



ALTEX Proceedings

Horst Spielmann:
Welcome

Session I:
**International Activities,
Disease Models &
Human-on-a-chip**

Session II:
3Rs go 3D

Session III:
**ESNATS Conference:
Use of Human Embryonic
Stem Cells for
Novel Toxicity Testing
Approaches**

Session IV:
**EU Dir 2010/63 &
Ethical & Legal Aspects**

EUSAAT
European Society for
Alternatives to Animal Testing



esnats

LINZ 2013

18th European Congress
on Alternatives to Animal Testing

EUSAAT 2013

15th Annual Congress of EUSAAT

ESNATS Conference

Embryonic Stem cell-based Novel
Alternative Testing Strategies

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Session V:
**Consequences of
the 2013 EU Marketing
Ban for Cosmetics &
NanoTox**

Session VI:
**Specific Endpoints
of Toxicity**

Session VII:
**Tox21 (TT21C)
& Specific Endpoints
of Toxicity**

Session VIII:
**Implementing
Alternatives into
Basic Research**

Session IX:
**Drugs, Vaccines
& EU FP7 Projects**



The organisers and the Scientific Committee wish to thank the following sponsors:

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Welcome address

Dear friends and colleagues,

on behalf of EUSAAT, the European Society for Alternatives to Animal Testing, I welcome you to the “EUSAAT 2013 - Linz 2013” congress, which is actually the 15th Annual Congress of EUSAAT and the 18th congress on alternatives in Linz. This year we are happy that the EU FP7 project ESNATS, European Stem cell-based Novel Alternative Testing Strategies, will hold its annual conference in 2013 in Linz during the EUSAAT 2013 conference. We are sure that the ESNATS conference will in 2013 add to making EUSAAT 2013 most attractive.

On March 11, 2013 the vision most of us share finally became reality when the EU Commission announced the marketing ban for cosmetics testing in animals. As most of you will recall during the past 20 years at our congresses in Linz we have had long and fruitful discussions on all aspects of the increasing number of amendments of the EU Cosmetics Directive. The success in 2013 will stimulate our enthusiasm to search for and implement more non-animal methods in other areas of safety testing. Europe has been the international leader in making our first dream become reality. We will have to cooperate intensively with our colleagues in the USA, Japan and around the world to establish non-animal test approaches that are scientifically better than the current animal-based approaches.

During the past two decades the “Linz-Congress” has emerged in Europe as one of the major scientific events in the field of the 3Rs. EUSAAT 2013 is hosting presentations, discussions and exchange of new ideas for the benefit of alternative methods to animal experiments. The Scientific Committee has identified the most important subjects related to the 3Rs which are of interest to scientists in Europe.

The EUSAAT 2013 conference is focusing in oral and poster sessions on animal-free disease models, non-animal tools for basic biomedical research, -omics techniques and advanced 3D models including recent progress on developing a “human-on-a-chip”.

EUSAAT 2013 is providing a forum for discussing the new EU Directive 2010/63 on the protection of experimental animals, which is currently being implemented in EU member states. Colleagues from the EU Commission, from member states and from the animal welfare movement will discuss the high expectations that the public and the animal welfare movement have for improving the current situation on behalf of the experimental animals.

The EUSAAT Board is happy 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 EUSAAT Board and Scientific Committee want to thank all of the sponsors of EUSAAT 2013 on behalf of the participants.

Finally, we are also happy that the abstracts of the EUSAAT 2013 conference on the 3Rs are published in ALTEX Proceedings and we want to thank the editors of ALTEX that they have helped us to pave the way for future cooperation on behalf of implementing the 3Rs in Europe.

I finally want to thank my colleagues on the EUSAAT Board and in the Scientific Committee for their continuous support in planning EUSAAT 2013.

Horst Spielmann
President of EUSAAT



Preliminary Program

Sunday, 15.09.2013

13:00-13:15 Welcome Addresses

Session I - International Activities, Disease Models & Human-on-a-chip

- 13:15-13:35 Hajime Kojima, National Institute of Health Sciences, JP-Tokyo
Japanese project “ARCH-Tox” for the future chemicals management policy: research and development of *in vitro* and *in vivo* assays for internationally leading hazard assessment and test methods
- 13:35-13:55 Horst Spielmann, Freie Universität Berlin, DE-Berlin
Peer review panel evaluation of the ROS Photosafety Assay
- 13:55-14:15 Mario Fabri, University of Cologne, DE-Cologne
Vitamin D-dependent antimicrobial pathways in human macrophages
- 14:15-14:35 Günther Weindl, Freie Universität Berlin, DE-Berlin
Increased cutaneous absorption reflects impaired barrier function of reconstructed skin models mimicking keratinisation disorders
- 14:35-14:55 Eva-Maria Materne, TU Berlin, DE-Berlin
A multi-organ-chip platform for long-term maintenance and substance testing of human tissue co-culture
- 14:55-15:15 Dmitry Sakharov, Scientific Research Centre Bioclinicum, RU-Moscow
A dynamic multi-organ-chip containing enterocytes and hepatocytes for the absorption of orally administered drugs prediction and substance toxicity testing

15:15-16:30 Poster session I + coffee break and exhibition

16:30-17:00 Horst Spielmann, Freie Universität Berlin, DE-Berlin
Willi Halle Memorial Lecture

Session II – 3Rs go 3D

- 17:00-17:30 Ellen Fritsche, Leibniz Research Institute of Environmental Medicine, DE-Düsseldorf
Human Neural Progenitor Cell (hNPC) Aging is mimicked by 3D Cultures *in vitro*: an ‘Adverse Outcome Pathway’ gains importance at susceptible life stages
- 17:30-17:45 Jens Kelm, InSphero AG, CH-Zürich
3-dimensional organotypic *in vitro* model systems for liver, cardiac and neuro safety assessment
- 17:45-18:00 Silvia Angeloni, CSEM SA, CH-Neuchâtel
Blood-brain tumor and air-blood tissue barrier: two examples of how BioMEMS can improve the relevance of co-cultured *in-vitro* models of biological barriers
- 18:00-18:15 Frederique van Acker, TNO Triskelion, NL-Zeist
Cerium oxide nanoparticles air exposure: a comparison study using a human 3D airway model, A549 and BEAS-2B cell lines
- 18:15-18:30 Nicky Slawny, 3D Biomatrix, Inc., US-Ann Arbor
3D spheroid cell cultures as models in drug discovery
- 18:30-18:45 Martha Liley, CSEM SA, CH-Neuchâtel
Towards replacing *in vivo* tests of dental and orthopedic implants

19:15-20:45 Annual general assembly of EUSAAT



Monday, 16.09.2013 - special day
(at the same time session III of the 18th European Congress on Alternatives to Animal Testing)

ESNATS CONFERENCE - Preliminary Programme

“Use of Human Embryonic Stem Cells for Novel Toxicity Testing Approaches”

- 09:00-09:10 Horst Spielmann, President of EUSAAT, Freie Universitaet Berlin, DE-Berlin
Opening address
- 09:10-09:20 Jürgen Hescheler, Coordinator of ESNATS, University of Cologne, DE-Cologne
Overview of the ESNATS project
- 09:20-10:00 Marcel Leist, University of Konstanz, DE-Konstanz
The ESNATS hESC-based toxicity test battery
- 10:00-10:40 Jan Hengstler, IFADO, DE-Dortmund
The ESNATS hESC-based toxicity biomarker identification study
- 10:40-11:00 Jan Hengstler, IFADO, DE-Dortmund
**Hepatocyte *in vitro* systems to study metabolism:
stem cell derived hepatocytes as an alternative to primary hepatocytes**
- 11:00-11:15 Coffee break**
- 11:15-11:30 Joery de Kock, Vrije Universiteit Brussels, BE-Brussels
hSKP-derived hepatocytes for toxicity testing
- 11:30-11:50 Sieto Bosgra, TNO, NL-Utrecht
***In vitro-in vivo* extrapolation of prenatal (neuro)toxicity assay data by PBPK modeling**
- 11:50-12:10 Agapios Sachinidis, University of Cologne, DE-Cologne
Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of human embryonic stem cells
- 12:10-12:30 Karl-Heinz Krause, University of Geneva, CH-Geneva
Pluripotent stem cell-derived engineered neural tissues: what's new?
- 12:30-13:45 Lunch and posters**
- 13:45-14:30 **First panel discussion** (chair: Thomas Hartung, CAAT, US Baltimore)
How can the ESNATS data be used to define mechanisms of action and/or AOPs
- 14:30-15:00 Aldert Piersma, RIVM, NL-Bilthoven
Enhancing the readout of the embryonic stem cell test with molecular approaches
- 15:00-15:30 Ellen Fritsche, Leibniz Research Institute for Environmental Medicine, DE-Duesseldorf
Signaling pathways in human neural assays (title to be confirmed)
- 15:30-16:00 Robert Kavlock, US Environmental Protection Agency, US-Washington
Transforming the Conduct of Toxicology in the US: the Tox21 Program
- 16:00-16:30 Coffee break and posters**
- 16:30-17:00 Thomas Knudsen, US Environmental Protection Agency, US-Research Triangle Park
Predictive models and computational embryology
- 17:00-17:30 Beatriz Silva-Lima, EPAA stem cell group, BE-Brussels
EPAA calls for a “Stem Cells in Safety Testing” forum to keep fluent communication



17:30-18:00	Jos Kleinjans, University of Maastricht, NL-Maastricht Data infrastructure for chemical safety
18:00-18:45	Second panel discussion (chair: Michael Schwarz, University of Tuebingen, DE-Tuebingen) How can the ESNATS assays be applicable in companies/regulatory toxicology and what are necessary future developments
18:45-20:00	Continued discussion and poster session
20:00	Dinner and social evening (at the congress venue)

Tuesday, 17.09.2013

Session IV – EU Dir 2010/63 & Ethical & Legal Aspects

09:00-09:30	Susanna Louhimies, The European Commission, BE-Brussels Directive 2010/63/EU: Overview of the work of Commission Expert Working Groups on an EU Framework for Education and Training and on Project Evaluation
09:30-10:00	Katharina Kluge, Federal Ministry of Food, Agriculture and Consumer Protection, DE-Bonn Implementation of Directive 2010/63/EU into German law
10:00-10:15	Ursula G. Sauer, Scientific Consultancy - Animal Welfare, DE-Neubiberg Ethical review of projects involving non-human primates funded under the European Union's 7th Research Framework Programme
10:15-10:30	Norbert Alzmann, Messerli Research Institute, AT-Vienna A catalogue of criteria to objectify the harm-benefit analysis according to Austrian legislation
10:30-10:45	Marie-Jeanne Schiffelers, Utrecht University School of Governance, NL-Utrecht Factors influencing regulatory acceptance and use of 3R models & strategies to improve this process: The case of the EOGRTS
10:45-11:00	Mangala Gunatilake, University of Colombo, LK-Colombo Steps taken to implement 3Rs concept in research using animals in Sri Lanka
11:00-11:40	Coffee break, posters and exhibition

Session V – Consequences of the 2013 EU Marketing Ban for Cosmetics & NanoTox

11:40-11:55	Gamze Ates, Vrije Universiteit Brussel, BE-Brussels Retrospective analysis of the mutagenicity/genotoxicity data of the cosmetic ingredients present on the Annexes of the Cosmetic EU legislation
11:55-12:10	Helena Kandarova, MatTek In Vitro Life Science Laboratories, SK-Bratislava Ability of the reconstructed human tissue models to correctly predict phototoxicity and photopotency of topically applied substances and formulations: Review of available data
12:10-12:25	Martin Paparella, Environment Agency Austria, AT-Vienna Uncertainty of testing methods – What do we (want to) know?
12:25-12:40	Katy Taylor, BUAV/ECEAE, UK-London The ADAPT principles for regulatory authorities



- 12:40-12:55 Stefanie Schindler, Animalfree Research, CH-Bern
Mind the gap - developing and implementing integrated testing strategies into nanotoxicology
- 12:55-13:10 Cornelia Loos, University of Ulm, DE-Ulm
The chick chorioallantoic membrane (CAM) assay as a model for the development of antitumor nanotherapeutics

13:10-14:30 Lunchtime poster session - poster session II
(business lunch to be served at the congress venue)

Session VI - Specific Endpoints of Toxicity (part I)

- 14:30-14:50 Robert Landsiedel, BASF SE, DE-Ludwigshafen
Hurdles during the development and application of alternative methods: a perspective from the chemical industry
- 14:50-15:05 Judith Madden, Liverpool John Moores University, UK-Liverpool
Modelling local and systemic toxicity: Incorporation of *in silico* predictions in the development of adverse outcome pathways
- 15:05-15:20 Jan Willem Van der Laan, Medicines Evaluation Board, NL-Utrecht
Pharmacology and Carcinogenicity. Predictivity as a possibility to reduce the number of carcinogenicity studies
- 15:20-15:35 Katherina Sewald, Fraunhofer ITEM, DE-Hannover
Pre-validation of the *ex vivo* model PCLS for the prediction of acute inhalation toxicity
- 15:35-15:50 Rodger Curren, Institute for In Vitro Sciences Inc. (IIVS), US-Gaithersburg, MD
Further refinement of the Reconstructed Skin Micronucleus Genotoxicity Assay (RSMN)
- 15:50-16:05 Tzutzy Ramirez Hernandez, BASF SE, DE-Ludwigshafen
Inter-laboratory validation of the Yeast Estrogen and Yeast Androgen Screens for identification of Endocrine Active Substances

16:05-16:45 Coffee break, posters and exhibition

Session VII - Tox21 (TT21C) & Specific Endpoints of Toxicity (part II)

- 16:45-17:00 Robert A Coleman, Safer Medicines Trust, UK-London
Humanizing toxicity testing in the 21st century: Who should be responsible for introduction of human biology-based tests into regulatory process?
- 17:00-17:15 Carl Westmoreland, Unilever, UK-Bedford
21st century safety science and non animal approaches at Unilever
- 17:15-17:30 Marcel Leist, Univ. of Konstanz, DE-Konstanz
Use of transcriptome profiling in stem cell based test systems for reproductive toxicity
- 17:30-17:45 Julia Hoeng, Philip Morris international, CH- Neuchâtel
Assessing the effects of repeated cigarette smoke exposure using human organotypic systems reproducing the respiratory tract *in vitro*
- 17:45-18:00 Anne Krug, Univ. of Konstanz, DE-Konstanz
Integrating transcriptomics and metabolomics to identify pathways of toxicity of the parkinsonian toxin MPP+

18:30 Departure for the social evening

19:00 Social evening



Wednesday, 18.09.2013

Session VIII - Implementing Alternatives into Basic Research

- 09:00-09:15 Gerhard Püschel, University of Potsdam, DE-Potsdam
Cell culture-based *in vitro* method for determining the activity of the botulinum toxin
- 09:15-09:30 Karin Schütze, CellTool GmbH, DE-Bernried
Novel laser-based identification of cancer cells and monitoring of cell-agent interactions
- 09:30-09:45 Theresia Licka, University of Veterinary Medicine, AT-Vienna
Research in perfused limbs successfully replaces induction of laminitis in live horses
- 09:45-10:00 Günther Weindl, Freie Universität Berlin, DE-Berlin
Phase I biotransformation of testosterone by human skin and reconstructed skin tissues
- 10:00-10:15 Candida Nastrucci, Tor Vergata University, IT-Rome
The alternatives to animal experiments in basic research and education: Status and possibilities in the EU
- 10:15-10:30 Johannes Grillari, VIBT – BOKU, AT-Vienna
Immortalized primary-like human cells as novel model systems in nephrotoxicity

10:30-11:00 Coffee break, posters and exhibition

Session IX - Drugs, Vaccines & EU FP7 Projects

- 11:00-11:18 Maren Bernau, Livestock Center Oberschleissheim, DE- Oberschleissheim
Magnetic resonance imaging as an alternative method in safety-testing of veterinary vaccines – preliminary results in pigs
- 11:18-11:36 Otmar Schmid, Comprehensive Pneumology Center & Helmholtz Zentrum München, DE-Neuherberg
New perspectives for realistic and efficient *in vitro* screening for inhalable drugs
- 11:36-11:54 Jia Jia, Department of Clinical Pharmacology, University Medicine, Greifswald, Germany
Use of *in vitro* cell assays and noninvasive imaging techniques to reduce animal experiments in drug development
- 11:54-12:12 Francois Busquet, CAAT EU, BE-Brussels
The CAAT EU policy program in Brussels
- 12:12-12:30 Sunniva Förster, University of Konstanz, DE-Konstanz
Development and application of an algorithm to determine statistically-valid non-cytotoxic concentrations from imperfect *in vitro* cytotoxicity data sets
- 12:30-12:40 Closing remarks**
- 12:40 End of the 18th European Congress on Alternatives to Animal Testing**
- 13:00 Start of the post congress EUSAAT workshop**



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Abstracts of All Lectures and Posters

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A catalogue of criteria to objectify the harm-benefit analysis according to Austrian legislation

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The EU Directive 2010/63/EU (EU, 2010) prescribes a harm-benefit analysis as a part of the evaluation of animal experiments, which has to take ethical considerations into account (Article 38 (2) d). The Austrian Animal Experimentation Act (TVG 2012), that transfers this requirement into national law, has come into force on January 1, 2013. One of the new aspects in the harm-benefit analysis in Austria is that a catalogue of criteria has to be submitted by the applicant to the competent authority to provide (amongst other things) the basis for the project evaluation. The catalogue to be used is based on scientific criteria in order to objectify and standardize the harm-benefit analysis. A project to develop this catalogue has been launched at the Messerli Research Institute. It is funded by the Austrian Federal Ministry for Science and Research (BMWF).

Against the background of the main results of a symposium on harm-benefit analysis (March 27, 2013, Vetmeduni Vienna), the structure of the Austrian Catalogue of Criteria has been developed. In this presentation, we will outline the methodological structure that comprises three approaches: a) checklist, b) scoring and weighing procedure, c) comparative approach. These

three methodologies have advantages and disadvantages for the project evaluation. However, in order to provide a feasible methodology, all approaches are integrated in the Austrian Catalogue of Criteria according to their specific merits. The reasons for integrating the three methodologies and how they are built into the structure of the catalogue of criteria will be presented.

Acknowledgement

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Blood-brain tumor and air-blood tissue barrier: two examples of how BioMEMS can improve the relevance of co-cultured *in vitro* models of biological barriers

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In the last years cell culture models with various cells to mimic different organs have been successfully developed and they tremendously improved the relevance of the organs' *in vitro* models. In general the behaviour of the co-cultures in response to external stimuli result closer to physiology also thanks to their 3D arrangement and complexity. In the case of biological barriers, such co-culture models are of great interest to evaluate drug nanocarriers biokinetics, with a specific application in the clinical early screening of drug carrier absorption and in the risk assessment of novel (nano-)materials. In this case the relevance of their behaviour lies in their absorption capacity according to the specific transport mechanism leading to the translocation occurrence which needs to be accurately quantified.

We present here two novel *in vitro* co-culture models of biological barriers, namely the blood-brain tumor barrier (BBTB) and the air-blood tissue barrier, which have been designed using an innovative insert-like system for cell cultures which greatly contributed to increase the relevance of the investigated models. The insert-like device named SIMPLI-well, (Silicon Microporous Permeable Insert-well), is the issue of the CSEM's know-how in the conception of micro electro-mechanical systems for biological applications (BioMems).

The contribution to the relevance of the *in vitro* models lies in the following features: the reduced thickness of the permeable mechanical support (i.e. 500 nm) compares better than commercial available polymer based inserts (i.e. 10 mm) to the thickness in one case of the basement membrane of the cerebral vascular system and in the other with the basal lamina of the air-blood alveolar barrier. Then, the uptake of ultrasmall iron oxide nanoparticles (USPIO NP) and gold nanoparticles (Au NP), respectively, was shown according to a mechanism where the rate determining step is the cell layer crossing, being

the mechanical ceramic support almost transparent due to its extremely reduced thickness. Finally the thinness, the well-controlled distribution as well as size and shape of the pores are particularly suitable for the building up co-culture systems where different cell lines are grown on the opposite sides of the same mechanical support and are then kept separated while getting benefice of their mutual proximity (cell-cell signaling). Therefore, the *in vitro* BBTB was used to show for the first time the transfer of USPIO NP from human brain derived endothelial cell to human glioblastoma cells.

