK-Aachen), which represents partially activated HSC. HepaRG (BioPredic) is a bi-potent hepatic progenitor cell line able to differentiate into two distinct hepatic cell types, hepatocyte-like and biliary-like cells. HepaRG cells were treated with 1% DMSO for two weeks to differentiate. Thereafter, HepaRG cells were co-cultured with LX-2 cells for 1 (d1), 7 (d7) or 14 (d14) days. Cytotoxicity (LDH assay), albumin, collagen 1a1 (col1a1) protein expression (western blotting), total collagen (Sirius red) and secretion of albumin, TNF-α, and TGF-β (ELISA), were analysed over time. From d1 up to d7, col1a1 protein expression and total collagen increased significantly in co-culture models and remained widely unchanged up to d14. Compared with d1, expression and secretion of albumin decreased significantly in the HepaRG-LX-2 co-culture model from d7 to d14. Furthermore, the co-culture model led to a significant increase of tumour necrosis factor-α (TNF-α), transforming growth factor-β (TFG-β) in the cell culture supernatant over time, compared to d1 of co-culturing and to mono-cultures (LX-2, HepaRG). The HepaRG-LX-2 co-culture model exhibited an increase of inflammatory, fibrosis markers and a decrease of hepatocyte functionality after d7 of co-cultivation time. Therefore, the HepaRG-LX-2 co-culture model may be a useful tool to study the human liver fibrogenic process.

Acetaminophen Changes the Bioavailability of Substances Across an Intestinal Barrier Model

M.R. Lornejad-Schäfer,[1] C. Schäfer,[1] O. Höglinger,[2] S. Tollabimazraehno[3] and K.R. Schröder[1]
[1]BioMed-zet Life Science GmbH & zet — Centre for Alternative and Complementary Methods to Animal Testing, Linz, Austria; [2]School of Engineering/Environmental Sciences, Upper Austria University of Applied Sciences, Wels, Austria; [3]Center of Surface and nano analytic (ZONA), Kepler University Linz (JKU), Linz, Austria

Co-administration of drugs may affect the net intestinal absorption by different mechanisms. The aim of this study was to find out whether acetaminophen (APAP) changes the bioavailability of substances across the CaCo-2 barrier model via changes in cell membrane topography and transporter activity. The intestinal barrier model is based on CaCo-2 cells which were seeded onto inserts and cultured for a period of 21 days to induce differentiation, followed by APAP-administration for 24 hours. The cell cytotoxicity was measured by using the LDH assay. The effect of APAP on cell membrane topography and microvilli was investigated by using scanning electron microscopy (SEM). The cell transepithelial electrical resistance (TER) and capacitance were analysed by using two different impedance measuring systems. The membrane permeability was tested with different-sized molecules: 3–5kDa and 40kDa fluorescein isothiocyanate (FITC)-dextran. The activity of the efflux transporter multidrug resistance protein 1 (MRP1/ABCC1) was tested by using rhodamine 123. APAP concentration was determined by high-pressure liquid chromatography (HPLC) analysis. SEM analysis revealed

characteristic cellular surface profiles of APAP treated Caco-2 cells in the barrier model. The number of microvilli was significantly reduced. In addition, APAP increased the TER value (= membrane integrity), which was significantly correlated with the decreased permeability of small molecules, FITC-dextran (3–5kDa). Therefore, we assume that APAP affects the paracellular transport pathway. Furthermore, APAP decreased cell capacitance in a dose-dependent manner. This may be traced back to changes in transporter activity. When we measured the activity of the MRP1 transporter, we found that the transport of its substrates, rhodamine 123 and APAP, from the basolateral side to the apical side increased significantly after pretreatment with APAP. Hence, we assume that APAP induces the MRP1 transporter activity, which is responsible for the efflux of rhodamine 123 and APAP from the basolateral side to the apical side. We conclude that APAP may reduce the bioavailability of substances through different mechanisms: i.e. by changes in the cell membrane topography, reduction in the number of microvilli on cell membrane, and decreased paracellular and increased transcellular transport activity in the net intestinal absorption.