The *in vitro* model of the air-blood tissue barrier consisting of epithelial cells as well as monocyte-derived macrophages and dendritic cells was refined not only by replacing the conventional inserts by the novel one but also by complementation with endothelial cells as a fourth cell type.

Thanks to the improved BBTB, the system revealed as a convenient approach to evaluate nanotheranostics' delivery to brain cancers, then very interesting as clinical early screening trial.

Using the improved air-blood tissue barrier model to quantify the uptake of Au NP, we proved the whole novel *in vitro* model as excellent tool to "realistically" study the inhalation effects while reducing the number of animals used for risk assessment.

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Retrospective analysis of the mutagenicity/genotoxicity data of the cosmetic ingredients present on the Annexes of the Cosmetic EU legislation

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To evaluate the mutagenicity/genotoxicity of cosmetic ingredients at the regulatory level, usually a battery of three *in vitro* tests is used. This battery, however, produces high numbers of misleading positive results, as such imposing the need for *in vivo* follow-up testing. In Europe the use of experimental animals became impossible due to the implementation of an animal testing and marketing ban in the cosmetic legislation. Consequently, in the future potentially safe substances will be lost for the EU market. Strategies to try to improve the specificity of the existing assays include optimization of the cell type and cytotoxicity assay used and lowering of the top concentration applied. A reduction of the number of tests in the battery from three to two has also been suggested. In this study, the performance of the “standard” *in vitro* mutagenicity/

genotoxicity testing battery is analyzed retrospectively, setting up a database containing quality toxicological data on 249 cosmetic ingredients present on the Annexes of the cosmetic legislation (2000-2013). For all *in vitro* mutagenicity/genotoxicity tests carried out on these ingredients a lower specificity was found compared to the results published for chemicals. Misleading positives amounted up to 93%. The cell type and the top concentration used did not have a major impact on the specificity. For the cytotoxicity assays used, a consensus should be reached, as different cytotoxicity end points may lead to different testing concentrations. These results point to the urgent need for better regulatory strategies to assess genotoxicity of cosmetic ingredients present on the Annexes



A new organotypic 3-D human small intestinal tissue model reconstructed from primary human cells

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The intestinal epithelium controls entry of orally ingested nutrients/medicaments. Currently, the most common *in vitro* model for study of intestinal drug safety/absorption is a colon carcinoma cell line, Caco-2. These cells differentiate into monolayers of polarized enterocytes that express tight junctions, but lack mucus-secreting goblet cells and show inter-passage inconsistency. Others have developed small intestinal organoids which are not suitable for apical application of test articles. Here, we report an organotypic small intestinal epithelial tissue (SI) generated from primary human SI epithelial cells. Human SI epithelial cells and fibroblasts were expanded in monolayer culture and seeded on microporous membrane inserts to reconstruct 3D organotypic SI tissues. Tissue morphology, biomarker expression, and ultrastructural features were characterized by H&E staining, immunohistochemistry, and transmission electron microscopy, respectively. Drug transporter

expression was checked by RT-PCR. Tissue responses were examined following TNF-alpha exposure. Analysis of the SI tissue model revealed: 1) wall-to-wall epithelial growth, 2) columnar epithelial cell morphology similar to human SI, 3) expression of muc-2, CK19, and villin, and 4) formation of brush borders and tight junctions. RT-PCR results showed expression of efflux drug transporters MDR-1, MRP-1, MRP-2, BCRP and the metabolic enzyme (CYP3A4). Furthermore, treatment of the tissue model with TNF-alpha induced a proinflammatory response by inducing chemokines including IL-8 and GRO-alpha. In conclusion, the new human cell-based SI model will likely be a valuable tool for pre-clinical evaluation of drug safety/absorption as well as preclinical study of intestinal mucosal inflammation, microbiomes, and microbial infection mechanisms in the gastrointestinal microenvironment.



Novel 3-D reconstructed human tissues model of psoriatic skin

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Psoriasis is a chronic skin disease that affects about 2% of the global population. Availability of well characterized *in vitro* psoriatic tissue model will enhance our understanding of the disease and the development of therapeutic strategies. In this study, an *in vitro* three-dimensional psoriatic-like tissue model was reconstructed, culture conditions were optimized, and the model was characterized using H & E staining (morphology), immunohistochemistry (proliferation of basal cells as demonstrated by the Ki67 staining profile, psoriasin expression), RT-PCR (gene expression levels), and ELISA assays (cytokine analysis). Results showed that the reconstructed psoriatic 3-D tissue model has phenotypic and architectural similarity to the *in vivo* counterpart in that the tissue model showed over expression of biomarkers associated with psoriasis including human beta

defensin-2 (HBD-2), psoriasin, and CXCR2. Similar to the *in vivo* situation, cytokine analysis of culture supernatants from the psoriatic-like tissue model showed increased release of IL-6 (7 fold), IL-8 (5.5 fold), and GRO- α (3.8 fold) by the psoriatic tissue model when compared to normal reconstructed epidermal tissues. Treatment of the psoriatic-like tissue with IL-4 or calcipotriol showed a dose dependent decrease in HBD-2 gene expression and IL-6 release (3.0-4.3 fold). In conclusion, the psoriatic tissue model mimics the *in vivo* counterpart in terms of tissue morphology, tissue structure, gene expression (CXCR2, beta defensin, and psoriasin), and cytokine release (IL-6, IL-8, GRO-a). The model can serve as a valuable tool to study the biology of psoriasis and for preclinical assessment of toxicity and proinflammatory effects of therapeutic candidates.



Human 3D corneal models for a detailed quantification of the initial depth of injury as an indicator for cellular damage in the human eye

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Currently, the definite prediction of all GHS categories for the eye-irritating potential of chemicals in one single test system relevant to the human eye is not possible. Instead, chemicals can only be classified according to the GHS in the framework of an extensive battery.

Our study aims at the complete replacement of the Draize Eye Irritation Test by a new test system which is based on biotechnologically produced hemi-cornea equivalents (Engelke et al., 2013). The sophisticated structure of the hemi-cornea model comprises both an epithelium and stroma compartment. Hence, this two-compartment model offers the potential to analyze the initial depth of injury (DoI) after substance application and to discriminate between damages induced in the epithelium and/or the stroma. We developed different approaches for the analysis of the corneal DoI in the hemi-cornea model:

1. A collagen membrane is inserted between stroma and epithelium during production as an artificial Bowman's membrane. This membrane allows the detachment of the epithelium from the stroma and the individual quantification of the cell viability in both compartments after chemical treatment.
2. A TUNEL assay is performed on cryosections of the hemi-cornea in order to label all cells which have undergone apoptosis after topical treatment with the respective test

substances. The TUNEL assay can be combined with the detection of cleaved-caspase 3, another biomarker for apoptosis. The border between the area of fluorescent-labelled cells and non-labelled cells indicates the DoI.

3. In order to specifically label the viable cells within the treated tissues, the MTT viability assay is combined with image analysis on cryosections. Quantitative interpretation of the cryosection images is based on ImageJ software analysis tools. By using this method we can distinguish between substances of all three GHS categories by means of their depth of injury.

By using one single method or a combination of the above-mentioned methods we aim at the quantification of the initial depth of injury within epithelium and stroma of the hemi-cornea as a stand-alone test system for the reliable prediction of the eye-irritating potential of substances according to the GHS system.

The project was funded by the German Federal Ministry of Education and Research (FKZ 0316010 A-C).

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Magnetic resonance imaging as an alternative method in safety-testing of veterinary vaccines – preliminary results in pigs

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As an important part of the licensing procedure for veterinary vaccines safety-tests in animals are mandated by the European Pharmacopoeia and further European regulations for immunobiologicals (European Pharmacopoeia, 2008; Regulation, 2009). These tests depend on a large number of animals, because every animal species and class, for which the approval has to be obtained, needs to be tested. Normally, after vaccination, the animals have to be killed in frequent intervals to undergo a pathologic examination. In order to reduce the number of animals – according to the 3R concept of Russel and Burch (1959) – this study aimed at testing magnetic resonance imaging (MRI) as an alternative method in detecting and evaluating vaccination-caused inflammatory reactions in live pigs. MRI is the method of choice for soft tissue imaging in human medicine. It offers the potential of non-invasive 3D imaging and can be used for follow up examinations (Kuo and Carrino, 2007; Schedel et al., 1992; Walker, 2008).

For this study 32 pigs (12-weeks of age) were divided into two groups and vaccinated into the left side of the neck (VN) using two licensed vaccines. An MRI scan was performed in the sedated animals at day 1, 3, 8, 15, 22 and 29 after vaccination, using an open low-field MRI system (Siemens Magnetom Open; 0.2 Tesla). T1- and T2-weighted Spin-Echo sequences were chosen representing two directions of acquisition (coronal and axial). In order to detect the whole extent of the expected inflammatory reaction, a contrast agent (Gadobutrol) was used in 50% of the animals of each group. As Gadobutrol is not listed in the annex by law (Regulation, 2009), its use in farm animals is not permitted. Therefore, all Gadobutrol-treated animals had to be euthanized at the end of the study and underwent a pathological examination.

In order to compare the volumes of the inflammatory reactions caused by vaccination, the MRI images were evaluated using the Able 3D Doctor Software[®] (Lexington, MA, USA; FDA approved). According to signal intensity altered tissue structures were bordered semi-automatically and their volumes were calculated; on the one hand within the VN on the other hand the – regarding the grey scales – corresponding structures within the control (right) part of the neck (CN).

For statistical evaluation an F-Test and a paired t-Test ($p \leq 0.05$) were performed using the statistic software SAS 9.3 (Institute Inc., Cary, NC, USA) in order to compare the tissue-volumes for all sequences. The results showed significant differences between VN and CN. As well differences regarding

the extent of an inflammatory reaction were observed between both vaccines.

These preliminary results demonstrate that *in vivo* MRI is suitable for safety-testing of veterinary vaccines to monitor and to evaluate inflammatory reactions. The number of animals could be reduced by replacing the need for intermediate pathologic examinations. By evaluating additional vaccines and age categories of pigs a scoring system could be developed. This could be used to document the extent of inflammatory reactions caused by immunologic products and would offer a tool for safety evaluation and benefit-risk assessment of veterinary medicines.

Acknowledgement

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***In vitro-in vivo* extrapolation of prenatal (neuro)toxicity assay data by PBPK modeling**

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In the replacement of animal studies by *in vitro* and *in silico* methods, a prominent role has been attributed to (physiologically based) pharmacokinetic ((PB)PK) modeling. The prediction of the kinetics of compounds can be a goal in itself, ultimately to replace pharmacokinetic and tissue distribution studies. In addition, new toxicity testing paradigms based on *in vitro* assays require a link between concentrations the cells are exposed to and the (external) doses leading to those concentrations in target tissues *in vivo* (Adler et al., 2010; Hartung et al., 2011).

Developmental and reproductive toxicity are among the most demanding areas of toxicology in terms of laboratory animal use, and at the same time most challenging to develop alternative methods for (Adler et al., 2010; Hartung et al., 2011). In the past years, a steady progress has been made in establishing new methods, assays and batteries of assays to address developmental toxicity, in the ESNATS project specifically neurotoxicity. PBPK modeling has contributed to the development of these new test systems and helped to gain confidence in the predictive value of their outcomes. Sensible study designs with *in vivo* relevant concentrations were defined, results have been compared between test systems with differing protocols, and effective concentrations observed *in vitro* have been correlated to those predicted based on *in vivo* toxicity information from historical animal and/or human data (Krug et al., 2013; Piersma et al., 2013).

Drawing from examples from EU projects ESNATS, Chem-Screen and ReProTect, an overview is given of the approach to *in vitro-in vivo* correlation and extrapolation that was applied in these projects (Krug et al., 2013; Piersma et al., 2013; Verwei et al., 2006; Louisse et al., 2010). This approach is based on

1) (PB)PK modeling to relate doses from different routes and duration of exposure in different species to the target site concentration, and 2) the correction for differences in protein binding and lipid partitioning between *in vitro* medium and plasma/extracellular fluid *in vivo*. A perspective is given on applying these methods for predicting safe human exposure levels from *in vitro* test results, and the challenges on the road towards animal-free toxicity testing are discussed.

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Coupling of the BALB/c-3T3 cell transformation assay to a metabolic activation system and to the soft agar colony formation assay: determination of two endpoints in a single *in vitro* test system

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The analysis of the carcinogenic potential of chemicals plays an important role in toxicology. The optimization of well-established *in vitro* methods for this purpose could lead to a significant reduction of the amount of experimental animals used for carcinogenicity testing in the near future. The aim of this study was to further optimize the BALB/c-3T3 cell transformation assay, which is routinely performed in up to 42 days. This method is well suited for high-throughput screening applications and allows a quantitative analysis of the aforementioned carcinogenic potential of different chemicals. The principle of this test method is based on the fact that BALB/c-3T3 cells (murine embryonic fibroblasts) loose cell-cell contact growth inhibition upon treatment with different carcinogenic compounds. This leads to the development of so-called foci, which can be distinguished by characteristic changes in cell morphology and growth behaviour. However, a major drawback of the "classic" BALB/c-3T3 cell transformation assay is the difficulty to detect chemical compounds that initially require metabolic activation to gain their full genotoxic potential. Hence, without prior metabolic activation, many chemicals will not be detected as carcinogenic.

In the present study the BALB/c-3T3 cell transformation assay was linked to a metabolic activation system to overcome the aforementioned disadvantage and to generate an *in vitro* method, which can mimic the *in vivo* situation as closely as possible. Moreover, this test system was coupled to the soft agar colony formation assay, another *in vitro* method used for the detection of the transforming potential of agents. In the soft agar colony formation assay one can investigate the ability of

chemically treated cells to grow in an anchorage-independent manner. This type of growth is a characteristic of malignantly transformed cells. By coupling the two above-mentioned assays two biological endpoints related to malignant cell transformation can be determined in a single *in vitro* assay.

In the initial step of the study, the metabolism of the well-known genotoxic agents benzo[a]pyrene, aflatoxin B1 and N-nitrosodimethylamine by two different metabolic activation systems was investigated. The so-called S9 mix was shown to be suited to activate the genotoxic compounds, while the cell line HepaRG[®] also led to the formation of foci, but to a much lower extent. All three compounds led to a concentration-dependent increase in the number of foci formed, and this increase was observed in a non-cytotoxic concentration range.

In a next step cells exposed to aflatoxin B1 as an initiating agent and TPA as a tumor promoting agent in the classical BALB/c-3T3 cell transformation assay were successfully transferred into the soft agar colony formation assay on day 22, in the case of wanting to test different tumour promoters the transfer was performed on day 28 to 30. The results show that a coupling of the BALB/c-3T3 cell transformation assay to the soft agar colony formation assay is possible. By performing the soft agar colony formation assay in 96-well microtiter plates a high number of compounds can be tested in parallel.

Acknowledgment

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The Lush Prize – Supporting Animal Free Testing

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The Lush Prize was created to expedite the replacement of animal tests in product safety testing by rewarding and funding strategic projects and interventions anywhere in the world. It aims to raise public awareness of ongoing safety testing, promote the movement for change and focus pressure on toxicity testing for consumer products in a way which complements the many projects already addressing medical testing issues. The £250,000 Lush Prize makes awards for projects or interventions in a number of categories and is a 1R prize, focussing on replacement only. It is a partnership between Lush Cosmetics and the Ethical Consumer Research Association and has been developed in consultation with a panel of judges with particular expertise in relevant fields, and the general public through inviting comments and interactions via the project website and inviting two 'lay persons' onto the judging panel. The prize has a dual focus: A longer term aim is to offer the 'Lush Black Box Prize' in any one year of the full £250,000 Lush Prize fund for a key breakthrough in human toxicity pathways research (21st century toxicology). A more immediate aim is to highlight the various areas in which change needs to happen for animal testing to be eliminated and reward those driving change in these areas, whilst on a strategic level encouraging a multidisciplinary approach to tackling the issue. With this in mind in interim years the £250,000 annual award is broken down into five smaller awards for 'outstanding contributions' in the following sectors:

Science Prize – 21st Century Toxicology Research, a new approach to safety testing which is exciting regulators, toxicolo-

gists, campaigners and companies globally. It has become possible because of advances in biology, genetics, computer science and robotics.

Training Prize – training researchers in non-animal methods, as many scientists and regulators concerned with chemicals testing are not aware of the full range of non-animal methods available, or are not trained in using them. Establishing training programmes around the world makes a huge difference to progress.

Lobbying Prize – policy interventions to promote the use of alternatives as scientific innovation needs to go hand-in-hand with policy change to ensure that end-users of new testing approaches – industry and regulators – are receptive and responsive to the new methods.

Public Awareness Prize – public-awareness raising of ongoing testing, as despite years of campaigning, animal testing has yet to be consigned to the history books where it belongs. However, partial legislative victories have led to the common misconception that animal testing for cosmetics no longer takes place.

Young Researcher Awards – to five post-doctoral researchers specialising in alternatives research, with toxicology centred on animal testing for so long many scientists concerned about animal use are deterred from entering the field. Those who do can find that access to funding for working on non-animal tests can be a barrier. We want to change this and encourage young scientists to develop a career in toxicology without harming animals by offering £10,000 bursaries to allow them to advance in this area.

Hurdles during the development and application of alternative methods: a perspective from the chemical industry

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The safety of consumer products needs to be assured before products enter the market. For this purpose, the toxicological potential of those products must be determined. By law, the toxicological testing often requires animal studies. According to our commitment to the ethical principle of animal protection we try to develop and apply 3R methods whenever it is possible. However, during the process of development, establishment, and application of those methods many problems can be encountered. Some of these hurdles are technical issues, published alternatives which do not fulfill requirements towards high reproducibility in different laboratories, and an insufficient validation procedure (not enough compounds

tested to accurately determine the applicability domain of the test system). Most often, however, alternative methods are not validated fast enough and regulatory acceptance takes too much time. Therefore, the use of alternative methods for regulatory purposes is often delayed and do not match legislative and political goals (e.g. cosmetic directive and REACH). Herein, we report several examples of alternative methods that have encountered technical issues, lack of relevance relative to the *in vivo* situation but also on methods that would be ready for application but for which formal validation and regulatory acceptance is still lacking, and regulatorily accepted methods that have little value for the hazard assessment.



Why a CAAT Europe policy program in Brussels?

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Up to 12 million laboratory animals are used per year in the EU for scientific purposes compared to 550 million farm animals consumed each year in the very same EU. These two numbers put in perspective how difficult it can be to raise the attention of a sensitive and technical topic such as alternative test methods for laboratory animal experiments at the European Parliament. It is however necessary since the European Union is responsible for more than 80% of the national laws. Until 2012, only animal welfare organisation and Industry were discussing permanently the 3Rs on safety testing in Brussels at the European Parliament, but nobody from the Academia. Since February 2012, CAAT-Europe launched its own policy program in order for the Members of the European Parliament (MEPs) 1) to be aware on

the 3Rs center in their own member states 2) to inform them of the multiple initiatives from the US and/or at the EU level 3) to disseminate the concept of PoTs and AOPs 4) to explain research project such as the human toxome project or SEURAT-1 5) to become an reliable source of information for science in safety testing. In this lecture, the CAAT EU policy program will be further explained and will summarise the activities of lobbying, dissemination and collaboration achieved so far. It will describe the functioning of the legislative process using the EP parliament own initiative report on "Endocrine disrupters for the protection of public health" to illustrate how 3Rs can be disseminated at the policy level.

Alternative methods in education: what do students think?

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From AY 2006-2007 to AY 2012-2013, the University of Thessaloniki, School of Veterinary Medicine and the University of Milan, Faculty of Veterinary Medicine collected 276 and 119 completed questionnaires respectively, on the use of alternative methods in education (Daneshian et al., 2011).

The majority of Italian students (80%) were attending their 3rd year in Veterinary Medicine and only a few were studying Animal Husbandry and Welfare and Veterinary Biotechnology Sciences. In Greece, the survey was conducted more uniformly and involved Veterinary Medicine students in different years of study.

Both Greek and Italian students underlined the relevance of practical classes to their future careers. Even if students believe that opportunities for active participation in animal-based classes are very important, they desire a balance between technology-based alternatives and animal-based practical classes. Moreover, Greek students (60.1%) would like to have the opportunity to participate in independent learning based on alternatives including computer simulations, manikins/models and high quality videos.

In Greece, practical classes with alternatives were offered mainly in anatomy, physiology and pharmacology, whereas few students received practical training with alternatives in toxicology. Conversely, Italian students received more alternative-based practical training in toxicology.

Students expressed their desire to have more teaching based on alternatives in the veterinary curriculum. However, 61.2% of Greek students believe that the use of alternatives in veterinary education should be optional, whereas 55.4% of Italian students believe it should be compulsory.

In contrast to Italian students (47.9%) who have not formed an opinion on the matter yet, Greek students (62.5%)

declared that they would not refuse to participate in practical classes involving the use of animals, even if alternatives were available.

Although students believe that practical classes based on alternatives are a valuable part of the veterinary curriculum and provide opportunities for the development of practical skills/techniques, they do not consider practical training with alternatives to be as good as traditional methods. Indeed for the majority of the veterinary students who took part in this survey, animal-based practical training is essential, interesting and justifiable in relation to the knowledge and skills acquired. Moreover, for Italian students (47.9%) practical classes with animals are encouraged by the veterinary curriculum.

Most of the students from both Universities believe that alternative learning methods can prepare or reinforce existing knowledge required in veterinary clinical years and be an effective supplement to traditional veterinary training and teaching. Most importantly, the majority of students interviewed think that the development of new technology-based as opposed to animal-based alternatives in veterinary education will improve teaching and learning.

Finally, 58.8% of students believe that animals should be used only if the scientific objectives are valid, there is no other alternative and pain and suffering are kept to a minimum and expressed their desire to participate in a working group aimed at developing alternatives to animals in veterinary education.

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Development of a neural teratogenicity test: comparative microarray analysis of two different hESCs lines following exposure to valproic acid

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Human embryonic stem cell (hESCs) lines are increasingly used as cell model for the development of alternative toxicity testing assays, due to their ability to recapitulate during differentiation some of the early gastrulation processes. In the context of the ESNATS project (www.esnats.eu) the focus has been pointed on prenatal neurodevelopmental toxicity taking advantage of the tendency of hESCs to easily differentiate *in vitro* towards the neural lineage. While this tendency is extensively reported in the literature, there is also evidence that different hESCs lines exhibit different propensity to originate specific sublineages, potentially making some lines more suitable for toxicity testing.

The aim of this study was to compare by microarray analysis the effect of the exposure to valproic acid (VA) on neural differentiation of two hESCs lines (HUES1, H9), in order to evaluate the similarities and differences of the induced transcriptomic changes. To this aim both cell lines were differentiated, through an embryoid body stage (Zhang et al., 2001) and plated in neural induction media. During the process of neural rosettes formation the cells were exposed to different concentration of VA, known to induce perturbation of neural development *in vivo*. At the end of the 8 days of exposure samples were analysed by Affymetrix HG-U133 Plus 2.0 array technology and the data compared after K-mean clustering and ANOVA calculation. Overall the results indicate, as expected from the literature, that VA acts in both lines principally on two targets: the central nervous system (CNS) and the neural tube derivatives, particularly on the neural crest cells migration. On the basis of these observations, two wide classes of genes were differentially expressed in both lines in respect to the untreated control: those acting on cellular adhesion molecules and cell migration processes and those belonging to the CNS. In addition to that there were also genes linked to or acting together with the histone deacetylase complex, that

is known to be inhibited by VA, causing most of the dysregulations seen following the exposure. Analysing the data it was clear that there was a difference in the effective concentration of VA between the two lines (on average ten folds lower for HUES1), probably due to the different propensity of the lines to differentiate in neural crest cells. We have already demonstrated (Colleoni et al., 2010) that neural rosettes from HUES1 lines and their surrounding cells are prone to generate neural crest precursors by default, while it is known from the literature that neural rosettes derived from H1/H9 lines have a default tendency to generate CNS precursors. Since VA acts more specifically on neural crest cells, it was expected to see transcriptomic changes at lower concentrations in neural rosettes derived from HUES1 line.

Taken together these data indicate that this neural teratogenicity test has predicted the same perturbation of neural differentiation in both lines, with some differences linked to their intrinsic default differentiation pattern. Finally the knowledge of the differentiation tendency of each cell line is important for a correct interpretation of the results and for producing reliable data.

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Ologen[®] Collagen Matrix: a new dermal scaffold for skin tissue engineering

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Acute and chronic human skin wounds have a large impact on patient's quality of life as well as on the global cost of health care. This problem is expected to further increase with ageing of the human population, illustrating the urgent need for novel chronic wound treatment strategies such as tissue engineered skin. Various epidermal, dermal and bilayered products are currently available for skin wound healing purposes, either cellular or acellular. In this study we investigated for the first time the suitability of the biodegradable porous collagen-glycosaminoglycan (C-GAG) ologen[®] Collagen Matrix (ologen[®] CM) for full thickness skin tissue engineering purposes. We generated ologen[®] CM-based human skin equivalents (HSEs) by seeding ologen[®] CM with dermal human fibroblasts and epidermal human keratinocytes. Static and centrifugal seeding approaches were compared.

Primary human skin cells successfully attached to the ologen[®] CM and resulting HSEs showed healthy and intact human skin morphology, proliferation, differentiation and basement membrane formation. This was shown by normal presence of proliferation marker Ki67, early and late differentiation markers keratin 10 and involucrin in the epidermis and basement membrane components collagen type IV and laminin 332 at the dermal-epidermal junction of ologen[®] CM-based HSEs. In conclusion, the C-GAG content of ologen[®] CM combined with centrifugal seeding of human dermal fibroblasts provides a functional dermal substitute closely representing native human dermis. This opens opportunities for successful applications in full thickness human skin tissue engineering.



Investigation into the inflammatory response of the H292 lung epithelial model to cigarette smoke particulate generated using different smoking regimes and reduced toxicant prototypes

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Introduction

Cigarette smoking is the primary risk factor in the development of COPD, a complex, multifaceted disease characterised by specific pathologies such as emphysema, small airway disease and chronic bronchitis. These pathologies are driven by an abnormal and persistent inflammatory response. To assess whether changes in product design which reduce toxicant levels are effective at a biological level, *in vitro* models are used to measure disease related endpoints. The aim of this work was to determine whether the NCI-H292 lung epithelial model can be utilised to assess the effects of smoke particulate from different products and smoking regimes. This was achieved by exposing the cells to particulate matter (PM) extracts and measuring levels of COPD associated inflammatory mediators.

Methods

PM extracts were prepared by smoking cigarettes, capturing the particulate fraction of smoke onto a Cambridge filter pad and eluting in dimethyl sulphoxide. Extracts were generated from 3R4F reference cigarettes, 1 mg, 6 mg and 7 mg reduced toxicant prototype cigarettes and a 3R4F reference cigarette modified with a carbon filter. 3R4F reference cigarettes were also tested using two different smoking regimes; ISO 3308: 2000, defined as a 35ml puff, 2 second duration and 60 second interval (35/2/60, vents open) and an intense smoking regime used within R&D (55/2/30, vents open). NCI-H292 cells were cultured to a fixed passage number

of 89 then seeded in test plates and grown to confluence. Twenty four hours prior to exposure, serum free media was used to replace culture medium. Subtoxic doses of PM were prepared in serum free media and cells exposed for a period of twenty four hours. Media was collected and analysed using electrochemiluminescence. The proteolytic enzymes MMP-1, MMP-3 and MMP-9, inflammatory chemokine IL-8, cytokine IL-6 and vascular endothelial growth factor were all measured using the Meso Scale Discovery system.

Results

All mediator levels increased proportionally with increasing concentration of PM extract, however the slope of the response for all samples remained similar and non-significant when products were compared with their controls. Differences in the magnitude of inflammatory mediator secretion were observed between the different smoking regimes ($p=0.004$), and also the different cigarette types ($p<0.001$) but this was not observed for all prototypes.

Conclusions

Our results show that the NCI-H292 PM exposure model can be used to test variations in cigarette product design and smoking regime. The effects of cigarette modifications on particulate matter derived from these new products can be determined at a biological level and used to guide further investigation using more complex test and smoke exposure systems.



3D spheroid cell cultures as models in drug discovery

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Three-dimensional (3D) cell cultures have great potential as predictive tools in drug discovery. 3D cell cultures possess many features that mimic the *in vivo* microenvironment that are lacking in traditional two-dimensional (2D) cell cultures, such as physiological cell-cell and cell-extracellular matrix (ECM) interactions and mass transfer gradients. Thus, with the implementation of 3D cell cultures into the drug screening process, more physiological data can be obtained long before animal testing. 3D spheroids make excellent simple models of avascular tissues and tumors, as well as embryoid bodies, because they are reproducible, versatile, and well-characterized. Importantly, spheroids possess physiological cell-cell contacts, secrete their own ECM, have nutrient, drug, and oxygen mass transfer gradients, and are often used as 3D models of many types of avascular tissues, tumors, and embryoid bodies.

This talk will describe the uses of Perfecta3D Hanging Drop Plates, which are 96- and 384-well plates that facilitate the formation, culture, and testing of spheroids and stem cell aggregates without the aid of coatings or matrices. First, we will describe the uniformity of spheroid formation. Second, we will cover data on comparing anti-cancer drug testing data in spheroid and 2D formats (Tung et al., 2011). The ability to form patterned cocultures will be discussed, including those relevant to migration and invasion studies (Hsiao et al., 2012). New data will be presented on tactics to inhibit spheroid formation in a cancer model. Lastly, we will talk about utilizing these spheroid-formation plates to form and study embryoid bodies.

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Further refinement of the reconstructed skin micronucleus genotoxicity assay (RSMN)

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Cosmetics Europe (former COLIPA) initiated in 2007 a global, multi-laboratory validation project that used reconstructed 3D human skin tissues to assess the genotoxic and carcinogenic potential of substances with known positive and negative genotoxic potential. RSMN was considered as a possible second-tier *in vitro* assay that could replace banned *in vivo* assays as a follow-up to verify false-positives from standard *in vitro* genotoxicity assays. RSMN was evaluated for assay transferability, inter-laboratory reproducibility, and accuracy of the prediction based on approximately thirty substances with known genotoxic potential. The aggregate results of the most recent phase of the RSMN project showed that three out of eight substances expected to be true positives, all tested only in one of the participating laboratories, were found to be

negatives. In addition, while showing very good specificity (about 90%) for both “real negatives” and substances that are prone to generate false-positive results in standard genotoxicity assays, four substances showed positive responses in at least one participating laboratory. Based on these results, the assay procedures in the next phase of the project will be refined. The goals of the next phase of the project are: 1) further assessment of predictive capacity in order to understand the reasons for the false-negative results obtained in previous testing; 2) extension of number of chemicals, especially true positive chemicals, to increase statistical power and acceptance of data; 3) assessment of substances that require metabolic activation for genotoxicity; and 4) investigating automated scoring to improve both the statistical power and throughput of the assay.

hSKP-derived hepatocyte-like cells for toxicity testing

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Hepatocytes in the liver represent the central place where pharmaceuticals are biotransformed. As such, they are highly susceptible to potential toxic effects of mother compounds and their metabolites, leading to so-called Drug Induced Liver Injury (DILI). Currently, DILI is an important cause for drug attrition and of acute liver failure. Furthermore, the correlation between animal liver safety studies and humans is less than 60%, demonstrating that the available models for assessing liver toxicity are not performing adequately. Hence, the pharmaceutical industry urgently needs novel, preferably *in vitro* test systems that are more suitable to predict adverse liver responses in humans. Since primary human hepatocytes are very scarce and their large-scale *in vitro* use is hampered by their inability to proliferate in culture, other cell sources need to be explored. Stem cells could be suitable as they have the ability for self-renewal and the potential for multi-lineage differentiation. Within the ESNATS project, we primarily focused on human skin-derived precursor cells (hSKP). hSKP are a multipotent postnatal stem cell population that resides within the dermis throughout adulthood and shares several properties with embryonic neural crest cells. As such, they are able to generate Schwann cells, catecholaminergic neurons and mesenchymal cell types including adipocytes, chondrocytes and osteocytes. They can be easily isolated from small skin biopsies and expanded in large quantities, qualify-

ing them as an interesting cell source for toxicity testing. Our findings obtained within the ESNATS project showed that, upon exposure to hepatogenic growth factors, hSKP acquire specific features of hepatic progenitor cells as well as typical characteristics of adult hepatocytes. Indeed, hepatic differentiated hSKP (hSKP-HPC) express hepatic progenitor cell markers (EPCAM, NCAM2, PROM1) and adult hepatocyte markers (ALB) as well as key biotransformation enzymes (CYP1B1, FMO1, GSTA4, GSTM3) and influx and efflux drug transporters (ABC4, ABCA1, SLC2A5, SLC39A8). Using a toxicogenomics approach, we could demonstrate that hSKP-HPC respond in a comparable way as cultured primary human hepatocytes to the exposure to acetaminophen, a well-known hepatotoxicant. More specifically, enrichment of the toxicological gene classes of 'liver damage', 'liver proliferation', 'liver necrosis' and 'liver steatosis' was observed in both exposed *in vitro* models. As such, hSKP might represent a suitable preclinical model for *in vitro* developmental hepatotoxicity screening of new chemical entities. At present, the predictive capacity of the hSKP-HPC model towards acetaminophen-induced toxicity is further evaluated by comparison to other relevant *in vitro* hepatic cell lines including HepaRGTM and HepG2, as well as human liver samples of patients suffering from acetaminophen-induced acute liver failure.



The way forward in case of a false positive *in vitro* genotoxicity result for a cosmetic substance?

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The currently used regulatory *in vitro* mutagenicity/genotoxicity test battery has a high sensitivity for detecting genotoxicants, but it suffers from a large number of irrelevant positive results (i.e., low specificity) thereby imposing the need for additional follow-up by *in vitro* and *in vivo* genotoxicity tests. This has a major impact on the cosmetic industry in Europe, seen the imposed animal testing and marketing bans on cosmetics and their ingredients. Afflicted but safe substances will be lost. Using the example of triclosan, a cosmetic preservative, proof-of-principle for the potential applicability of a toxicogenomics-based *in vitro* assay as a potential follow-up test for positive

in vitro genotoxicity results, is described. Triclosan shows a positive *in vitro* chromosomal aberration test but is negative during *in vivo* follow-up tests. Toxicogenomics analysis unequivocally shows that triclosan is identified as a compound acting through non-DNA reactive mechanisms. This study illustrates the potential of genome-wide transcriptomics data in combination with *in vitro* experimentation as a possible weight-of-evidence follow-up approach for de-risking a positive outcome in a standard mutagenicity/genotoxicity battery. As such, in the future a substantial number of compounds wrongly identified as genotoxicants could be saved.

***In vitro* toxicology of air samples: Breathe or not to breathe?**

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Outdoor air pollution represents one of the major problems in many countries. Airborne pollutants have been often associated with many adverse health effects including endocrine disruption. In our work, we have selected three specific effects that have been described in several studies to be related to air contaminants and could be associated with the increased occurrence of diseases. Dioxin-like activity, estrogenicity and antiandrogenicity were assessed in air samples using H4IIE-luc (rat hepatocarcinoma), HeLa9933 (human cervical tumor) and MDA-kb2 (breast cancer) reporter gene cell models, respectively.

Endpoints as endocrine disruption cannot be studied only by chemical analyses because the environmental samples contain very complex mixtures of chemicals and their overall biological effects result from interaction among the pollutants. Thus, it is necessary to employ bioassays to detect the overall toxic effects of the mixtures.

In our study, we have described specific biological effects of air samples obtained by active sampling from two localities in Czech Republic. Air samples were collected for one year at a traffic-burdened urban site in Brno and a village site about 8 km from Brno city to address the influence of city agglomeration as an air pollution source on the surrounding area.

Interestingly, the dioxin-like and estrogenic activity data indicate that samples from village site produced several times greater dioxin-like and estrogenic activity than samples from traffic-burdened sites. In some of the samples we have observed significant androgenic potential as well.

Results demonstrate utility of environmental air sampling, *in vitro* biotests in assessment of specific biological effects of air and last but not least monitoring of safe levels of contaminants.

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Vitamin D-dependent antimicrobial pathways in human macrophages

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T cell-mediated activation of antimicrobial pathways in macrophages is crucial for effective control against intracellular pathogens, such as *Mycobacterium tuberculosis*. In mice, the macrophage antimicrobial response against *M. tuberculosis* is principally mediated via the induction of nitric oxide, yet the role of nitric oxide in the human macrophage response against *M. tuberculosis* remains controversial. However, activation of human macrophages via T cell-derived IFN- γ induces an antimicrobial pathway that is dependent on the intracellular conversion of 25-hydroxyvitamin D (25D) to the bioactive 1,25-dihydroxyvitamin D (1,25D). 1,25D-mediated activation of the vitamin D receptor (VDR) results in the induction of the antimicrobial peptides cathelicidin and DEF4 that contain vitamin D response elements (VDREs) in their promoters. Cathelicidin also mediates the vitamin D-dependent induction of autophagy, which is required to overcome the phagosome maturation block in infected macrophages and leads to phagolysosomal fusion and finally antimicrobial activity.

Importantly, mice, which are nocturnal animals as opposed to humans, are not a relevant model to study the vitamin D-dependent antimicrobial response, given that the cathelicidin gene contains three VDREs in the human promoter but none in the mouse. In addition, the human promoter of DEF4 contains one VDRE, whereas there is no mouse homolog for this gene. In summary, these findings support the existence of distinct antimicrobial pathways in murine vs. human macrophages.

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Testing neurodevelopmental toxicity on differentiating human embryonic stem cells

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Ambiguous prognostic values of developmental toxicity studies in animals have emphasized the need for a complementary human assay. Since *in vitro* directed differentiation of human embryonic stem cells reproduces the successive inductive events that occur *in vivo*, it represents a promising versatile system for teratogenicity testing. However, evidences have accumulated that show heterogeneity in gene expression profiles among different ESC lines, either resulting from donor genotypes or linked to the establishment of cell lines. The effect of such heterogeneity on *in vitro* toxicity testing results has not yet been evaluated. On the other hand, individuals are known to differ in their sensitivity to a given toxicant and proper testing of developmental effect of chemicals should imply reproduction and comparison of data obtained with different cell lines. The main goal of our work is to analyze the variability of response of different ESC lines to neurodevelopmental toxicants *in vitro* and to identify the sources of that variability.

We are using 4 hESC lines created in our laboratory (CHES2, CHES3, CHES5 and CHES6) to compare their response to several neuroteratogens, i.e. cyclophosphamide (CPA), valproic acid (VPA) and nicotine, known to affect respectively neural induction, neural tube formation and neural cells growth during early CNS development *in vivo*. These 4 hESC lines are used to evaluate the effect of toxicants on two concomitant aspects of neuroectodermal cells development toward neural cells: their ability to form mature neural tube-like structures (rosettes) expressing SOX1 and their differentiation into neural precursors express-

ing nestin. Our data show that each cell line exhibits a unique response profile. However, based on their different sensitivities to that panel of toxicants, two groups of lines can be clearly separated, CHES2-CHES5 and CHES3-CHES6. Cell lines belonging to the same group may nevertheless exhibit slightly different response to a particular toxicant or when a particular readout is used to quantify the deleterious effect.

In parallel, we analyzed the differentiation behavior toward the neural lineage of the 4 hESC lines. Although, exhibiting a similar degree of differentiation efficiency toward neural cells, the hESC lines greatly differ in their tendency to spontaneously commit into the neural lineage during stem cell maintenance. As a consequence, cell lines starting with the higher degree of spontaneous neural pre-differentiation exhibit an increased degree of heterogeneity in differentiating cultures. That behavior correlates with distinctive neurodevelopmental response to the 3 toxicants as the 2 same groups of cell lines can be clearly identified based on differentiation behavior (CHES2-CHES5 differentiating in a much more asynchronous manner than CHES3-CHES6). In contrast, variability of response inside each group could not be correlated to obvious differences in differentiation properties.

Our results stress the importance of assaying several ESC lines for *in vitro* developmental toxicity testing. Experiments are in progress to try to evaluate genetic and non-genetic contributions to response variability to the action of toxicants.