Targeted Contamination of Medical Devices as Quality Assurance for Pyrogenic Contamination

S. Stoppelkamp, K. Stang, M. Post, S. Fennrich and H-P. Wendel
Clinical Research Laboratory, Department of Thoracic, Cardiac and Vascular Surgery, University Hospital of Tübingen, Tübingen, Germany

Any medical device which will come into contact with blood needs to be tested for its haemocompatibility as well as pyrogenic activity, since contamination and surface contact can start pro-inflammatory reactions and activation of cascades within the haemostatic system. ISO 10993-4 regulates quality assessment for haemocompatibility and the interaction of medical devices with blood, whereby endotoxins interfere with haemostasis but are not specifically determined. While the *Limulus* amoebocyte lysate test as a replacement *in vitro* assay for the rabbit pyrogen test detects endotoxins only, the novel PyroDetect test is able to detect all pyrogens, i.e. endotoxins and non-endotoxins such as Gram-positive and fungal pyrogens. For injectables, it has already been incorporated into the European Pharmacopoeia. We now aim to adapt this *in vitro* assay for the control of the pyrogenic burden of medical devices to amend the existing guidelines. Initial testing of a complex medical device (stent system) composed of various materials revealed no interference with the assay set-up, ensuring the suitability of the assay. Subsequent experiments employing stents (for handling purposes) showed that conventional contamination methods of devices, used as control for surface contamination, led to unspecific and low-level read-outs, which are misrepresentative of actual contaminant levels. Employing a novel deposition method for targeted contaminations specific recovery of different pyrogens (LPS and LTA) was achieved. Due to the detection of ubiquitous pyrogens, the novel PyroDetect test is ideally suited for *in vitro* quality assurance of injectables. We show here that our modified *in vitro* pyrogen test can also specifically detect pyrogenic contamination on surfaces, and thus would be a crucial amendment to the quality assurances of medical devices. We therefore propose the integration of the *in vitro* pyrogen test into the ISO regulations.

Characterisation of Human Airway Epithelia to Be Used in Carcinogenicity Studies

K. Sommer,[1] B. Kriechbaumer,[1] S. Constant,[2] D. Breheny[3] and K.R. Schröder[1]
[1]BioMed zet Life Science GmbH, Linz, Austria; [2]Epithelix Sàrl, Geneva, Switzerland; [3]British American Tobacco, Southampton, UK

MucilAir™ is an *in vitro* test-system of primary human respiratory epithelium, with a shelf-life of about one year. It reflects the natural human respiratory epithelium, since it contains not only the epithelial cells with cilia in motion but also goblet cells constantly producing a surfactant covering the tissue. The long shelf-life suggests that the model can be used for *in vitro* assays to study long-term effects. Specifically, we are interested in analysing this system for its potential to detect all phases of carcinogenesis, starting with initial events causing DNA lesions, promoting substance effects, and progressive outcome at the end of this process. Known tumour initiators like MNNG and MNU, producing point mutations as a consequence of O6-alkylguanine adducts, and TPA as tumour promoter, are used to characterise the test system in this respect. The DNA-damaging action of MNNG and MNU could be proven with indirect parameters like the phosphorylation of histones upon DNA double-strand breaks by using western blotting and immunofluorescence analysis. MTDs were defined for these substances and for TPA as a prerequisite for repeated dose applications. For manifestation of the induced lesions, at least two rounds of replication are necessary. Therefore, the differentiated cells of the quiescent epithelia had to be forced into mitosis, since furthermore the correct time point for the treatment had to be found. TPA did not force the cells of the epithelia into proliferation, but regenerative growth after wounding led to an increase in the mitosis rate. Interestingly, this did not correlate with the expression of the proliferation marker, Ki67. We found that, after wounding, the mitosis rate increased, peaked at day 7 and remained constant for another 8 days. We suggest that this period is ideal for treating the tissue with carcinogens. On the basis of MucilAir, we have established a test system in so far that we can identify MTDs for repeated application and have demonstrated a proper time point for the administration of compounds. What remains an open question is how to demonstrate fixed point mutations. Sanger sequencing of hot spot regions was not the appropriate tool to use, assuming that such rare mutations occur only incidentally. Several approaches to how to increase the probability of gaining fixed point mutations will be analysed by using modern techniques such as Next Generation Sequencing (NGS) with its high coverage of up to 98%, by using specific cancer panels.