Genotoxicity of nanosized TiO₂ particles in human reconstructed full thickness skin models

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Due to the widely use of nanomaterials in skin care products the examination of the potential risk to human health is of a great importance. Many studies have shown that through intact rodent skin nanomaterials do not penetrate. With the aim to reduce *in vivo* experiments 3D human reconstructed skin models might be used for absorption and effects monitoring of chemicals.

We used human reconstructed full thickness skin models (hEFT) to check for potential genotoxic hazard of manufactured nanomaterials by using the comet assay. Our hypothesis was that a fraction of small nanoparticles in polydispersed materials as investigated by the NANOGENOTOX consortium might penetrate stratum corneum layer and reach viable epidermal cells.

In more than hundred 3D human reconstructed full thickness skin models all TiO₂ nanomaterials (NM102-NM105) investigated for DNA damage in different doses were negative at each dose level in the Comet Assay. In contrast the chemical control MMS consistently generated significant increases in DNA-damage parameters such as percent DNA in comet tail and tail moment at the 3-h incubation time. Indices of DNA damage decreased after prolongation of incubation times, showing that this viable skin model – consisting of stratum corneum, epidermis, and dermis – has DNA repair.

We postulate that nanomaterials that agglomerate resulting in realistic nanoparticle sizes above 20 nm will not enter the viable fully differentiated human skin models and consequently would not exert genotoxic effects in this test system. Our results comply with the studies with pig (Monteiro-Riviere et al., 2011) and rodent skin (Adachi et al., 2010; Kimura et al., 2012).

It is proposed that investigations of skin penetration on 3D skin models will be continued investigating exposure scenarios towards certified stably monodispersed nanomaterials of defined realistic sizes between 1 nm and 20 nm when available.

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The antiproliferative, proapoptotic and antitumor activity of huIFN- α against CaCo-2 cells can be enhanced with 10% PBS holocene grain wash out

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During the holocene era withdrawal of pleistocene glacier occurred. In this time holocene sands near river Drava (Croatia) appeared. These sands contain the fairly uniform holocene minerals. When these minerals were grained they show the quite unusual biological/ microbiological activity. The grained holocene minerals were analysed and the following analytes were found: SiO₂ (88.71%), Al₂O₃ (5.37%), Fe₂O₃ (1.08%) and among elements the Aurum (Au) with 12.1%. It is also important the high percentage (36.1) of Rubidium (Rb) and 75.4% of Strontium (Sr). Other elements were present in the level of 1-5%. Previously it was found that the 10% Holocene grain PBS washout and Human Interferon - α N3 affect the growth of CaCo-2 cells (Kesteli, 2007; Filipič, 2009, 2013). The presented experiments are aimed to measure the effect of 10% PBS (Phosphate Buffer saline) washout of holocene grain on the HuIFN- α (Natural and recombinant) antiproliferative, proapoptotic and antitumor activity on the CaCo-2 cells *in vitro*. The following interferons were used: (1) Natural: HuIFN- α N3 (IMZ - Zagreb, Croatia), Alfaferon (Alfa Wasserman, Alanno, Italy), Egiferon (Trigon, Budapest, Hungary). (2) Recombinant: Human recombinant α A Interferon (EMD Chemicals Inc., San Diego, CA 92121 USA), Roferon-A (IFN Alfa-2a), Roche and Intron A (IFN Alfa-2b) (MSD Italia Srl, Rome, Italy). The interferons were analysed by RP-HPLC at 280 nm. CaCo-2 cells were treated as follows: (a) Cell control, (b) Interferon + 10% PBS (1:1.1:2.2:1), (c) Interferon, (d) 10% PBS. The comparison with the non-treated cells was calculated. The 50% cell growth inhibition test was used. The apoptotic cells were isolated using the »BioVision: Apoptotic cell isolation kit«. In the cells the level of Glutathione and in the cell supernatant the level of Alkaline phosphatase. 10% Holocene grain washout shows the AP (Antiproliferative)

activity. This can be enhanced up to three times by HuIFN- α N3, but not with the HuIFN- α 2a or HuIFN- α 2b. In case of proapoptotic activity the 10% Holocene grain washout shows 26.52% of apoptotic cells, while this % was increased to 49.85 with HuIFN- α N3. With HuIFN- α 2a this was 22.80, and with HuIFN- α 2b was 42.60%. Both, the Glutathione and Alkaline phosphatase levels were decreased similarly (up to 22.4%) but more, when natural IFNs were used. In general, it can be concluded, that 10% PBS holocene grain wash-out somehow synergizes with the HuIFN- α N3 in highest level of AP activity, while this cannot be found with HuIFN- α 2a or HuIFN- α 2b. It is also important to note the parallelism of these effects with the percentage of apoptotic cells and in the level of Glutathione and Alkaline phosphatase.

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HuIFN- α N3 inducing capacity of the Newcastle disease virus (NDV) ZG1999HDS

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Newcastle disease virus (NDV) ZG1999HDS was isolated from the flock of 16150 broiler chicken that suffer the respiratory and causing death of 77,29% of chickens up to 28 days. It was isolated from lung tissue and not from other organs. Virus proved to be lentogenic causing death because of the tropism of the virus for respiratory system (Biđin, 2010). It was sequenced and genetically characterised. (Nedeljkovic, 2011). The phylogenetic analysis based on the F gene and also full genome sequence analyses show that strain NDV ZG1999HDS belongs to genotype II of class II NDV and is related to NDV strains LaSota (Hitchner B1) The maximum identity of nucleotide sequence was found between NDV ZG1999HDS isolate and two velogenic strains isolated in Egypt and China. Its cytolitic characteristics were investigated *in vitro* on tumor cell cultures and *in vivo* on mice and compared with the impact of the strain LaSota (Mazija et al., 2011). The ZG1999HSD strain statistically more inhibit the growth of tumour cells and tumours compared with the LaSota NDV strain. It is lentogenic and immunogenic for chickens. The experiments were aimed to determine the HuIFN- α N3 inducing capacity of the NDV ZG1999HDS *in vitro* in human leukocytes. The human buffy coats were used. From them the leukocytes-lymphocytes were isolated. To measure the interferon induction, the leukocytes-lymphocytes were cultivated in the Eagle's medium with 5% Human Serum Albumine in the concentration of 2×10^7 cells/ml The interferon induction was performed with the ZG1999HDS in the concentration: 3,2, 6,4, 9,6 and 100 HA/ml and with Sendai virus (Cantell strain) in the concentration: 6,4, 12,8, 19,2 and 100 HA/ml. The induction with 100 HA/ml was enhanced with the 100 HuIFN- α N3 priming or with the addition of the 10% PBS washout of the holocene minerals. The interferon samples were analysed by Platinum ELISA (eBioscience, Vienna, Austria) and with the RP-HPLC. The following results were obtained: (1) NDV ZG1999 HDS: 3,2 HA/ml = 629,62 pg/ml, 6,4 HA/ml = 287,03 pg/ml, 9,6 HA/ml = 296,79 and 100 HA/ml =

383,33 pg/ml. (2) Sendai (Cantell strain): 6,4 HA/ml = 300 pg/ml, 12,8 HA/ml = 287 pg/ml, 19,2 HA/ml = 390,74 pg/ml and 100 HA/ml = 884,25 pg/ml. When the HuIFN- α N3 100 I.U./ml priming was performed with 100 HA/ml of NDV ZG1999HDS, 9064, 81 pg/ml was obtained, and when the same priming was performed with 100 HA/ml of Sendai virus (Cantell Strain), 8287 pg/ml of IFN was obtained. The addition of the 10% PBS washout of the Holocene minerals, in case of NDV strain ZG1999HDS, 7601 pg/ml of IFN, and in case of Sendai virus (Cantell strain), 8246 pg/ml of IFN was obtained. The RP-HPLC profile show the difference in the NDV ZG1999HDS and Sendai virus (Cantell strain) induced interferons. The NDV ZG1999 HDS induced IFN lacks the IFN-subtype 14 and has smaller amount (concentration) of IFN-subtype 1. It can be concluded, that the NDV ZG1999HDS has the interferon inducing capacity similar to the Sendai virus (Cantell strain), despite the interferons differs in the subtype 14 and concentration of the subtype 1.

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Development and application of an algorithm to determine statistically-valid non-cytotoxic concentrations from imperfect *in vitro* cytotoxicity data sets

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Many *in vitro* test systems for chemicals or bioactive agents measure sophisticated functional or biochemical endpoints. However, such data can be hard to interpret, or be even meaningless, when cells are dying under the chosen assay conditions. Therefore, the determination of the non-cytotoxic concentration range for each test compound is crucial. A simple and widely used approximation is to define concentrations below those that trigger a 10% reduction in cell viability as non-cytotoxic (EC10 of the full cytotoxicity curve). However, a statistically valid approach to define non-cytotoxicity is based on three conditions: first, a benchmark response (BMR) level (e.g. 10% cell death) needs to be defined; second, the benchmark concentration (BMC), i.e. the concentration of the test compound at which the BMR is reached, needs to be defined. This requires selection of a mathematical model, and its adaptation to the data points of the cytotoxicity curve. Third, the lower limit of the confidence interval of the BMC (BMCL) needs to be calculated, because only concentrations smaller than the BMCL can be considered to be non-cytotoxic with a defined minimum confidence level. An automated procedure to calculate the BMCL corresponding to a BMR of 15% cell death (BMCL15) has been developed here. For many data sets of average quality this BMCL15 corresponded to the EC10, but it provided the additional advantage of taking the overall data quality into account and thereby increases the reliability of

the cytotoxicity border. The concept of BMC and BMCL is an adaptation of the benchmark dose (BMD) concept developed for *in vivo* toxicology studies. Several software programs are available for the purpose of BMD calculations, but the adaptation to *in vitro* tests is difficult for bench scientists, and the concept has still not found its way into most of the toxicology laboratories. A further reason that the BMC concept is not widely implemented yet is that this statistical approach has to be adapted to real-life problems that are typical for complex cellular assays. For instance, the data may not cover the full range of toxicities, data variation may be large, assays with different test concentrations may need to be pooled and normalizations may result in imperfect baseline data. We show here that the software solution developed by us automatically copes with such types of concentration-response data from various stem cell-based assays from the ESNATS consortium. To define cytotoxicity in these test systems it was crucial to develop a reliable and reproducible method that allows inter-laboratory comparisons. An interdisciplinary programming approach was chosen by combining biological assumptions with a statistical BMC estimation concept. Using this approach it was possible to determine the highest non-cytotoxic concentration for all compounds tested in the different stem cell based test systems.



NPC-derived neurospheres serve as test systems for early neurodevelopmental toxicity: an interspecies comparison of toxicity pathways

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Neurospheres are three dimensional (3D) cell culture models consisting of neural progenitor cells (NPCs), which proliferate in culture and migrate and differentiate into neurons and glia 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 adverse effects in human vs. rodent neurospheres reveals some species-specific differences.

Besides compound screening, we investigate mechanisms of action of selected chemicals for the identification of relevant DNT “Toxicity Pathways” contributing to the “Adverse Outcome Pathway” concept. By employing different compounds in the neurosphere assay, we so far identified intracellular signalling pathways like thyroid hormone, Nrf2 and epigenetic modification by histone deacetylases as well as pathways guided by cell surface receptors like integrins and the FGF receptor as modulators of human NPC development. Some of

those are specific to humans, while others do not exert species-specificities. In addition, ligand binding to the Arylhydrocarbon receptor (AhR) does not affect function of human NPCs, while it interferes with mouse NPC development. Resistance of human NPCs towards AhR modulation is due to a missing AhR expression in human fetal brains at this developmental stage.

To make the “Neurosphere Assay” applicable for compound testing, we established automated sorting and plating of spheres in a 96-well format as well as high content image analyses for a medium throughput evaluation of endpoints.

In summary, 3D neurosphere cultures are useful for identifying toxicity pathways relevant for human DNT and they are applicable for medium-throughput chemical testing. 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.

Neurospheres as a predictive 3D *in vitro* model for DNT testing and pathway investigation in a species-specific context

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Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. So far, DNT testing is performed by animal experiments in rats, but such *in vivo* testing is time consuming, expensive and uses large numbers of animals. Moreover, species differences between rat and human entail a problem for extrapolation. Thus, alternative tests are needed to provide faster and cheaper methods for DNT testing. Therefore we established the human and rat neurosphere model as a primary neural three-dimensional *in vitro* cell system for developmental neurotoxicity testing. In the “neurosphere assay” we are able to detect the potential of chemicals to disturb basic processes of brain development (proliferation, migration, differentiation) and distinguish them from general cytotoxic effects.

In order to determine the predictivity of the neurosphere assay for DNT testing, we tested a training set of nine chemicals in the rat and human neurosphere assay to find out if the assay distinguishes between DNT positive and negative compounds. Moreover, we examined the functional outcome of automated sorting and plating with a COPASTM Large Particle Flow Cytometer in the neurosphere assay to meet the needs of medium throughput screening.

The study revealed that (i) the neurosphere assay was able to discriminate between positive and negative compounds with a predictive value of 88% and that (ii) rat and human neurospheres often differed in their sensitivity to the testing compounds being in general, the rat neurospheres more sensitive than the human ones. For example, rat neurospheres reacted 5 to 10 times more sensitive than human neurospheres to the antiepileptic drug sodium valproate (VPA). Investigations of the VPA analogs sodium butyrate and valpromide on two prominent cellular effects of valproate revealed that this species difference is not mediated by voltage gated sodium channels and that HDAC inhibition does not contribute to effects of VPA on all endpoints. (iii) Automated sorting and plating allows performing the “Neurosphere Assay” in a medium throughput.

In conclusion, (i) 3D neurospheres can be used for DNT testing, (ii) the comparison of human and rat data opens up the opportunity to investigate toxicodynamic differences between the relevant test species rat and humans in an experimental approach for usage in human risk assessment and (iii) industrial application is possible due to automation of the method.



Human neural progenitor cell (hNPC) aging is mimicked by 3D cultures *in vitro*: an “Adverse Outcome Pathway” gains importance at susceptible life stages

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The “Adverse Outcome Pathway” (AOP) concept is currently promoted by the OECD because it puts existing information, e.g. the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization, into a framework that is applicable for risk assessment. Here, I present an AOP for neurotoxicity, which links an excess of reactive oxygen species (ROS) to impaired neurogenesis. This exercise gathers existing information on the molecular and key events involved in ROS-induced disturbance of this process, which is fundamental during development as well as for neuronal regeneration in the adult.

In the elderly, cognition declines during the process of aging, also referred to as normal brain aging, which is the

adverse outcome. One reason for this loss of organ function is decline in regenerative capacities of NPCs. We have mimicked this decline of NPC function during the aging process with human neurospheres *in vitro* and show that the key player in adaptation against ROS, the transcription factor Nrf2, is playing a causal role in this age-related susceptibility towards oxidative stress.

In summary, we describe an AOP for neurotoxicity and show that we can mimic the cellular responses of this AOP with a 3D *in vitro* model. Moreover, we identify a susceptibility factor for a vulnerable life stage, which is Nrf2 in NPCs of the aging brain.



Use of animals and alternatives in Military Medical Training by NATO Nations

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Among the 28 member nations of the North Atlantic Treaty Organization (NATO), a variety of training methodologies – including simulators, moulage scenarios, didactics and live animal laboratories – are used to prepare military personnel to treat injured civilians and soldiers. For ethical, educational, practical, legal and economic reasons, the propriety of animal use for this purpose has come into question. This lecture

discusses the results of a survey which found that more than three-quarters of NATO nations do not use animals for military medical training. We will also address comparative research on the efficacy of animals and non-animal training methods, associated legal issues, and recent developments in efforts to curb animal use in the European Union and North America.



Genotoxicity of nanosized Titanium dioxide particles in 16HBE14o-cells

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Bronchial and alveolar cells are both targets for granular dusts and fibers up to an aerodynamic size of 10 μm . In the first phase of NANOGENOTOX partners performed an exploratory investigation of the sensitivity of different lung cell lines against polydispersed manufactured nanomaterials (MNs). BfR was in charge of the assessment of the potential genotoxicity for different types of titanium dioxide (TiO_2) in transformed with SV40 large T-antigen replication origin deficient human bronchial epithelial cells (16HBE14o- cells). HBE cells widely and rapidly accumulate TiO_2 as aggregates in cytosolic vesicles. Interestingly, intracellular nanomaterials accumulation is assumed to be dissociated from cytotoxicity (Belade et al., 2012).

In this study we performed cytotoxicity analysis using impedance analysis and comet assay following exposure to different forms of TiO_2 nanomaterials (NM102-NM105) at different doses for 3 h and 24 h. Two experiments were performed for each TiO_2 MN. Cytotoxicity was only evident at the highest dose. However, indications for DNA damage were not observed at any dose. A dose-response relationship for MMS as positive chemical control was established for the parameters % DNA in tail and Olive tail moment. The level of DNA damage obtained with the positive control chemical MMS (22 $\mu\text{g}/\text{ml}$) was always clearly increased at the 3-h and 24-h

exposure. Nanomaterials exposure was performed according to the NANOGENOTOX dispersion protocol using ultrasonication of test-item stock solutions. Particle size distribution in dilutions of exposure medium was followed by dynamic light scattering. Particle size distribution changes considerably with dose. Only the lowest dose (2 $\mu\text{g}/\text{ml}$) shows nanoparticles according to the ISO definition.

HBE cells appear not to be the most sensitive cell line to investigate the potential genotoxicity of nanomaterials. Future work in NANOGENOTOX therefore selected the more sensitive cell line BEAS 2B instead.

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Immortalized primary-like human cells as novel model systems in nephrotoxicity

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Nephrotoxicity of chemicals or therapeutics can be caused by damage to all parts of the kidney including 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 screening of kidney protective substances that e.g. can be co-administered during chemotherapeutic treatment of cancer patients to diminish kidney injury.

So far, the short replicative life span and the limited access to human RPTECs has limited their use in the field of *in vitro* toxicology. We recently established the telomerase immortalized renal proximal tubular epithelial cells from tissue and alternatively also from urine as a non-invasive source of cells. The cells were used as a model system to study cisplatin nephrotoxicity. In addition, we tested different exotoxins on the cells as model system.

Taken together, we present a novel non-invasive source of human, primary like cells that represent relevant and standardizable model system for nephrotoxicity studies.



Steps taken to implement 3R's concept in research using animals in Sri Lanka

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The objections of Animal activists and welfare committees for subjecting animals to painful procedures without ethical clearance in 2006 led to the development of "Guidelines for Ethics Review of Research Proposals involving Animals in Sri Lanka" which is a detailed document covering all the areas related to animal research including necessity of applying 3R's concept. Further, when reviewing research proposals by the animal ethics committees, adherence of researchers to this concept was checked and advised on improvement.

Through the establishment of Sri Lanka Association for Laboratory Animal Science in 2012 we are taking steps to advance scientific understanding and knowledge of the use,

care and welfare of laboratory animals and promote 3R concept (refinement, reduction and replacement) which is the first general objective of the association and to be the monitoring body for the animal experiments conducted in the country to enhance welfare of animals use in research.

We are keen on introducing methods of replacement in research and include the topic "Alternatives for Laboratory Animal Research" with necessary examples in the workshops we conduct for the benefit of researchers who are new to the field. One best example is use of Rapid Fluorescent Focus Inhibition Test (RFFIT) instead of mouse inoculation test to check the development of antibodies following anti-rabies immunization.

***In silico* models for dermal absorption from complex formulations**

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Dermal exposure is a relevant parameter for risk assessment of chemicals, cosmetics and pesticides. Here, we present potential *in silico* models for prediction of dermal absorption based on realistic exposure scenarios in complex mixtures.

The calculations were based on 342 individual dermal absorption *in vitro* experiments using human or rat skin samples for 56 chemicals (mainly pesticides) in more than 150 different mixtures containing up to 20 ingredients like water, organic solvents, surfactants or thickeners. The first approach was based on the Abraham solute descriptors, mixture factors (MFs) as suggested by Riviere and Brooks and the logarithmic maximal permeability coefficient (logmaxKp) as response (Abraham et al., 1999; Riviere and Brooks, 2005). Additionally, an indicator variable for the species (SpI) was introduced. In a second approach class variables – which bundled substance-specific information – were used in combination with mixture factors. Validation was performed in accordance with the OECD Guidance document for QSAR models.

The final validated Abraham-based model comprised the solute excess molar refractivity of the penetrant, SpI and topological polar surface area of the mixture ($R^2:0.38$, $Q^2\text{Ext}: 0.41$). Despite the low correlation, the model was suitable to estimate Marzulli classes of penetration for unknown penetrants in specific mixtures (Marzulli and Brown, 1969). Furthermore, precise prediction of mixture effects on well-known substances was possible with the substance-based approach ($R^2:0.75$, $Q^2\text{Ext}: 0.73$). Taken together, both applications are suitable screening tools in early stages of product development.

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Dynamic culture of human liver equivalents inside a micro-bioreactor for long-term substance testing

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This study is meant to contribute to the development of a liver equivalent, which comprehends the functionality of a hepatic organoid, as well as its incorporation into a dynamic miniature culturing system.

Cell culture systems imitating liver functionality are considered as a tool for evaluating the toxicology and pharmacology profiles of any substance. However, classic culture systems fail to retrain liver-like functionality. Assembling cells in a spherical aggregate is one of the many possibilities tissue engineering offers for lifelike cultivation, which was shown to be superior to standard two-dimensional cell culture in manifold publications. Here, hepatocytes were aggregated with hepatic stellate cells. Underlining the superiority of this method, within aggregates cells generated several components of the extracellular matrix, exhibited signs of zonation, and were capable of extensively producing members of the cytochrome P450 family. Liver-specific parameters were hence effectively recreated *in vitro*.

Beyond that, attempts were made to cultivate the aggregates dynamically in our preliminary human-on-a-chip model bearing near physiologic fluid flow and volume ratios on a miniature

scale. Due to the very low proportion of medium volume to cell volume, the cells are enabled to shape their environment through the secretion of autocrine and paracrine signals. The control over the microfluidic circulation allows us the creation of a specific physical microenvironment characterized by a pulsatile laminar flow.

The liver-mimicking aggregates were inserted into our chip-bioreactor and cultured for up to 14 days. The production of albumin, urea, and lactate, as well as the consumption of glucose and the release of LDH into the culture medium was analysed daily and related to static controls. End-point analyses comprised live-dead stainings as well as immunohistochemistry of marker proteins and RT-PCR of selected marker genes.

It could be shown, that the micro-bioreactor system is capable of supporting long-term co-cultures of human liver equivalents. Cell polarity was restored as shown by the expression of specific transporters, tight junctions and the formation of rudimentary bile canalicular like structures. Vitality was markedly increased compared to static controls. Our findings indicate the utilisation of our hepatic aggregates as liver equivalents in co- or multi-organoid culture systems for long-term substance testing.



Tissue engineered *in vitro* human airway models (EpiAirway) of asthma and COPD

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Asthma and chronic obstructive pulmonary disease (COPD) are the two leading chronic respiratory diseases in the US. However, reliable *in vitro* human models are not widely available to researchers attempting to understand asthma and COPD pathogenesis and develop therapeutic interventions for these diseases. Here we report on a program to create and maintain a human cell bank derived from airway epithelium of diseased individuals, and production of tissue engineered *in vitro* human models of asthma and COPD from the cells.

Tracheal and bronchial tissues are obtained from non-transplantable organs donated from normal, asthmatic or COPD individuals with IRB approval and informed consent. Epithelial and mesenchymal cells are isolated from the tissues, cryopreserved and maintained in a cell bank. As needed, cell are recovered and utilized to produce *in vitro* tissue engineered models.

Sixteen lung samples have been processed to-date. The airway histopathology of each donor was evaluated. Airway epithelial cells, fibroblasts and smooth muscle cells isolated from the lung tissues (7 normal, 6 asthma and 3 emphysema donors). Cells were characterized morphologically and by immunocytochemistry. Tissue engineered models were cultured

at the air-liquid interface and consist of well-differentiated human tracheal/bronchial epithelium (EpiAirway™) as well as human airway epithelium co-cultured with donor-matched human airway mesenchymal cells (EpiAirway-FT™). H&E stained paraffin sections show development of pseudostratified epithelium with mucociliary phenotype similar to *in vivo* proximal airway epithelium. Transmission electron microscopy shows development of tight junctions and transepithelial electrical resistance measurements demonstrate barrier function. The cultures exhibited *in-vivo* like responses including stimulation of mucus production by TH2 cytokines and secretion of various cytokines and chemokines after stimulation with innate immune challenges and TH1/TH2 cytokines.

These *in vitro* human models of asthma and COPD provide important unique attributes that animal models cannot provide, including the ability to address human individual variability and genetic factors and a means to determine mechanisms of human virus elicitation of asthma and COPD exacerbations. These models will provide researchers important new tools for investigating the role of airway epithelium and mesenchymal cells in asthma and COPD pathogenesis and development and testing of new therapeutic treatments for these diseases.



Overview of the ESNATS project

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ESNATS aims at developing a novel toxicity test platform based on embryonic stem cells (ESC), especially human ESC, to accelerate drug development, reduce R&D costs, propose a powerful alternative to animal tests and increase human safety. To this aim, ESNATS has developed a unique battery of toxicity tests using human and mouse ESC lines subject to standardized culture and differentiation protocols. The test battery focuses on prenatal development with emphasis on the nervous system and covers reproductive toxicity, neurotoxicity, metabolism and toxicokinetics.

The ESNATS testing strategy is based on standardized ESC-based test systems validated in a feasibility study. This strategy includes two parts- “The Biomarker study” and “The Test Battery”; the functional readouts of which will be compared with the gene expression patterns identified. This will help the assessment of the predictive capacity of the ESNATS developments.

The biomarker study aims to identify gene expression signatures by gene array analysis and to establish an algorithm that allows the identification of compounds that act by a certain toxic mechanism or induce a specific phenotype. As a proof of principle, the already published transcriptomics results indicate that the test compounds valproic acid and methylmercury in-

duce a “common response” which can be distinguished from “compound-specific” responses. Thus the ESNATS assay battery allows classification of human developmental neurotoxins or reproductive toxicants on the basis of their transcriptome profiles (Krug et al., 2013).

The test battery aims to assess different aspects of prenatal toxicity such as functional impairments and changes in the differentiation capacity after exposure to selected reference compounds of pharmaceutical interest and unknown prenatal toxicity.

Conclusion: The overall development and implementation of the test strategy can be described through the biomarker study and the test battery approach. Both the studies are novel and demonstrate the proof of principle that human ESC can be used for toxicity testing applicable for reproductive toxicity, neurotoxicity and toxicokinetics and identify relevant biomarkers.

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Molecular recognition force microscopy/spectroscopy

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In molecular recognition force microscopy (MRFM), ligands are covalently attached to atomic force microscopy tips for the molecular recognition of their cognitive receptors on probe surfaces. A ligand-containing tip is approached towards the receptors on the probe surface, which possibly leads to formation of a receptor-ligand bond. The tip is subsequently retracted until the bond breaks at a certain force (unbinding force). In force spectroscopy (FS), the dynamics of the experiment is varied, which reveals a logarithmic dependence of the unbinding force from the loading rate. These studies give insight into the molecular dynamics of the receptor-ligand recognition process and yield information about the binding

pocket, binding energy barriers, and kinetic reaction rates. Applications on isolated proteins, native membranes, viruses, and cells will be presented. We have also developed a method for the localization of specific binding sites and epitopes with nm positional accuracy. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule is oscillated at a few nm amplitude while scanning along the surface. In this way, topography and recognition images are obtained simultaneously. Finally, we will show how high-speed bio-AFM is able to film dynamical biological processes on the nano-scale.



Assessing the effects of repeated cigarette smoke exposure using human organotypic systems reproducing the respiratory tract *in vitro*

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The aim of this study is to develop human bronchial and nasal epithelium culture models that are relevant to investigate the impact of cigarette smoke (CS) observed *in vivo* in respiratory tract tissues in contact with inhaled CS.

We used two organotypic cultures generated from primary cells derived from non-smoking donors that contain fibroblasts and epithelial cells in order to reproduce as closely as possible the *in vivo* situation. To mimic the smoking behavior of a moderate smoker during one day, human tissue cultures (bronchial and nasal epithelium) were exposed repeatedly and directly at the air/liquid interface (using the Vitrocell® System) to two dilutions (10% and 16% vol/vol dilution with air) of the whole smoke generated by one cigarette or to humidified air (sham). CS exposure was repeated four times with one hour intervals between each cigarette. Various endpoints (e.g., gene and microRNA expression, CYP activity, pro-inflammatory markers release, differential cell counts, cytotoxicity measurement) were then captured to assess the baseline (time 0) and early responses of the tissues after exposure (4 hours) as well as the recovery phase (24 and 48 hours). Computational methods such as Gene Set Enrichment Analysis were applied to identify the biological perturbations induced by CS exposure in both tissue cultures. A comparison with *in vivo* datasets from nasal and bronchial epithelial cells obtained from smokers and non-smokers was also undertaken to investigate how close the effect obtained *in vitro* can reflect the *in vivo* situation.

At the highest CS dose (16%), the global gene expression changes in both tissue cultures are more intense early after exposure and decrease during the recovery phase. Gene Set Enrichment Analysis performed on datasets from both nasal and bronchial tissue cultures captured early after exposure (0 and 4 hours), indicates an induction of genes related to xenobiotic metabolism and inflammation as well as a decreased expression of genes involved in pathways related to fatty acids breakdown and protein synthesis/degradation. *In vivo/in vitro* comparison of CS effect on gene expression changes related to xenobiotic metabolism shows a good correspondence between nasal *in vivo* dataset and CS-exposed nasal tissue culture 24 hours after CS exposure. A similar result is observed for CS-exposed bronchial tissue cultures (4 hours) compared to four different datasets derived from bronchial epithelial cells obtained by brushings from smokers and non-smokers. Essential genes involved in the xenobiotic metabolism (e.g. CYP1A1 and CYP1B1) were equally found to be transcriptionally activated in both CS-exposed nasal and bronchial tissue cultures (0 and 4 hours).

We describe for the first time the impact of whole CS exposure on a human nasal organotypic *in vitro* model. By using computational approaches and by capturing systems biology endpoints, various biological perturbations triggered by repeated exposure to CS were observed in both nasal and bronchial *in vitro* models.

Improving cadaveric models for surgery teaching and training

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In Veterinary Medicine, surgical skills should be ideally developed in an environment that could be transferred to live surgery in the operating rooms, giving students the ability to face their first experiences with a live animal. Several models are available for surgical training, including cadaveric specimens. However, three main issues are often appointed as obstacles: few corpses available for education, lack of bleeding and improper tissue preservation and consistency. Bleeding during surgery is always a cause for concern among novices, and learning how to prevent and correct it is essential for every surgical procedure. Therefore, students must be very familiar with hemostasis maneuvers before taking part their first surgical experiences, refining their techniques without harming animals. Besides bleeding, adequate tissue consistency makes any surgical training more efficient, which can be obtained with proper cadaver preservation, giving even more reality to the procedure in a specimen closer to the real patient. This poster aims to describe a preserved cadaveric model that simulates bleeding and evaluate the acceptance by veterinary students. For that, ethically sourced cadavers were obtained, and preservation process used modified Larssen solution infusion (Silva et. al., 2004), and cryopreservation at -8°C . When used in the surgical technique training, they were unfrozen and connected to a pump system that infuses a blood substitute, simulating the circulatory

system. When tissues were incised and different surgical techniques performed, bleeding simulation occurred, making possible to train all steps of a surgical procedure, from skin incision to skin suture. Each cadaver can be used at least 3 times, reducing the number of specimens needed for classes or training. The system was tested by veterinary students ($n=20$), and their feedback was assessed by a questionnaire. Proper bleeding simulation, more proximity to reality, suitable anatomy and topography visualization and tranquility to practice were main subjects pointed out by evaluators. 85% of students affirmed that the method of training in cadavers that simulate bleeding made them more confident for live surgery. 100% of students classified the system as good/optimum, and they would choose this method for surgical classes before performing procedures on live animals that would benefit them.

Acknowledgment

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Use of *in vitro* cell assays and noninvasive imaging techniques to reduce animal experiments in drug development

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Membrane transporters can be major variables for disposition, efficacy and safety of many drugs. The function of drug transporters can be explained using probe drugs like talinolol or digoxin; however, to analyse the distribution of such compounds tissue samples from experimental animals have to be taken for each time point, resulting in a high number of animals. Therefore noninvasive imaging tools like magnetic resonance imaging (MRI) have been developed to quantify multidrug transporter function indirectly by visualization of substrate transport. Evidenced by the pharmacokinetic results obtained in multidrug resistance-associated protein 2 (Abcc2)-deficient rats, the liver specific contrast agent gadoxetate acid (Gd-EOB-DTPA, Primovist[®]) seems to be mediated by Abcc2, which is also expressed in small intestine. To get comprehensive data on interactions of gadoxetate acid with transporters expressed along the entero-hepatic absorption route, we measured its affinity to human organic anion transporting polypeptides (OATP) 1A2, 1B1, 1B3, -2B1, to the Na⁺/taurocholate cotransporting polypeptide (NTCP), to the apical sodium dependent bile acid transporter (ASBT), to the organic cation transporter (OCT) 3 and to the efflux transporters ABCC2 and ABCC3 in stably transfected cells. Moreover, we evaluated its pharmacokinetics after oral and intravenous administration in rats using T1-weighted MRI and a validated LC-MS/MS method, respectively.

Gadoxetate acid was demonstrated to be an *in vitro* substrate for OATP1B1 (Km: 1.2 mmol/l; Vmax: 6.3 pmol/mg×min), OATP1B3 (Km: 0.5 mmol/l; Vmax: 7.4 pmol/mg×min), NTCP (Km: 0.04 mmol/l; Vmax: 1.4 pmol/mg×min), OATP1A2

(Km: 1.0 mmol/l; Vmax: 101.3 pmol/mg×min), ABCC2 (Km: 1.0 mmol/l; Vmax: 86.8 pmol/mg×min) and ABCC3 (Km: 1.8 mmol/l; Vmax: 116 pmol/mg×min), but not of OATP2B1, ASBT and OCT3. After oral administration to wild type animals, gadoxetate acid was considerably absorbed from the small intestine (bioavailability ~17%) whereas in Abcc2-deficient rats the oral bioavailability increased to ~25%. In Abcc2-deficient rats after intravenous application the liver enhancement was significantly prolonged compared to wild type rats (AUC₀₋₉₀, 36.4 vs. 14.8 AU×min, p=0.003; T_{max}, 48.6 vs. 6.0 min, p=0.001) and gadoxetate acid was exclusively excreted into urine.

Our data show that cell-based *in vitro* assays have the potential to replace *in vivo* animal testing and provide reliable data. Moreover, the non-metabolized gadoxetate acid may have some potential to be used as a probe-contrast agent to evaluate transporter-mediated mechanisms along the entero-hepatic absorption route for drugs by functional visualization *in vivo*.

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InterNICHE outreach and replacement of animal experiments in education in Uzbekistan and Kyrgyzstan

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InterNICHE performed a successful 2-month series of seminars, multimedia exhibitions and further meetings in the Central Asian states of Uzbekistan and Kyrgyzstan in 2012. This outreach provided the first major exposure to replacement alternatives in countries that are isolated from the international community and that have a reputation for conducting severe animal experiments. Up to 170 teachers and students attended each of the 7 outreach events that were held over 1-2 days at medical, veterinary, agricultural, biological science and pedagogical faculties. Meetings were held with Deans, Rectors and Heads of Department to discuss further collaboration. Resources including models and software alternatives were distributed to teachers. Despite existing small-scale use of alternatives, most teachers were not aware of the range and quality of humane tools and approaches. There was considerable openness to enhancing education and training with alternatives, and val-

uable opportunities for future progress were identified. The experience provided many lessons in how to work effectively in the countries, and potential follow-up outreach can use these to consolidate the initial successes and to achieve more replacement. Following the strategy of InterNICHE and its partner organisations signing formal agreements with universities in Russia and Ukraine in order to end animal experiments, new agreements have been signed with departments and faculties in Uzbekistan and Kyrgyzstan during 2013. Further donations of alternatives are being made as part of the agreements. The positive results of the outreach and its follow-up reflect teachers' understanding of the pedagogical, ethical and economic advantages of alternatives, and a growing acceptance of replacement of animal experiments in education in former Soviet countries. They also demonstrate the potential for collaborative action in countries often described as challenging.



A 3R test of biocompatibility of a new intensive patch by using the reconstituted three-dimensional human skin model EPISKIN-SM™ (SKINETHIC) to replace animal use

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Objective

In the present study the skin irritant potential of the test item Overnight Intensive Patch (OIP) from Merz Pharmaceuticals GmbH Germany, was analysed. OIP is a non-invasive medical device for repeat overnight treatment of newly formed scars upon completion of wound healing. The Patch contains cepalin and allantoin, which in combination with occlusion further aid in improving cosmetic results during treatment.

The EPISKIN-Standard Model™ (EPISKIN-SMTM), a reconstituted three-dimensional human epidermis model, was used as a replacement for the Draize Skin Irritation Test (OECD TG 404) to distinguish between UNGHS and EU CLP "Category 2" skin irritating test substances and not categorized test substances ("No Category") which may be considered as non-irritant. This skin model consists of normal (non-cancerous), adult human-derived epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis. The NHEK are cultured on chemically modified, collagen-coated cell culture inserts. A highly differentiated and stratified epidermis model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum. For the purposes of 3R, this test replaces animal experiments with rabbits.

Extraction of the Test Item

The test item OIP was extracted in compliance with ISO 10993-10, -12. The protective foils were removed before extraction. The test item OIP was extracted in a surface/volume ratio of 6 cm²/

mL under agitation for 72 ± 2 h in Phosphate Buffered Saline (PBS) at 37 ± 1°C. The extraction procedure did not reveal any abnormalities in the extraction medium or the test item. The test extract was stored for max. 24 h at 4°C before treatment.

Pre-Experiments: To check the non-specific MTT-reducing capability of the test item 10 µL of the test item extracts was mixed per 2 mL MTT medium and incubated for 3 h at 37 ± 1°C in the dark. If the mixture turns blue/purple, the test item extract is presumed to have reduced MTT. To check the colouring potential of the test item 10 µL of the test item extracts were mixed per 90 µL aqua dest. in a transparent recipient for 15 min.

Experimental Procedure: Upon receipt of the EPISKIN-SMTM, the tissues were transferred into 12-well plates containing 2 mL prewarmed maintenance medium per well. The 12-well plates were incubated in a humidified incubator at 37 ± 1°C, 5.0% CO₂ for at least 24 h. After this pre-incubation the tissues were treated with each dose group in triplicate, starting with the negative control. Start time was recorded with dosing of the first tissue. Then the tissues were incubated at room temperature for 15 ± 0.5 min. Afterwards, the tissues were washed with PBS to remove any residual test item extract. Excess PBS was removed by blotting bottom with blotting paper. The inserts were placed in a prepared 12-well plate containing 2 mL prewarmed fresh maintenance medium and post-incubated at 37 ± 1°C, 5.0% CO₂ for 42 ± 1 h. After this incubation period the plates were placed for 15 ± 2 min on a plate shaker.



***In vitro* phototoxicity screening assay for systemically administered pharmaceuticals using a reconstructed skin model EpiDerm**

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According to current regulatory guidelines, photosafety testing is required for new compounds if they absorb light in the range of 290-700 nm or partition into the skin or eyes. The only approved non-animal *in vitro* phototoxicity assay, the 3T3 Neutral Red Uptake (3T3 NRU-PT), is sometimes over-sensitive in predicting the *in vivo* photosafety hazard to humans and thereby eliminates valuable, new active pharmaceutical ingredients (API) from further development, even though they are safe to humans. EpiDerm™, a normal human 3-dimensional (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 utilized EpiDerm to develop an *in vitro* assay for screening the phototoxicity of pharmaceuticals following systemic administration (sPHO). Test articles (n=42) were added into the culture medium and allowed to partition into the epidermal tissue. Tissues were exposed to

solar radiation and phototoxic effects were determined by the comparing the tissue viability of UV irradiated vs. non-irradiated tissue models, using the MTT assay. A prediction model (PM) 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 $\geq 30\%$ when compared to identical concentrations in the absence of irradiation (-UVR); a material is non-phototoxic if the decrease in tissue viability is $< 30\%$. The PM resulted in high sensitivity (91.7%) and specificity (100.0%) for 42 test materials (24 phototoxic/18 non-phototoxic). Results of sPHO assay were compared to in-house 3T3-NRU-PT assay. The current protocol extends phototoxicity testing using EpiDerm for risk assessment to systemically administered chemicals and medications and will provide the pharmaceutical industry with an *in vitro* screening method to assess the phototoxic risk of new API.