Session X: Good Cell Culture Practice

Use of Human Platelet Lysates in Stem Cell-based Alternative Testing Strategies

C. Rauch, E. Feifel, J. Wechselberger and G. Gstraunthaler
Innsbruck Medical University, Innsbruck, Austria

The supplementation of basal culture media with animal serum — mostly fetal bovine serum (FBS) — is common practice, and is essential for cell growth and proliferation *in vitro*. The major function of serum is to provide culture media with hormones, cytokines, growth factors, attachment factors, etc. The most potent mitogenic factors present in serum are derived from activated platelets. However, the use of animal serum in cell culture bears a number of disadvantages: serum is an ill-defined medium supplement with serious lot-to-lot variations; serum may contain adverse factors (e.g. microbial contaminants, endotoxins, haemoglobin); serum is harvested via puncturing of the beating heart of unanaesthetised bovine fetuses, causing severe suffering of fetal animals. Recently, we were able to show the capacity of human platelet α-granule lysates to replace FBS in a variety of human and animal cell culture systems. Thus, lysates of human donor platelets may become a valuable non animal-derived substitute for FBS in cultures of mammalian cells and in human and animal stem cell technology. Stem cells may become the future human-based alternative to animal testing and *in vitro* toxicology. New stem cell-based test systems are continuously being established, and their performance under animal-derived component-free culture conditions has to be defined in prevalidation and validation studies. In order to accomplish these tasks, human bone-marrow stem cells (BMSC) and adipose-derived mesenchymal stem cells (ADSC) were expanded in media supplemented with platelet lysates. Proliferation monitoring by direct cell counting, resazurin and WST-8 assays, confirmed the growth-promoting effect of platelet lysate, comparable to high FBS. Furthermore, we established culture conditions in which the BMSC and ADSC maintained their undifferentiated state, as proven by CD73, CD90 and CD105 expression and the lack of negative marker, CD45. Preliminary tests to determine whether BMSC and ADSC can be differentiated toward osteogenic, chondrogenic and adipogenic phenotypes under platelet lysate-supplemented growth conditions, were also successful.

Human Platelet Lysates as a Serum Substitute in Renal Epithelial Cell Culture for Toxicity Screening *In Vitro*

C. Rauch, E. Feifel and G. Gstraunthaler
Innsbruck Medical University, Innsbruck, Austria

Cultured renal epithelia form monolayers of highly differentiated, polarised cells. When grown on permeable supports, an apical compartment and a basolateral compartment are separated by the cultured epithelium, which enables the study of epithelial transport *in vitro*. Some requirements must be met by the *in vitro* system: e.g. retention of the polar architecture and junctional assembly of the epithelial cells, presence of vectorial transport, sidedness of cellular substrate uptake routes, and retention of segment-specific metabolic and transport properties. Thus, it is obvious that culture conditions, culture media and supplements have a significant impact on *in vitro* epithelial function. In this respect, the quality of fetal bovine serum (FBS) seemed to be of major importance. In early studies, a high lot-to-lot variablity of FBS was found that substantially influenced the differentiation of *Xenopus laevis* A6 epithelia and the generation of a transepithelial potential difference (PD) and resistance (TEER). As a result, the batch-testing of FBS was required. TEER is a multifactorial property of trans-