Optimization of the EpiOcular eye irritation test for hazard identification and labelling of chemicals in response to the requirements of the EU Cosmetic Directive and REACH Legislation

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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 utilizes the normal human cell-based EpiOcular tissue model, has been developed. The EpiOcular-EIT utilizes two separate protocols, one specifically designed for liquid chemicals and a second, related protocol for solids. Over 100 substances were tested during the development of the assay. The EpiOcular-EIT can discriminate between ocular irritants (“I”, GHS cat 1 and 2) and non-irritants (“NI”, no category) with 100.0/70.0 and 83.6% sensitivity/specificity and accuracy (SS&A) for liquids and with 95.0/78.9 and 87.2% SS&A for solids. Currently the assay is involved in a formal, multi-laboratory validation study sponsored by the Cosmetics Europe (formerly COLIPA) under the auspices of the European Centre for the Validation of Alternative Methods (ECVAM) to assess the relevance and reliability of the assay with the goal of bringing it to formal validation. Analysis of a larger dataset using the solids protocol (Kolle et al., 2011) indicated that there might be a need for improvement of the assay's sensitivity. Therefore, the EIT

test method for solids was further optimized by MatTek. The optimized EIT protocol for solids, which now utilizes a longer exposure time, was optimized using the 39 test articles for which results had been previously published (Kaluzhny et al., 2011) and discriminated between ocular “I” and “NI” with 100.0/66.7 and 84.6% SS&A. When the optimized protocol was used on an expanded set of 67 solid chemicals 93.2/65.2 and 83.6% SS&A were obtained, which meet all the acceptance criteria for validation.

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Analysis of the validated EpiDerm Skin Corrosion Test (EpiDerm SCT) and a prediction model for sub-categorization according to the UN GHS and EU CLP

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Skin corrosion refers to the production of irreversible damage to skin manifested as visible necrosis through epidermis and into dermis. In 2004, OECD adopted two ECVAM-validated reconstructed human skin model assays for testing skin corrosion (OECD TG 431). However, OECD TG 431 does not satisfy international labelling guidelines for transport of dangerous goods since none of the methods were validated for sub-categorization. UN-GHS utilizes 3 corrosion sub-categories (1A-very dangerous, 1B-medium danger, 1C-minor danger). Labeling a chemical as 1A has important consequences for transport, 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 utilized for assessing the packaging sub-classes. *In vitro* method that discriminates between 1A and 1B/1C classes will therefore have substantial impact on reducing animal tests

for this purpose. The current study evaluates whether EpiDerm SCT can discriminate between UN-GHS classes 1A, 1B/1C and non-corrosives (NC) based on the MTT viability assay. Data obtained during ECVAM validation study (Liebsch et al., 2000) indicated sensitivity of 100% for class 1A. In the current study with >80 chemicals, sensitivity for class 1A was obtained in a range of 77-87% depending on cut-off chosen following 3 min exposure. None of 1A chemicals was under-predicted as NC. Specificity for NC chemicals was 80%. As demonstrated by results of this study, EpiDerm-SCT allows a partial sub-classification of corrosives into sub-category 1A, 1B/1C, and NC. Adoption of the new prediction model based on 3 min endpoint into validated EpiDerm SCT design would allow identification of severely corrosive substances and would lead to significant reduction in animal use for corrosion sub-group package labelling.



Ability of the reconstructed human tissue models to correctly predict phototoxicity and photopotency of topically applied substances and formulations: review of available data

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Phototoxicity is defined as an acute toxic response that is elicited after initial exposure of the skin to certain chemicals and subsequent exposure to light, or that is induced by skin irradiation after the systemic administration of a chemical substance. The assessment of phototoxicity is necessary for all substances that sufficiently absorb certain parts of UV and visible light and that are intended for human use (including pharmaceuticals, cosmetic ingredients, and food additives).

None of more than ten different animal tests used in the past for predicting acute phototoxicity in humans have been scientifically validated. However, it was proven in an international EU/ECVAM/COLIPA validation study that the phototoxicity of chemicals can correctly be predicted by the *in vitro* phototoxicity test (3T3-NRU-PT) which involves the use of the permanent mouse fibroblast cell line, Balb/c 3T3. This test gained regulatory acceptance in all EU Member States in June 2000 as Method No. 41 in Annex V to Directive 67/548/EEC, and was accepted as the new OECD Test Guideline (OECD TG 432) in 2004.

Due to its excellent sensitivity, the 3T3-NRU-PT is regarded as a basic method for identifying acute phototoxicity and is widely used by the pharmaceutical and cosmetic industries. If a chemical provides a negative result in the 3T3 NRU-PT, no further testing is required in most instances. However, if the

result is positive additional testing may be required to obtain combined information on the phototoxicity and bioavailability (i.e. photopotency) of the compound in the targeted tissues (e.g. skin or eye).

Ideally, a photopotency test should be performed *in vivo* in human volunteers, but this is often not acceptable for ethical reasons, especially if the chemical may be a photoallergen. Reconstituted human skin models could therefore offer an effective means of avoiding the need for confirmatory testing *in vivo*, especially since such models are characterised by having both viable, metabolising primary skin cells and skin barrier functions. Based on the promising outcome of an ECVAM pre-validation study on the EpiDerm™ model (Liebsch et al., 1999) and evidence provided by further follow-up studies with commercially available skin models (EpiDerm, EPISKIN, SkinEthic) the use of reconstructed epidermis models for topical phototoxicity testing has been implemented into the draft ICH Guidance Document on Photosafety Testing (S10).

The presentation will provide overview of available data obtained with commercially available reconstructed human tissue models and will discuss further steps necessary for regulatory acceptance of the 3D-human skin models phototoxicity assays at the OECD level (i.e. for testing of chemicals and cosmetics).



Transforming the Conduct of Toxicology in the US: the Tox21 Program

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The last two decades have produced dramatic technological advances in molecular biology and computer science. The Tox21 consortium of U.S. federal organizations (the National Institute of Environmental Health Sciences/National Toxicology Program, the Environmental Protection Agency's National Center for Computational Toxicology, the National Institutes of Health Chemical Genomics Center, the Food and Drug Administration) are evaluating how best to incorporate these advances into testing strategies in order to broaden scientific knowledge of exposure-related disease mechanisms and ultimately to develop *in vitro* predictive models for *in vivo* biological response. EPA's ToxCast program is a major element of the Tox21 program. The Tox21 chemical library of nearly 10,000 chemicals and its accurate characterization is a central pillar of the effort. The library is unprecedented in its scope, structural diversity, use scenarios, and chemical reactivity characteristics in relation to toxicology and, thus, is ideally suited to extensively probe target interactions, pathways and toxicity mechanisms. The program is in the process of profiling the library across a variety of *in vitro* assays in 14 point concentration-response format. Replicated experi-

mental designs facilitate the evaluation of intra-array reproducibility based on 88 compounds duplicated within each assay plate and the entire library is run in triplicate. The activity profiles generated so far have been analyzed in terms of structure-activity relationships and biological relevance to assess their potential to serve as response signatures for in-depth toxicological testing prioritization, toxicity mechanism interpretation, and extrapolation to *in vivo* toxicity endpoints. Finally, a targeted testing is working to evaluate prediction models and prioritization schemes developed from Tox21 data. The Tox21 Program has now assembled a diverse and fully QC'ed collection of ~10,000 chemicals, begun profiling them across a variety of *in vitro* assays in concentration-response format, devised a variety of analysis algorithms and display tools, and initiated a variety of targeted testing programs to assess the physiological relevance and predictive value of the *in vitro* signatures generated. While data generation in Tox21 is continuing, increasing emphasis is being put on additional types of chemicals, chemical mixtures, incorporating metabolism and cell-cell interactions, and data integration. This is an abstract of a proposed presentation.



3-dimensional organotypic *in vitro* model systems for liver, cardiac and neuro safety assessment

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Evaluation of toxicological effects with *in vitro* cell models is recognized as the way forward in future toxicity testing. To maintain *in vivo*-like functionality, *in vitro* model systems have to mimic the 3-dimensional tissue structure and cell composition as close as possible. Similar to organs cells require their 3-dimensional organization to maintain their function. Two-dimensional cell models do not mimic the complex environment necessary for tissue functionality, 3-dimensional cell cultures recapitulate *in vivo* complexity more closely. Three of the major organs related to severe toxicological effects have been recapitulated *in vitro* base on the same design principles:

Human liver microtissues were created from primary human hepatocytes and non-parenchymal cells (NPCs), comprised of Kupffer cells and endothelial cells which were well integrated into the microtissues. Inflammatory stimuli activated the Kupffer cells, which secreted cytokines and thereby triggered hepatotoxicity of immune-related toxic compounds such as Trovafloxacin. The human liver microtissues were stable for >4 weeks and are thus suitable for long-term toxicity studies, as shown for acetaminophen, diclofenac and troglitazone. In addition, gene-expression profiles showed marked differences

between 2D- and 3D-culture of primary human hepatocytes, highlighting the importance of cellular contacts.

Human myocardial microtissues were generated from human cardiomyocytes derived from induced pluripotent stem cells (iPSC). Morphological characterization of cardiac microtissues displayed cell-specific cytoskeletal structure and cell-cell contacts. The cardiac microtissues exhibited spontaneous and continuous contractile activity over at least three weeks in culture. Electrical field pacing resulted in electrical excitability at 2Hz frequency. Myocardial microtissues were further profiled for their response to known cardiac toxic drugs.

Rat brain microtissues were composed of whole cell populations of the cortex. Glia and neurons identified by GFAP and beta-III tubulin respectively. The model is stable for at least 4 weeks in culture. Astonishing, myelination of axons was observed after 7 days in culture.

In summary, scaffold-free 3D microtissues are a versatile tool for establishing novel, organotypic *in vitro* model systems. These model systems are a valuable component in the overall tox testing tool box to improve and accelerate the de-risking process in drug-development.

Study on development for the alternative methods for photo-toxicity and photo-genotoxicity tests using mouse keratinocytes and human 3D skin model

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Photo-toxicity is defined as a toxic response from substances including drugs, cosmetics applied to the body which is either elicited or increased after subsequent exposure to light. The *in vitro* 3T3 NRU photo-toxicity test (OECD TG 432) has been widely used to identify the phototoxic potential of test substances in response to ultraviolet (UV) irradiation, due to its simplicity for measurement to the relative cell viability in terms of photo-cytotoxicity. However, it is known to have poor *in vitro-vivo* correlation, which is nearly 85% of photo-toxicity positively determined substances *in vitro* turn to be proven negative in *in vivo* confirmative testing. Therefore, it is necessary to develop a new *in vitro* photo-toxicity test method. In this study, we tried to develop a new *in vitro* photo-toxicity test method using ARE-luciferase transfected JB6 cell (JB6-ARE). After establishment of JB6-ARE cell lines, the radiation sensitivity of the cell for selecting radiation dose were evaluated. A dose of 5 J/cm^2 was determined to be a non-cytotoxic to JB6-ARE cells and to be sufficiently potent to exert on the phototoxic reaction. The effect of seven test substances (i.e., chlorpromazine, ketoprofen, terbinafine hydrochloride, L-histidine, sodium lauryl sulfate, hydrochlorothiazide, 4-aminobenzoic acid), which are known as phototoxic or non-phototoxic chemicals, on the

photo-toxicity were evaluated by measuring cytotoxicity and luciferase activity. In cytotoxicity assay using JB6-ARE cells, the results of five test substances were identical with the results of *in vitro* 3T3 NRU test and its known photo-toxicity information, except for hydrochlorothiazide and 4-aminobenzoic acid had no correlation between test results and known photo-toxicity information. Furthermore, results of luciferase assay using JB6-ARE cells were highly comparable to the known photo-toxicity information. These results showed that this test using JB6-ARE cells have highly productivity and sensitivity compared to *in vitro* 3T3 NRU photo-toxicity assay. In addition, photo-genotoxicity tests method using human 3D culture skin models were established by measuring DNA impairment using Comet assay. To evaluate the test method, four chemicals were used as photo-genotoxicity positive (i.e., chlorpromazine, ketoprofen, 2-phenylbenzimidazole) and negative control (i.e., sodium lauryl sulfate). The results from Comet assay in human 3D skin models were comparable to the results using keratinocytes. These results revealed that the photo-genotoxicity test method using human 3D skin models might be challenged as an alternative method for evaluating for photo-genotoxicity using human 3D skin models.



Confocal μ -Raman spectroscopy of living cells

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Introduction

Eukaryotic cells consist mainly of water and the 30-40% of bioorganic materials, for instance lipids, peptides, carbohydrates and nucleic acids which are highly heterogeneous. The distribution of these components varies in different cell types and cell states. Over the past few years Raman spectroscopy got more and more attention as non-destructive and non-invasive *in vitro* method for the characterization of cells. It can provide information in living cells surrounded by cell culture media about cell viability, cell differentiation and tumorigenicity.

The aim of this study was to develop an easy and reproducible method for characterization of Caco-2 cells using Raman spectroscopy.

Material and methods

Caco-2 cells were cultured in DMEM supplemented with 20% FCS, 1% MEM non-essential amino acid solution, 1% HEPES buffer and 1% Penicillin/Streptomycin. Media exchange was carried out every 2-3 days. Cells were seeded onto different substrates (Silicon, Calcium Fluoride and polymeric polystyrene) which were carried out two to 30 days after seeding, depending on the desired proliferation or differentiated state of cells. Prior to Raman imaging of the cells, medium was replaced by FCS and phenol red free medium. Cells were imaged alive. The Raman spectra and images were taken with a Witec α 300R Confocal Raman Microscope equipped with a UHTS spectrometer with a CCD detector. Laser power on the cells was between 15 and 31 mW. Integration time for Raman images was 0.5 seconds. Data analysis was done with Witec Project 2.08 and Microsoft Office 2007.

Results

We found suitable substrates (CaF₂ and Silicon) and media for confocal Raman imaging of cells and also could reveal differences between proliferating and differentiated Caco-2 cells with respect to their lipid content. Differentiated Caco-2 cells seem to have more lipid enclosures than Caco-2 cells still in proliferation. We could also see the distribution of Cytochrome C inside cells with the help of confocal Raman microscopy (532 nm laser).

Discussion

Raman spectroscopy is an excellent tool to discriminate and allocate different macromolecular components inside cells without destructing organic material. The ratio formation is a suitable method for distinguishing between cell types (McEwen, et al., 2013) and cell states. We could reveal differences in lipid distribution of proliferating and differentiated Caco-2 cells that can be used for cell characterization.

Conclusion

The Raman spectroscopy mapping of cells may be a reliable non-invasive method to identify and characterize the molecular changing of the cell components in living state.

Reference

McEwen, G. D., Wu, Y., Tang, M., et al. (2013). Subcellular spectroscopic markers, topography and nanomechanics of human lung cancer and breast cancer cells examined by combined confocal Raman microspectroscopy and atomic force microscopy. *The Analyst* 138, 787-797.

The diXa project

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The EU nowadays witnesses increasing demands with regard to chemical safety. In particular, animal-based test models need to be replaced preferably by robust, non-animal assays *in vitro/in silico* which better predict human toxicity *in vivo*, are less costly, and are socially better acceptable. Consumer's and patient's health will benefit and competitiveness of EU's chemical manufacturing industry will be increased. For developing such assays, FP6/FP7 Research Programmes are exploiting the revenues of data-dense genomics technologies. However, till date, there is no infrastructure foreseen which aims at capturing all data produced by toxicogenomics (TGX) projects, in a standardized, harmonized and sustainable manner. Data may thus evaporate. The lack of such an infrastructure also prevents innovative breakthroughs from meta-analyses of joint databases and systems modeling.

Driven by these needs of the TGX research community, diXa focuses on networking activities, for building a web-

based, openly accessible and sustainable e-infrastructure for capturing TGX data, and for linking this to available data bases holding chemico/physico/toxicological information, and to data bases on molecular medicine, thus crossing traditional borders between scientific disciplines and reaching out to other research communities. To advance data sharing, diXa ensures clear communication channels and delivers commonly agreed core service support to the TGX research community, by providing SOPs for seamless data sharing, and by offering quality assessments and newly developed tools and techniques for data management, all supported by hands-on training. Through its joint research initiative, by using data available from its data infrastructure, diXa will demonstrate the feasibility of its approach by performing cross-platform integrative statistical analyses, and cross-study meta-analyses, to create a systems model for predicting chemical-induced liver injury.



Implementation of Directive 2010/63/EU into German law

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Directive 2010/63/EU on the protection of animals used for scientific purposes was meant to be implemented in national law by the Member States by 10.11.2012. Because of delays in the legislative process, it was not possible for this deadline to be met in Germany. The protection of laboratory animals in Germany has hitherto been almost entirely regulated by the Animal Welfare Act. With the implementation of Directive 2010/63/EU, the Animal Welfare Act now only contains the fundamental principles. Further regulations are contained in a new Animal Welfare/Experimental Animal Ordinance.

Legislators in Germany had already addressed the protection of laboratory animals in great detail with the result that the existing national requirements went beyond the previously applicable Directive 86/609/EEC in some aspects. Apart from the obligation to implement Directive 2010/63/EU, the alignment of national legislation was also meant to include the retention of any existing, more extensive regulations which had proven effective, as Article 2 of the Directive allows. These would thus need to be meaningfully integrated within the new framework laid down by the Directive. One retained feature is the animal welfare officer who in future will also perform the tasks of the designated veterinarian in accordance with the Directive. The animal welfare commissions were also retained. These support the authorities in reaching decisions on the authorisation of projects and must include representatives of animal welfare organisations. The previous notification process, which was possible for certain projects, was retained in line with the Directive's simplified administrative procedure. However, due to the requirements laid down by the Directive it is now different than before.

The implementation of the Directive entails some changes in Germany too. The broadening of the definition of animal testing procedures must be mentioned here, along with the expansion of the regulations to cover certain animals prior to birth or hatching. New instruments include the animal welfare bodies to be established in the facilities, the retrospective assessments to

be conducted by the authorities for certain experiments, and the publication of non-technical project summaries. In Germany, the tasks of the national committee as stipulated in Article 49 of the Directive will be performed by the Federal Institute for Risk Assessment (BfR). This houses the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET), which was founded in 1989. Within the context of the Directive's implementation, ZEBET was also nominated as the national contact point in accordance with Article 47(5) and was tasked with publishing the non-technical project summaries.

The Directive also calls upon the Member States to contribute towards the development of alternative methods to animal testing. In this area, the German government has already shown above-average commitment in the past. Worthy of special mention here are the research funding priority, the founding of ZEBET and the annual presentation of an animal welfare research prize. This commitment will continue in future.

References

- Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off J L 276*, 20.10.2010, 33–79.
- Third Amendment to the Animal Welfare Act, Germany, 2013 Animal Welfare/Experimental Animal Ordinance



Predictive models and computational embryology

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Multiscale modeling and simulation is an important approach for discovery and formalization of biological design principles in complex adaptive systems such as the embryo. Cell-agent based modeling or “virtual embryo” models bring together *in vitro* data from alternative research platforms with biological information on dynamic cellular behaviors in a computational systems biology context. Such models can be used to predict the potential impact of chemical perturbations on higher-order biological organization and function. One schema involves a knowledge-driven adverse outcome pathway (AOP) framework utilizing information from public databases, standardized ontologies, semi-automated literature mining tools, and curated MeSH annotations to map relationships of adverse developmental outcomes to potential key events involving genes, proteins, molecular pathways, and chemicals. A data-driven approach

identifies significant statistical linkages between molecular pathway targets (e.g., retinoic acid receptor and TGF-beta signaling) for distinct developmental features such as disruption of blood vessel development, cleft palate, male urogenital defects and limb defects. Specifically, the target-feature associations are mined from 3.2 million *in vitro* data points in the high-throughput screening (HTS) and *in vivo* toxicity profiling data from ToxCast and ToxRefDB databases. Multicellular *in silico* computer models are engineered with CompuCell3D.org architectures to predict systems-level responses and dose effects. The capacity of these *in silico* models to engage the normal biology and simulate the behavior of a complex system steps us closer to *in vitro* profiling environmental chemicals for potential adverse effects on *in vivo* development. This abstract does not necessarily reflect US EPA policy.



Japanese Project “ARCH-Tox” for the future chemicals management policy: research and development of *in vitro* and *in vivo* assays for internationally leading hazard assessment and test methods

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In 2011, Japan’ Ministry of Economy, Trade and Industry (METI) launched a new 5 years research project, entitled as “ARCH-Tox”, with the goal of promoting the 3Rs in 28-day repeated dose oral toxicity studies, which are used to screen for compliance with Japan’s Chemical Substances Control Law. This project includes the following two sub-projects.

1. Tox-Omics Project: Development of methods to detect multiple-toxic effects using gene expression analysis

Tox-Omics project will attempt to analyze changes in gene expression in animals tested in 28-day repeated dose studies. This result contributes to establish methods for prediction or detection of carcinogenicity, neurotoxicity, or other effects of chemical substances in major organs.

2. Tox-*In vitro* Project: Development of *in vitro* assays to detect toxicities, including target organ toxicity and metabolic function

This sub-project will attempt to establish *in vitro* test methods simulated *in vivo* toxic effects for the speedy and efficient assessment of hepatotoxicity, nephrotoxicity, and other endpoints in repeated dose studies.

We believe that the successful completion of these projects will help further worldwide application of the 3Rs to safety evaluation of chemicals in systemic toxicity testing.

Peer Review Panel Evaluation of the ROS Photosafety Assay

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A reactive oxygen species (ROS) assay was developed for photosafety evaluation of pharmaceuticals. The multicenter validation study were indicative of satisfactory transferability, reproducibility, and predictive capacity of the ROS assay using Atlas Suntest CPS/CPS plus solar simulators and Seric SXL-2500V2 solar simulator. In 3 or 4 participating laboratories, 2 standards and 42 coded reference chemicals, including 23 phototoxins and 19 non-phototoxic drugs/chemicals, were assessed by the ROS

assay validation studies. The Japanese Center for the Validation of Alternative Methods (JaCVAM) convened an independent scientific peer review panel to evaluate the validation status of the ROS Assay in accordance with established international criteria. The panel concluded that the assay had excellent reproducibility both within and between laboratories for the 42 reference chemicals evaluated in the validation studies.



Alternative method in practice: postvalidation experience of the skin sensitization *in vitro* test strategy

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Several *in vitro* methods including dendritic cell line activation (e.g. MUSST and h-CLAT), keratinocyte activation (e.g. LuSens and KeratinoSens) and *in chemico* (e.g. DPRA) assays have been described as promising animal-free tools to qualitatively predict skin sensitizing potential. While these methods are currently undergoing evaluations in the different stages of formal validation, testing strategies have been proposed based on the combination of these assays. Yet to use suggested methods and prediction models for the diverse industrial sectors, such as the cosmetic, industrial chemical, pharmaceutical and maybe even the agrochemical sector, the scope of the substance classes tested as part of the initial validation exercise needs to be extended. Typically in a first validation phase novel alternative methods are evaluated against their gold standard *in vivo*

assays using model substances selected from literature for their well described toxicological endpoint effects. The substances tested in the first validation phase, do, however, usually not reflect the typical test substance portfolio of the different industrial sectors. Therefore in this study we present the post-validation evaluation of 40 additional substances with available *in vivo* skin sensitization data from various substance classes including acrylates, surfactants, isocyanates, plant extracts, and agrochemical formulations in state of the art *in vitro* methods to assess skin sensitization. This additional data provides valuable information to understand the predictive capacity in terms of the applicability domains and may also help to manage expectations what can be achieved with those assays.

***In vitro* toxicity testing: influence of different cell lines and endpoints**

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Our accredited testing laboratory is focused on the study of the toxicity of various materials (primarily medical devices) *in vitro* on cell culture models. The testing is carried out according to EN ISO 10993 Biological evaluation of medical devices – Tests for *in vitro* toxicity. Tests such as Test of Direct Contact and Test of Extract are used. This technical standard allows the use of different cell lines and detection endpoints for above mentioned tests.

Therefore, we compared three methods to evaluate Test of Extract: Photometric MTT test, Neutral red intake with photometric and with fluorescent detection. Our study confirmed the same level of toxicity detected by all three endpoints in the Test of extract.

Moreover, the comparison of two cell lines, which are frequently used for Test of Direct Contact, mouse embryonic fibroblasts (NIH 3T3) and human osteosarcoma (MG-63) cell line, was carried out. We found toxic response to be consistent between the samples tested.

The main criterion of quality, however, is to achieve the same results as compared to other testing laboratories. Since centrally organized proficiency testing of laboratories for our area of testing was not organized due to the very small number of interested laboratories, we conducted an interlaboratory comparison with two other accredited laboratories in the Czech Republic.

In our interlaboratory comparison based on Test of Direct Contact, carried out by three independent laboratories, we found conformity between all participating laboratories.

Acknowledgment

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The role of dioxygenases in human embryonic stem cells

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DNA methylation is a major epigenetic modification in the mammalian genome. It involves the addition of a methyl group at the 5' position of cytosine in CpG dinucleotides resulting in the formation of 5-methylcytosine (5-mC). DNA methylation is essential for the mammalian development as it plays vital roles in various biological processes such as regulation of gene expression, X chromosome inactivation and genome imprinting. A recently discovered epigenetic modification also present in the mammalian genome is 5-hydroxymethylcytosine (5-hmC) (Kriaucionis et al., 2009; Tahiliani et al., 2009) which is generated by oxidation of 5-mC by the TET family (TET1-3) of enzymes that belong to the 2-oxoglutarate (2OG)- and Fe(II)-dependent dioxygenases superfamily. 5-hmC is expressed at different levels in mammalian cells. It is present in the brain, some immune cell populations and undifferentiated embryonic stem cells (ESCs).

It has been reported that depletion of TET1 but not TET2 and TET3 in mouse ES cells (mESCs) impairs ES cell self-renewal and ES cell proliferation (Ito et al., 2010). Further, it has been shown that TET1 in synergy with NANOG enhances the efficiency of reprogramming (Costa et al., 2013). Another dioxygenase known to be 2-oxoglutarate (2OG)- and Fe(II)-dependent and is a critical regulator of mESC maintenance is JMJD2C. It has been shown to be positively regulated by OCT4 and that it regulates NANOG expression in mES cells (Loh et al., 2007). Upon depletion of JMJD2C in mESCs, the pluripotency markers OCT4 and NANOG were down-regulated and markers of different lineages were expressed.

To investigate the biological function of dioxygenases in human ES cells (H9 and RH1), we generated knockdown by short interference RNAs (siRNA) for each one of the human TETs and JMJD2C and verified the knockdown efficiency by quantitative RT-PCR. Knockdown of TET1-3 and JMJD2C resulted in morphological changes and induction of differentiation, reduced levels of the pluripotency markers OCT4 and NANOG as well as decreased levels of 5-hmC expression. Collectively, our data support a role for TET1, TET2, TET3 and JMJD2C for human ES cell maintenance.

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Defining compound subcytotoxic effects on epigenetic determinants of human embryonic stem cell renewal and lineage commitment

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It is well established from *in vitro* studies on animal and human cells that environmental factors, including subcytotoxic exposure to diverse chemicals can induce epigenetic alterations on chromatin correlated with and/or contributing to aberrations in genomic instability, development and cell and tissue function (Baccarelli and Bollati, 2009; Arita and Costa, 2009).

The aim of our study was to assess putative subcytotoxic effects of compounds known to disrupt epigenetic determinants on pluripotent stem cell renewal and/or cell differentiation. This was achieved by the evaluation of subcytotoxic concentrations of four compounds (5-azacytidine, cadmium chloride, sodium arsenite and valproic acid) known to affect development on two established human embryonic stem cell (hESC) lines (H9 and RH1).

Following 7 days of continuous treatment under normoxic atmospheric culture conditions (5% CO₂, in air), subcytotoxic treatments with compounds resulted in statistically significant reduction of the steady state levels of mRNA transcripts for pluripotency markers OCT4, NANOG and SOX2 by quantitative RT-PCR, qualitative confirmed by reduced immunostaining for OCT4 and NANOG. We also found compound specific changes in the mRNA expression levels of the TET1-3 and

JMJD2C dioxygenases, novel genes expressed by hESC possessing a conserved and unique methylation pattern of genes associated Cytosine-Guanine Islands, and germinal lineage markers as well as genomic incorporation of 5-hydroxymethylcytosine. Interestingly, the effects of the 5-azacytidine and sodium arsenite on undifferentiated hESCs were not apparent if treatments were applied under hypoxic conditions (0.5% O₂, 5% CO₂, 94.5% N₂) as shown by quantitative RT-PCR and immunocytochemistry for OCT4 and NANOG, unlike cadmium chloride (a well-known chemical inhibitor of HIF-1a). Based on the evidence of this study, we believe that dioxygenases TET1-3 and JMJD2C, and our hESC epigenetic biomarkers could constitute valuable markers with which to evaluate epigenomic toxicity of compounds known to perturb development.

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Pluripotent stem cell-derived engineered neural tissues: what's new?

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Successful engineering of neural tissues from human pluripotent stem cells (hPSC) is an important prerequisite for many *in-vitro* applications of hPSC in the neuroscience area. Our group has previously described a method to generate engineered neural tissues (ENTs) from hPSC through an approach involving neurosphere-based early neural commitment, followed by neural maturation on air-liquid interface. This model has provided a first useful engineered neural 3D tissue, consisting of radially organized neural tubes (which include proliferating neural precursors), areas of doublecortin-positive migrating neurons, as well as areas of differentiated post-mitotic neurons. During my presentation, I will summarize further developments of this model as well as applications for *in-vitro* testing:

- i) optimization of early steps in neural tissue engineering: cellular starting material, small molecule-guided differentiation, optimization of media and supplements
- ii) induction of gliogenesis: NSC maturation is a key factor for induction of astrocytes and oligodendrocytes

- iii) inclusion of microglia-like cells: primary human monocytes develop microglia-like features upon inclusion in engineered neural tissue
- iv) regionalization of ENT development: generation of a dopaminergic tissue
- v) hCMV infection: a model for fetal CMV encephalitis in humans
- vi) interaction of glioblastoma with normal neural tissues: tissue invasion and induction of a unique gene expression pattern
- vii) characterization of neurotoxic and neuroactive compounds: electrophysiology and gene expression

In summary, engineered neural tissues have developed from an academic observation to an emerging tool for applied stem cell research. Key developments necessary for a future widespread use of this technology include standardized large scale production, long shelf life, as well as quantitative read-outs.

Integrating transcriptomics and metabolomics to identify pathways of toxicity of the parkinsonian toxin MPP+

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Chemicals targeting the nervous system may favour the risk of the development of brain disorders such as autism or Parkinson's disease (PD). Therefore test systems are needed to first of all identify neurotoxicants and in a subsequent step to classify them according to their mode of action. Individual "omics" technologies are of rising interest in the field of toxicology for classifications, but they cannot unravel all the complexities of toxicant effects on human beings. As new alternative strategy, we integrated two "omics"-technologies, transcriptomics and metabolomics, to obtain a more precise picture of all cellular processes in an established model system of basal ganglia neurotoxicity. We exposed human dopaminergic neurons to the parkinsonian toxin MPP+, to test the usefulness of this combined "omics" technology. With the combination of transcriptomics and metabolomics we wanted to answer the question, which of the events triggered by MPP+, including mitochondrial respiration deficits, oxidative stress and energy failure, was most upstream. After 24 h of treatment with 5 μ M MPP+, ATP depletion was rather minor, but pronounced changes were already seen on the transcriptome level. Gene ontology comparison did not support our expectation of altered glycolysis, but revealed changes which were mainly involved in cellular amino acid and amine metabolic processes (upregulated), as well as reactive oxygen species metabolic processes (upregulated) and DNA conformation changes (downregulated). On the metabolic level changes of glutathione, homocysteine or methionine sulfoxide confirmed

the role of oxidative stress as initial pathway of toxicity. Combining data of both approaches, the transsulfuration pathway seemed to be strongly implicated in MPP toxicity. This pathway contributes to the conversion of methionine to cysteine, which is the rate limiting amino acid for glutathione synthesis. Intermediates of this pathway, such as S-adenosyl-methionine and S-adenosyl-homocysteine are important contributors in DNA methylation processes, possibly explaining the changes on DNA conformation. Our data suggests that combined omics analysis is more sensitive for pathway identification than individual approaches and we found that effects on DNA level as well as oxidative stress are observable before strong mitochondrial respiration deficits or energy failure were detected.

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Use of transcriptome profiling in stem cell based test systems for reproductive toxicity

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Neurodevelopmental toxicity is usually characterized by relatively subtle changes of cellular phenotype that result in altered connectivity and network function of the nervous system. One approach to characterize in a relatively comprehensive way the changes of cells exposed to toxicants, is whole genome transcript profiling. Many developmental toxicants exert effects even after their exposure has ceased. A potential explanation are epigenetic changes, left as “memory traces” in the genome. We have used here several human embryonic stem cell (hESC)-based models of early neural development to study transcriptional and epigenetic changes exerted by several different mercurial compounds and histone deacetylase inhibitors (HDACi). For instance, one system (UKN1) modelled the directed differentiation of pluripotent hESC to PAX6-positive neural precursors, and another system (UKK) modelled the same period of early tissue specification, but allowed generation of cells of all germ layers. In all systems, transcriptome profiling clearly distinguished mercurials from HDACi and negative control compounds. The transcriptome patterns were used to predict the

modes of action of unknown compounds in a “blind testing” part of this study. The transcriptome patterns were also analysed for underlying biological themes. The overrepresentation of transcription factor binding sites in different transcriptome sets indicated an astonishing overlap of the damage response to different toxicants, but also indicated “pathways” triggered only by one class of toxicant, but not by another. The idea of exploring pathways of toxicity on the basis of the altered transcriptome patterns was followed further by time-series analyses and epigenetic profiling. Preliminary results suggest that “pathways-of-toxicity” may not be constant for a given toxicant, but that they are highly dependent on the model system and the time point of exposure.

Background reading of previous work

Krug, A. K., Kolde, R., Gaspar, J. A., et al. (2013). Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach. *Arch Toxicol* 87, 123-143.

Screening of a large group of medical substances and environmental pollutants in an embryonic stem cell-based test battery

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To further explore the utility of the ESNATS test battery to pre-screen/prioritize potential developmental toxicants, a wider list of substances has been tested in this study. Twenty-seven compounds, comprising several modern pharmaceutical substances and environmental pollutants have been selected in order to create a list of test compounds with wide structural heterogeneity. The selected panel contained also known DNT-positive and negative controls and substances showing general developmental toxicity. For each compound, a reasonable testing concentration range was determined on the basis of pilot experiments and data of cytotoxicity assay in different test systems (human embryonic kidney-293 (HEK293) cells and CGR8 murine embryonic stems). Moreover, physiologically-based pharmacokinetic modeling analysis, clinical monitoring data, and published epidemiological data were used to establish realistic test concentrations.

Tests were performed in four assays: the UKK assay models early germ layer formation. The UNIGE assay modeled early neuronal development, starting with neural stem cells, the UKN1 assay modeled early neural development, covering the period from pluripotent cells to neural stem cells. As fourth assay, the UKN2 (=MINC) was used and characterized in more detail. The Migration of Neural Crest cell (MINC) assay is able

to detect the effects of chemicals on one of the key events of nervous system development, i.e. neural crest cell migration. Disturbance of the neural crest cell (NCC) migration process by toxicants is known to lead to severe malformations in different species. Several factors; including genetics and exposure to chemicals can cause neural crest (NC) -related developmental defects. For each chemical, the MINC assay allowed the determination of the lowest observed adverse effect level (LOAEL) in absence of cytotoxicity; compounds showing a LOAEL inside of the toxicologically-relevant testing concentration range have been classified as DNT positive in our test system, and their concentration-dependent effect has been determined by generation of concentration-response curves. The results of this testing study showed impairment of the NCC migration after the exposure to 11 different chemicals of the test battery. Some of these positive compounds, as the environmental pollutants cyproconazole (5 μM), triadimefon (50 μM) and PBDE-99 (20 μM) have already been associated with developmental toxicity in *in vivo* and/or *in vitro* studies. More interestingly, others showed unexpected DNT effects, such as the drugs geldanamycin (16 nM) and IFN β (500 pM). These compounds were also tested in the other assays of the battery and a synopsis of the initial results will be presented.



Using the slug mucosal irritation (SMI) assay to predict discomfort caused by ophthalmic formulations

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Introduction

Some ocular pathologies may cause a lot of discomfort, irritation and pain. Ophthalmic formulations are applied to relieve these adverse effects and treat the disease. Therefore, it is important that these formulations do not induce discomfort or damage themselves. The Slug Mucosal Irritation (SMI) test was developed as an alternative for the Draize eye irritation test in rabbits. Previous research with shampoos demonstrated a strong relation between an increased mucus production (MP – expressed as % of initial body weight) in slugs and an increased incidence of stinging, itching and burning (SIB) sensations in human eyes. The current study aimed to investigate the usefulness of the SMI-test to predict the discomfort potential of ophthalmic formulations, since screening for this would be very helpful in the development and refinement of new and existing ocular formulations.

Methods

The stinging potency of several artificial tear solutions, anti-glaucoma formulations, non-steroidal anti-inflammatory drugs (NSAIDs), their active ingredients and preservatives was evaluated with the SMI-test by placing 3 slugs per treatment group 3 times on 100 μ l of the test item. After each 15-min contact period (CP), MP was measured. Evaluation of the results is based upon the total MP during the 3 repeated CPs. A classification prediction model is able to distinguish between 4 categories of

discomfort: no (total MP <3%), mild (total MP between 3% and 8%), moderate (total MP between 8% and 15%) and severe (total MP >15%). Results were compared with clinical data found in literature.

Results

Most of the tested formulations, as well as their ingredients, were very well tolerated by the slugs, inducing a total MP <3% and therefore resulting in a classification as causing no discomfort. When an increased total MP was measured (between 3% and 8%; mild discomfort), this could always be ascribed to the presence of benzalkonium chloride (BAC), which is added as a preservative. Several alternative preservatives (e.g. Polyquad[®], Oxyd[®], Purite[®], polyhexanide, thiomersal) appeared to be better tolerated by the slugs than BAC (total MP <3%). Results in slugs were in agreement with clinical data.

Conclusions

Results indicate that the SIB protocol of the SMI-test is a good tool to predict clinical ocular discomfort with reference to non- and mildly irritating formulations in humans, in a quick, reproducible and relevant manner. The concentration of BAC added to an ophthalmic formulation should be as low as possible (<0.01%); if possible another preservative should be added, but undose, preservative-free formulations appeared to be better tolerated.

Importance of reproducibility demonstration of the bio-engineered tissue models used for *in vitro* toxicity testing purposes

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Advances in tissue engineering enable scientists to closely mimic almost all tissues of the human body. However, for scientific as well as regulatory purposes, it is crucial that the reconstructed tissue models are reproducible not only within a given lot, but also between the lots produced over an extended period and at different production sites.

To demonstrate reproducibility of the epidermis tissues manufactured according to GMP protocols at 2 different production sites (Bratislava, Slovakia and Ashland, MA, USA), MatTek scientists undertook side-by-side evaluation of the barrier properties and tissue morphology of the EpiDerm model. Tissues were exposed to control chemicals (Triton X-100, 1%), and using the MTT assay, dose response curves were constructed, and an exposure time which reduces the tissue viability to 50% (ET-50) was interpolated.

The EpiDerm kits manufactured in the USA within a 4 month period averaged ET-50=5.91h, SD=0.8 and Exp. CV=14%. EpiDerm produced during the same period in EU provided highly comparable ET-50 = 6.1h, SD=0.7, Exp.CV=11.7%. Using light microscopy, histological H&E cross-sections showed stratified epidermis-like morphology that was reproducible both within and between lots. These data also fall into the historical ranges established by MatTek Corporation in 1996.