porting epithelia, comprised of transcellular and paracellular components. TEER is the most sensitive parameter of any impairment of epithelial barrier integrity and transport function. In prevalidation studies, it was shown that TEER is a reliable endpoint for *in vitro* nephrotoxicity screening. We recently reported the use of human platelet lysate (PL) as a non-animal replacement for FBS. PL preparation was optimised for growth factor content, as quantified by ELISA. The growth-promoting capacity of PL was tested on renal epithelial cell lines, for which growth characteristics, phenotypes, and differentiation endpoints are well established. PL supports growth, proliferation and differentiation, as assessed by dome formation, of proximal tubule-like LLC-PK1, HK-2 and distal tubule-like MDCK cells. Proliferation was monitored by determination of cell density, and by resazurin or WST-8 assays. Proliferation rates were comparable in culture media with either 10% FBS or 5% PL. In order to biochemically determine the mitogenic potential of PL, the stimulation of ERK1/2 MAP kinase was determined. Addition of PL to quiescent LLC-PK1 cultures resulted, like FBS, in specific phosphorylation, and thus activation, of ERK1/2. In addition, TEER was monitored in filter-grown LLC-PK1 and in low resistance and high resistance MDCK epithelia. Low resistance epithelia generated a TEER of 150–250Ω.cm^2 in PL-supplemented media, as seen with FBS, and high resistance MDCK retained their TEER of 8,000Ω.cm^2. PL is therefore a valuable, animal-derived component-free substitute for FBS in renal epithelial cell culture for *in vitro* nephrotoxicity testing.

Optimisation of Human Cornea Constructs For a Rapid Characterisation of Pharmaceutical Preparations, Excipients and Chemical Compounds

G. Guzman,[1] S. Reichl,[2] U. Bock[1] and E. Haltner[1]
[1]Across Barriers GmbH, Saarbrücken, Germany; [2]Institut für Pharmazeutische Technologie, Technische Universität Carolo-Wilhelmina zu Braunschweig, Braunschweig, Germany

Currently, there are many efforts to develop and validate an *in vitro* cornea model to investigate drug absorption of new or modified ocular drugs, the effects of their excipients, active transport, chemical compounds, and cosmetics, among others. However, prior to the validation of an *in vitro* model used to replace animal experiments, several requirements must be fulfilled, such as prevalidation of the model. Nevertheless, further characterisation and improvement of the cell culture conditions are necessary to achieve the final goal. Thus, the main objective of the present investigation was to optimise the protocols for culturing human cornea constructs (HCC), by testing collagen from a new supplier. The second aim was to increase the experimental window of the experiments, by conducting quality controls between days nine and eleven to increase the experimental window. The parameters measured were transepithelial electrical resistance (TEER), the permeation coefficients of sodium fluorescein as a poorly permeable marker substance and propranolol as a highly permeable marker substance. Human cornea constructs were developed by using immortalised human cornea epithelial (HCE-T) and human cornea keratocyte (HCK) cell lines. The cells were cultured until confluence in KGM serum-free medium at 37°C and 5% CO_2. To construct HCC, a suspension of HCK cells in collagen gel (collagen from rat tail, Becton Dickinson [BD]), and another suspension of HCK cells under optimised parameters (i.e. in collagen from rat tail, Sigma), were scattered on polycarbonate filters. After the polymerisation of the stromal equivalent, a suspension of the HCE-T cells was seeded onto it. In order to induce epithelial multilayer formation, HCC were exposed to the air–liquid interface between day seven and day eleven. The results were compared to the reference values that were previously obtained during the prevalidation of the human cornea model. The results suggested that collagen from BD is not suitable for the development of the human cornea constructs, and it was not possible to achieve the tightness of the model with either 1.2mg/ml or 2.6mg/ml of this collagen. One out of four collagen lots from Sigma did not meet the acceptance criteria. Permeability assays were conducted on days nine, ten, and eleven, with at least three different cell passages, after the modification of the protocol. The comparison of P_{app} of Na-FLU with the reference value

(prevalidation of the HCC) on day ten demonstrated no significant variation, whereas significant difference was only calculated for the P_{app} on day nine for propranolol, even when the mean P_{app} was between the ranges of the prevalidation of HCC. This demonstrated that modification of the protocol was conducted successfully. The screening of collagen should thus be performed before use, in order to guarantee the robustness and the reproducibility of the HCC. The most suitable collagen to successfully form HCC constructs is probably Sigma collagen from rat tail. The permeability coefficients and transepithelial electrical resistance investigated in HCC at days nine, ten and eleven, after modification of the protocol, met the acceptance criteria of the model.

EUSAAT–ETS
Joint Symposium

Embryonic Stem Cell Approaches Towards Biomarkers of Embryotoxicity

A. Piersma
RIVM/GBO, Bilthoven, The Netherlands

The embryonic stem cell test has been an object of extensive research over the past two decades. It provides an elegant model for studying the interference of chemicals and pharmaceuticals with embryonic cell differentiation. Whilst initially designed to monitor cardiac myocyte differentiation by counting contracting muscle foci as the readout parameter, innovative techniques in cell culture and molecular biology now allow the study of a variety of differentiation lineages, by using molecular markers of cell differentiation. These innovations have improved the understanding of mechanisms of cell differentiation, as well as the detection of differentiation-modulating agents. We have employed transcriptomics to characterise gene expression changes throughout the stem cell differentiation cascade in the assay. We thus defined a differentiation track, based on gene expression changes, and derived gene sets containing limited numbers of genes with which we were able to detect compound effects. In addition, we studied concentration–response relationships, and showed that morphologically observable effects, such as on differentiation and cell viability, were mimicked in specific gene expression responses. Comparisons of compounds, both within and between structural classes of chemicals, revealed class-specific gene expression signatures. These findings may help define the applicability domain of the embryonic stem cell test as to the classes of chemicals for which the test can be usefully applied. The possibility of appending the assay with the neural end bone differentiation routes, has given rise to enhanced detection of compounds that specifically affect one of these differentiation pathways. In due course, in a testing strategy design, several differentiation routes may need to be studied in a battery or tiered approach to optimise the detection of compounds interfering with embryonic cell differentiation. Challenges arise in discriminating subtle adaptive gene expression responses from more severe effects that may indicate adversity, and in defining the threshold of adversity in these *in vitro* assays. Most probably, a combined approach, using information from a variety of *in silico* and *in vitro* tests in an integrated testing strategy, will be needed to make optimal use of these approaches with a view to reducing animal experimentation in regulatory toxicology.

The Validated Embryonic Stem Cell Test (EST) and its Applicability Domain

C. Riebeling, A. Luch and A. Seiler
German Federal Institute for Risk Assessment, Centre for Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

The validated embryonic stem cell test (EST) is a system for testing developmental toxicity of chemicals *in vitro*. In a stand alone approach, it comprises three assays: i) the disturbance of mouse embryonic stem cell line D3 differentiation towards beating cardiomyocytes, ii) the chemical-mediated cytotoxicity to D3 cells, and iii) to the mouse embryonic fibroblast cell line 3T3. The half-maximal effective concentrations determined in the three assays are then used in a prediction model that computes a predicted embryotoxicity class for the individual test substance. Since its validation by the European Centre for the Validation of Alternative Methods (ECVAM), the original EST has been used in a number of studies that both underscored its potential, but also demonstrated several limitations. In the validation study, the EST showed an overall accuracy of 78% with 20 substances. Moreover, by using seven congeners of valproic acid, a teratogenic anticonvulsant, the EST nicely differentiated the structurally

related molecules and ranked them in the same order and in full accordance with data obtained in the NMRI exencephaly *in vivo* mouse model. In addition, during the ReProTect Project, funded within the 6th Framework Programme of the European Union, two sets of chemicals were tested. One set comprised reproductive toxicants, and all substances labelled as reprotoxicant according to the European Union Classification, Labelling and Packaging (CLP) Regulation were detected as developmentally adverse by the EST. By contrast, only 15% of substances were correctly identified in another set comprising a broad array of chemicals. Reviewing the misclassified substances of all sets unveiled several limitations of the EST, many of which also affect other *in vitro* and *ex vivo* alternative approaches to animal testing. The major limitation of the EST is its representation of early organogenesis only. In particular, substances affecting specifically neural or osseous tissues have to be detected by separate, more specialised assays. Secondly, the use of aqueous media restricts the testing of highly lipophilic substances, a shortcoming of most *in vitro* assays. In addition, some components of the media are necessarily supplemented at artificially high concentrations that counteract some of the tested substances' effects. A specific shortcoming of the validated EST is its readout of beating cell clusters, which is inhibited by muscle relaxants. Conversely, approaches where this physiological endpoint has been replaced by molecular markers, including our FACS-EST, remain unaffected by this class of substances. Moreover, some of the tested substances' effects, or lack thereof, depends on maternal tissues and their pharmacokinetic behaviour, influencing factors that cannot be provided by the EST. Finally, the *in vivo* classification of the substances is an important issue, requiring the consistent use of criteria and recognition that species specificity also applies to *in vitro* systems. In conclusion, many of the limitations revealed can be overcome by modifications and additions to the EST, making it a promising basis for a regulatory acceptable *in vitro* assay system for reproductive toxicity.

Identification of Thalidomide-specific Transcriptomic and Proteomics Signatures During Differentiation of Human Embryonic Stem Cells

A. Sachinidis
Centre for Physiology and Pathophysiology, Institute of Neurophysiology, University of Cologne, Cologne, Germany

Embryonic development is partially recapitulated under *in vitro* conditions by differentiating human embryonic stem cells (hESCs). In this context, it is repeatedly described that thalidomide is a developmental toxicant acting in a species-dependent manner, and therefore it serves as a prototypical model to study teratogenicity. In order to establish a human-relevant teratogenicity *in vitro* model, we combined -omics technologies such as transcriptomics, microarrays and proteomics (two-dimensional electrophoresis [2DE] coupled with Tandem Mass Spectrometry) with human embryonic stem cells (hESC), to demonstrate thalidomide embryotoxicity/teratogenicity in pharmacological dose(s). Applying the -omics technologies we were able to demonstrate differential expression of limb, heart and embryonic development-related transcription factors and biological processes. In addition, this study resulted in the identification of RANBP1, which was inhibited by thalidomide and normally participates in the nucleocytoplasmic trafficking of proteins. Interestingly, the inhibition of glutathione transferases (GSTA1, GSTA2) that specifically protect the cell from secondary oxidative stress has been observed. In summary, as a proof of principle, we demonstrated that a combination of -omics technologies, along with consistent differentiation protocols of hESCs, enabled the identification of canonical and novel teratogenic intracellular mechanisms of thalidomide.

Historical Perspective of the Embryonic Stem Cell Test (EST) Assay

H. Spielmann
Freie Universität Berlin, Berlin, Germany

In 1984, I developed the idea that pluripotent cells from the inner cell mass of mouse blastocysts could be used for *in vitro* embryotoxcity testing. Significant improvements in culture techniques, including the establishment of mouse embryonic stem cell (mESC) lines, allowed the development of the embryonic stem cell test (EST), in which differentiation of mESCs is used as a model to assess embryotoxicity *in vitro*. The test, which was successfully validated between 1998–2002, in a study funded by ECVAM, is used for modelling fundamental mechanisms in embryotoxicity, e.g. inhibition of growth (cytotoxicity) and differentiation. In addition, differences in sensitivity between differentiated (adult) and embryonic cells are taken into consideration. To predict the embryotoxic potential of a test substance, three endpoints are assessed: the inhibition of differentiation into beating cardiomyocytes, the cytotoxic effects on stem cells and the cytotoxic effects on 3T3 fibroblasts. A special feature of the EST is that it is solely based on permanent cell lines, so that primary embryonic cells and tissues from pregnant animals are not needed. Later, the morphological assessment of contracting cardiomyocytes, which is used in the validated method as endpoint for differentiation, was replaced by the molecular-based FACS-EST method, in which highly-predictive protein markers specific for developing heart tissue were selected. With these methods, the embryotoxic potency of a compound can be assessed *in vitro* within ten or seven days, respectively. Meanwhile, several groups have established mESC and also human ESC (hESC) based on *in vitro* embryotoxicity assays, in which differentiation of ESCs into nerve, cartilage and bone tissue is assessed by molecular markers. These improvements will be presented in the symposium, and also the use of ESC based assays in embryotoxicity testing in industry.

Development of the Neural Embryonic Stem Cell Test (ESTn): A Model for Screening (Neuro)Developmental Toxicity

P. Theunissen[1] and A. Piersma[2]
[1]RIVM / Utrecht University of Applied Science, Utrecht, The Netherlands; [2]RIVM/GBO, Bilthoven, The Netherlands

We developed a dynamic 11-day *in vitro* model for morphologically screening the effects of possible neurodevelopmental toxicants, by using neural cell differentiation of pluripotent murine embryonic stem cells. This neural embryonic stem cell test (ESTn) was designed to be complementary to the classical cardiac EST. In order to gain additional mechanistic insight and increase throughput of the ESTn, transcriptomics was used to characterise the model over time, and to assess the developmental toxicity of methylmercury at the molecular level at an earlier time point, as compared to the classical endpoint. In addition, transcriptomic concentration–response studies of various compounds, including anticonvulsants and triazoles, was performed. Inclusion of nontoxic and developmentally toxic concentrations in this system provided additional information on adaptive *versus* adverse compound-induced gene expression responses. A *de novo* analysis using transcriptomics data from 10 compounds, including 19 exposures in the ESTn, resulted in a gene set consisting of 29 genes, predicting neurodevelopmental toxicity with 84% prediction. Furthermore, a transcriptomic comparison between the classical EST and ESTn showed that these systems can complement each other in the detection of developmental toxicants. When combined, these transcriptomic experiments provided mechanistic insight for prediction of (neuro) developmental toxicity with the ESTn.

Simulating Developmental Toxicity in a Virtual Embryo

T. Knudsen
US EPA NCCT/ORD, Research Triangle Park, NC, USA

ToxCast is profiling 1060 chemicals across more than 650 assays that include a range of biochemical and cell-based platforms, mouse embryonic stem cells and developing zebrafish embryos. Virtual tissue models that integrate these data with biological knowledge and cellular dynamics of a developing embryo, provide a novel application to translate *in vitro* effects into mechanistic simulations of potential adverse *in vivo* outcomes. Through a virtual embryo framework, cellular agent-based models (ABMs) simulate morphogenesis as individual cells grow, divide, differentiate, adhere, migrate, and die. By integrating cellular dynamics with knowledge from molecular clocks, biochemical gradients, and gene regulatory networks, distinct anatomical features emerge as the simulation unfolds. Running many parallel simulations across dose–time–stage scenarios can begin to assess the range of effects, reveal the earliest signs of adversity, and generate new hypotheses about how cellular and molecular lesions propagate to higher levels of biological organisation. Proof-of-concept will be demonstrated for a simulation of embryonic limb-bud development integrating *in vitro* effects data captured from embryonic stem cells. This abstract does not necessarily reflect US EPA policy.

High-throughput Design in Industry: Novel Automation Approaches for Improved Predictivity in Early Safety Assessment

C. McGinnis
F. Hoffmann-La Roche AG, Pharmaceuticals Division, Basel, Switzerland

Higher Throughput Design in Early Safety Assessment is becoming increasingly important in order to run cost-effective, streamlined profiling studies of lead drug candidates. Reducing the cost of failures has also become paramount. Hence, the quality and predictivity of such high-throughput (HT) studies are expected to be as high, if not higher, than their low-capacity, manual equivalents. At Roche, considerable investments were made in recent years to develop and implement customised, high-quality automation solutions that helped to consolidate previously global screening groups to one functional excellence unit. This presentation will describe several fully automated cell-based toxicity assay platforms for several endpoints, with a particular emphasis on the Embryonic Stem Cell Test. Proprietary, specially designed labware — the 'HDCP plate' — and robotics solutions were required. The results of a thorough analysis of the historical value of this assay, as well as comparison between the manual, ECVAM-validated test and the Roche approach, will be presented in detail.

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