This study demonstrates, that following GMP-production rules, it is possible to produce reconstructed tissues of high quality and reproducibility, as required by regulatory guidelines (e.g. OECD TG 431 and 439). Furthermore, it is questionable whether an Open Source Model, recently suggested as an alternative to commercially available tissues, would sustain comparable levels of reproducibility.



Moving forward: a new paradigm for drug discovery

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In the EU, and indeed most other developed countries, the testing of new clinical entities (NCEs) on animals is required by law in order to determine the safety of a new drug before it can be tested on humans. However, the effectiveness of this process is much disputed, given the frequency with which animal testing fails to accurately predict the reactions and interactions of a drug in humans, leading to toxic insult and even death in patients. There are perhaps two explanations for this failure rate – firstly, that the current paradigm of drug discovery is flawed, and secondly that the methodologies employed as part of that paradigm are insufficient to determine the safety and efficacy of new drugs. With each new drug costing an average of \$ 6bn to get to market, and with NCE output decreasing annually despite annual R&D spend increases, the pharmaceuticals and CROs are beginning to recognise the limits in cost and effectiveness of current drug discovery approaches.

Alternative methods to traditional testing are required. New EU legislation (Directive 2010/63/EU) states that, where there is an alternative to animal experimentation, that alternative must be used. These methods are on or near to market, and promise to deliver more accurate and sensitive tests for toxicity than animal testing, but early widespread adoption of these new technologies is delayed by the structure of the validation strategy – with

an estimated investment of 10 years and € 5m for ECVAM validation of a new assay for example, change is not likely to happen quickly.

If not ethical concern over animal testing, the economic climate is likely to be a key motivating factor in helping drug companies move away from traditional methods and towards new alternatives to current practices, because animal testing is expensive in addition to its limited effectiveness. Alternative approaches promise to reduce the cost of pre-clinical screening, as well as reducing and eventually replacing animal use. Advances in *in vitro* cell culture technology, combined with quantitative systems pharmacology and new computational approaches to drug discovery, will pave the way for a future of cheaper and more accurate compound testing, better hit rates, and safer and more effective drugs.

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Research in perfused limbs successfully replaces induction of laminitis in live horses

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Introduction

Laminitis is a common, inflammatory, and usually severely painful failure of tissues within the hoof of the horse. The scientific investigation of the disease in clinical cases is often insufficient due to the number of pathogenetic factors involved. In order to remedy this, many studies have been carried out in horses in which the disease was induced using mainly carbohydrate overload and black walnut extract and horses were subsequently sacrificed at the time of severe pain development. Even in the years 2010-2013 20 such animal experimental studies were identified. The use of perfused limbs of healthy horses allows an even more standardised approach for investigation of the pathomechanisms of this disease, and obviates the need for animal experiments.

Objective

To illustrate how the examination of isolated equine distal limbs perfused with autologous blood has led to clinically relevant findings

Sample and Procedure

Up to now four studies have been carried out in perfused forelimbs, with a total of 10 forelimbs (comprising the metacarpus and digit) were collected from cadavers of healthy adult horses after routine slaughter for food production used for interventions, and 9 forelimbs were used for non-interventional control perfusions. Interventional perfusions were carried out with hyperinsulinaemic autologous blood (n=5), and with endotoxin added to the autologous blood (n=5). Limbs were perfused for up to 10 hours, and metabolic variables were monitored. At the end of the perfusion histology of the tissues of the hoof was carried out.

Results

In the control limbs neither changes from physiological metabolic parameters nor structural damage to the tissues was noted. Exposure of the perfused limbs to endotoxin (LPS) led to metabolic changes and significant changes in the laminar tissue similar to the ones seen in laminitis. Short-term hyperinsulinaemia lead to increased vascular resistance in the equine digit and

increased expression of ET-1 in the laminar tissue, a pathway not yet investigated in *in vivo* laminitis. Also, histology revealed significant changes in the tissue presence and distribution of ET-1 receptors following this short term hyperinsulinaemia.

Conclusions and Relevance

As horses are slaughtered at abattoirs for human consumption, limbs and autologous blood become available as unused by-products without any additional intervention in the live horse. Due to the selection of healthy limbs, the level of control over and monitoring of factors such as perfusion rate, oxygenation, and blood pressure as well as a detailed histology after the end of perfusion only a relatively small number of limbs has already led to important new insights into the pathomechanisms of laminitis. The findings obtained in the perfused limbs have been estimated to have replaced about 10 experimental inductions of laminitis with subsequent euthanasia. While we accept that not all research into this disease is possible in the isolated equine distal limb, the use of this system should be encouraged wherever possible.

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Towards replacing *in vivo* tests of dental and orthopedic implants

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New orthopedic and dental implants must be extensively tested, for safety and for mechanical stability after implantation, before market entry. Currently, 2D *in vitro* tests of implant materials suffer from a number of severe limitations and are consequently used only to screen materials for acute toxicity and biocompatibility. If the number of *in vivo* tests in this field is to be reduced, improved *in vitro* tests with good predictive value with respect to osseointegration are needed.

We are currently developing improved *in vitro* testing methods for tissue scaffolds for bone replacement. A central part of this is the development, use and optimization of a bioreactor that allows the scaffolds to be tested for long periods of time (weeks) under physiological mechanical loads. Mechanical loading is essential for bone growth *in vivo*. Its inclusion in a realistic *in vitro* test is thus, also essential; its absence in existing 2D tests is one of their major limitations. The bioreactor has been designed to be small and easy to use. It is placed inside a standard incubator, which ensures suitable temperature and CO₂ levels for cell culture. A peristaltic pump is used to pass cell

culture medium through the bioreactor at a slow rate to ensure oxygenation of the sample over long incubation times. The scaffold is held inside the bioreactor by a piston which applies a pre-determined force, a force which can be periodically varied to mimic the variation of mechanical forces on bones within the body. Bone slices can be held on either side of the scaffold during the culture period in order to achieve a closer approximation to the physiological environment.

First results using the bioreactor show good growth of cells – SaOs-2 and mesenchymal stem cells – within scaffolds in the bioreactor. They also highlighted difficulties in imaging cells within the scaffold. We are now developing strategies for imaging, both in fluorescence microscopy and scanning electron microscopy. An additional challenge that must be addressed is the determination of local culture conditions – pH, oxygen, CO₂ – within the scaffold. Future work will focus on the test protocol and on correlating test data from the bioreactor with existing *in vivo* test data.

The chick chorioallantoic membrane (CAM) assay as a model for the development of antitumor nanotherapeutics

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Activation of the mammalian target of rapamycin (mTOR) has been implicated in cancer development, proliferation and angiogenesis. Therefore, mTOR is representing a promising therapeutic target. Here we show by using *in vitro* and *in vivo* approaches that amino-functionalized polystyrene nanoparticles (PS-NH₂) inhibit, whereas carboxy-functionalized particles (PS-COOH) activate mTOR in leukemia cells. Thus, PS-NH₂ inhibited proliferation and induced G₂ cell cycle arrest in three different leukemia cell lines, whereas PS-COOH did not. Consistent with mTOR inhibition, PS-NH₂ inhibit activation of downstream targets of mTOR such as Akt and p70 ribosomal S6 Kinase 1, followed by overexpression of the cell-cycle regulator p21Cip1/Waf1, and degradation of cyclin B1. Initially, both particles induce autophagy in leukemia cells.

However, in leukemia cells treated with PS-NH₂, autophagy eventually switched to activation of caspase 3 and induction of apoptosis. In contrast, primary macrophages do not exhibit activated mTOR signaling and they are relatively resistant to the PS-NH₂-induced cytotoxicity. For *in vivo* testing, leukemia cells were xenografted on chick chorioallantoic membranes (CAM). In this model, in agreement with the *in vitro* data, PS-NH₂ likewise inhibited proliferation, angiogenesis, and induced apoptosis in the leukemia xenografts. These data indicate that the chick chorioallantoic membrane assay can be successfully used to analyze *in vivo* effects of the nanoparticle-based antitumor therapeutics designed to control activation of mTOR signaling pathway and to influence proliferation, viability, and drug resistance of tumor cells.



Directive 2010/63/EU: Overview of the work of Commission Expert Working Groups on an EU Framework for Education and Training and on Project Evaluation

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In 2012, the Commission established an Expert Working Group to develop a common education and training framework for the EU to fulfil the requirements under Directive 2010/63/EU. The common framework aims to assure the competence of all persons involved in the use, care and breeding of animals for scientific procedures, and to facilitate free movement of personnel.

The focus of the new Directive shifts from requirements on educational/training background to the demonstration and maintenance of individual competence. To respond to these new requirements, the framework establishes a flexible, output-based (Learning Outcome) modular training structure together with principles and criteria for supervision, competence assessment and continued professional development. Principles for a mutual approval/accreditation framework are equally required as the basis for mutual acceptance of training carried out elsewhere.

Agreement at EU level on general principles will assist those developing training courses to work towards common, acceptable standards. This in return should result in a wider offering of available training courses to promote the aims of availability, accessibility and affordability.

In 2013, the Commission convened an Expert Working Group to develop guidance on project evaluation and retrospective assessment under Articles 38 and 39 of the Directive. Project evaluation aims to ensure that the principle of the Three Rs are fully implemented and that the potential harms inflicted on animals can be justified by the expected benefits of the project. Harm-benefit analysis is a central element of a well-conducted project evaluation. The developed guidance looks at the key factors for identification and weighing of benefits and harms, and elements to take into consideration when assessing the likelihood of success.

Human epidermis reconstructed from UVB-irradiated keratinocytes mimics premature ageing in human skin

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Striving to find alternatives to *in vivo* testing in the animal is highly desirable and has become a focus of pharmacological and toxicological research. The use of epidermal or full-thickness skin equivalents has given promising results so far. In this context, disease models are of particular interest for the development of pharmaceutical applications, as they can mimic the pathological changes observed in diseased human skin and point to new drug targets. Prematurely skin ageing is a consequence of e.g. diabetes and arteriosclerosis, but also of extreme UV exposure. A construct that features the characteristics of prematurely aged skin including the changes in permeability and penetrability would be of great value. To induce premature ageing, we used UVB-exposed juvenile epidermal keratinocytes (30 mJ/cm²) for the construction of our epidermis model. Non-irradiated and UVB-irradiated cells, respectively, cultivated for 14 days (air-lift) form a stratified epidermis as indicated by the expression

of epidermal differentiation markers (keratin 14, keratin 10, involucrin). Staining of the senescence marker β -galactosidase is remarkably enhanced in UVB-exposed keratinocytes. Interestingly, the respective construct clearly differs in morphology when compared to constructs from normal keratinocytes. In addition, the tight-junction protein claudin-1, indicating appropriate barrier function of the epidermis, is differentially distributed between the two constructs. Moreover, different expression patterns of senescence-associated genes (VEGF-C, p16, IFI27, cyclinD2) are observed and the formation of the pro-inflammatory cytokines IL-1 α and IL-8 is strongly enhanced in the model of premature skin ageing. Further studies are needed to unravel the functionality of the epidermal barrier with respect to the uptake of xenobiotics including e.g. irritants.



Modelling local and systemic toxicity: incorporation of *in silico* predictions in the development of adverse outcome pathways

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Reliable and justifiable predictions of *in vivo* toxicity, using alternatives to animal testing, require an in depth understanding of the relevant biological processes involved in eliciting a particular response. Traditional *in silico* methods, such as (quantitative) structure-activity relationships have been largely unsuccessful in predicting complex toxicological endpoints. However, newer approaches, such as category formation and read-across, have shown more promise. A key factor in improving predictions of toxicity is breaking down more complex processes into individual components that may be more amenable to modelling. The development of the Adverse Outcome Pathway (AOP) approach has made a significant contribution to this new paradigm in prediction. AOPs provide a framework for organising and recording relevant chemical and biological information relating to toxicity pathways, by linking the chemistry underlying the process to the adverse effect.

Following exposure, the first step in an AOP involves the primary interaction between the chemical of interest and a biological macromolecule (e.g. proteins, DNA, membranes, etc.); this is termed the Molecular Initiating Event (MIE) which may lead to a downstream toxic effect (although this can be influenced by mitigating factors, adaptive mechanisms, etc.). Recently *in silico* tools have been developed that enable chemicals to be profiled as to their potential to elicit MIEs. These tools are based on identifying “chemotypes” i.e. structural features or “alerts”

within a molecule that are associated with specific chemical reactivity (or binding interaction etc.) and therefore potential toxicity. Profiling chemicals for the presence of such alerts can be used to form rationally-derived groups, or chemical categories, which can be used to predict toxicity by read-across.

This presentation will focus on recent work in developing alerts associated with specific chemical reactivity and their application in read-across predictions for toxicity (e.g. skin sensitisation, respiratory sensitisation and hepatotoxicity). Such predictions are fully transparent and justifiable from a mechanistic perspective; the alerts are freely available as SMARTS patterns. How such information can be used in combination with targeted *in vitro* studies, for example to identify regions of chemical space where the alerts are applicable, to develop AOPs and give greater insight into mechanisms of toxicity will also be presented.

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The COSMOS project – developing integrated computational approaches to predict repeated dose toxicity

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The COSMOS project (Integrated *In silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety) is developing databases and models to predict repeated dose toxicity, to support safety assessment of cosmetic ingredients without the use of animals. The project includes 15 partners from academia, industry, regulatory agencies and SMEs from across Europe and the USA. COSMOS is part of a cluster of six research projects within the Seventh Framework Programme SEURAT-1 (Safety Evaluation Ultimately Replacing Animal Testing) Research Initiative.

Modern computational approaches support toxicological safety assessment in a number of key areas and their role is anticipated to increase further in the foreseeable future due to changes in European legislation (e.g. implementation of the 7th amendment to the Cosmetics Directive). However, the prediction of long-term exposure to chemicals remains a challenge due to the scarcity of available toxicological data linked to high quality chemical structures and the complexity of the chemical and biological responses that ultimately lead to the adverse effects. The COSMOS project is making progress in overcoming these challenges by integrating a range of complimentary approaches. An inventory of cosmetic ingredients and related substances has been compiled, with emphasis being placed on those substances that are associated with unique chemical structures that have been systematically curated. The COSMOS database for repeated dose toxicity data has also been created, encompassing skin permeability and metabolite information, where available. These datasets are being used to evaluate the potential extension of the concept of Threshold of Toxicological

Concern (TTC) to cosmetics; factors relevant to extrapolating from the oral to dermal routes (such as differences in uptake and metabolism) are also being investigated.

Novel *in silico* approaches are being developed and applied. These include the use of category formation and read-across to predict toxicity and the development of novel profilers to identify structural features associated with specific mechanisms of toxicity. These approaches are providing information for the development of Adverse Outcome Pathways (AOPs) which provide a framework to link the initial interaction of a chemical with a biological macromolecule to an adverse effect. Physiologically-based pharmacokinetic (PBPK) models are being developed to predict target organ concentrations, along with multi-scale models to enable more accurate extrapolation from *in vitro* to *in vivo* concentrations. The KNIME open-access platform (www.knime.org) is being used to integrate the models and approaches being developed in the project. The KNIME software enables flexible workflows to be devised which can be used for example to access chemical inventories or databases, profile structures, calculate properties and report predictions. These will be freely accessible through KNIME as well as through a web portal. More information is available from www.cosmostox.eu.

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Animal Testing and Transparency – a Contradiction in Terms? Presenting a Project to Promote both, Transparency of Animal Testing and the Social Dialogue between Proponents and Opponents

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Animal testing becomes increasingly relevant for the public sphere. High numbers of animal experiments (about 50–100 million vertebrates worldwide were for example used in the course of animal experiments in 2005) (Taylor et al., 2008) are confronted with growing interest in animal protection by society. This interest is mainly caused by increasing importance of animals in society.

The public opinion and the demand for more animal protection are also reflected in legislation, underlining its importance. A current example is the new EU Directive 2010/63/EU (EU, 2010), which demands a harm-benefit analysis, including ethical considerations as part of the evaluation of animal experiments (article 38 (2) d).

Valid knowledge about animals and animal testing strongly depends on the availability of information. Insufficient transparency and restrictive information policy in the context of animal testing are a huge problem. Shaping the public opinion about such a sensitive and important topic (no matter what the individual opinion about animal testing will be) should not be based on sciolism and speculation. Therefore, it is necessary to inform people in an appropriate way.

The Messerli Research Institute at the University of Veterinary Medicine, Vienna, would like to contribute to the enhancement of transparency in the field of animal testing in a project, funded by the Austrian Federal Ministry for Science and Research (BMWF). In the course of this project, called 'Social Dialogue on Animal Testing' (SDAT), groups of visitors will be provided with comprehensive insight into different areas of research using animal experiments. Knowledge and relevant aspects from various disciplines will be prepared for the public. Topics of the SDAT will be, among others, the legal framework of animal testing, planning and realization of animal experi-

ments, education of people carrying out animal experiments and the 3Rs (Replacement, Reduction, Refinement). Furthermore, alternative methods, avoiding animal experiments, will be presented and ethical aspects of research, using animal experiments, will be discussed. The following activities are planned:

- a. Short presentations by scientists on their projects
- b. Guided tours for visitors in representative research and teaching institutions
- c. High quality information leaflets (readers) with a special focus on didactics
- d. Professional videos

In a concluding meeting, the participants' impressions and opinions will be collected and discussed. A final evaluation will analyze, if the event has been successful in terms of promoting transparency, transferring knowledge and supporting the opinion-forming process. Pilot events will take place end of 2013 and beginning of 2014.

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„Taking Ethical Considerations Into Account? Methods to Carry Out the Harm-Benefit Analysis According to the EU Directive 2010/63/EU“. Summary of a Symposium at the Messerli Research Institute

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The EU Directive 2010/63/EU (EU, 2010) requires a harm-benefit analysis as a part of the evaluation of animal experiments, which has to take ethical considerations into account (Article 38 (2) d). The Austrian Animal Experimentation Act 2012 (TVG, 2012)² that transfers this requirement into national law has come into force on January 1, 2013. One of the new aspects in the harm-benefit analysis in Austria is that the applicant has to submit a catalogue of criteria to the competent authorities as one basis for the project evaluation. This catalogue should be based on scientific criteria and objectify the harm-benefit analysis of animal experiments. A project to develop this catalogue by the end of December 2015 has been launched at the Messerli Research Institute. It is funded by the Austrian Federal Ministry for Science and Research (BMWF).

On March 27, 2013, the Messerli Research Institute organized an international symposium with several renowned experts in the field of (ethical) evaluation of applications for animal experiments. The aim was to establish a profound knowledge base as an optimal starting point for the development of the Austrian Catalogue of Criteria. For this purpose, previous experiences regarding the practical application of different catalogues as well as their advantages and disadvantages have been discussed:

First, the central aims of the Austrian Catalogue of Criteria and the integrated harm-benefit analysis have been framed as one of the main results of the symposium: a. improvement of scientific research/of its quality (among others via evaluation of methods) and b. protection of laboratory animals (among others via balancing the expected benefit of the animal experiment against its harm for the animals).

Second, a central proposal was to differentiate between the evaluation of an application and the evaluation of a whole research strategy. The latter one should be directed on a higher (national, political) level and not be part of the project evaluation by the competent authority. Furthermore, the compulsory retrospective evaluation of animal experiments was suggested

to be integrated in order to provide a basis for continually optimizing the catalogue of criteria.

Third, three different concepts of catalogues have been presented (checklists, scoring/weighting procedures and a comparative approach). A combination of all three methods in the Austrian Catalogue of Criteria is seen as a reasonable approach, making use of the advantages of all methodologies and compensating demerits. Besides different methodologies for the evaluation of applications for animal experiments, general desirable characteristics of the catalogue have been discussed. The catalogue has to be user-friendly. Considering its long-term usage, some flexibility is essential to provide a suitable methodology for the long term.

Finally, the importance and advantages of an independent, interdisciplinary and well-balanced committee to support the competent authority have been underlined. Besides representatives from local authorities and scientists, this committee should comprise animal welfarists and non-specialists/lay persons. The involvement of the public would also contribute to meet the increasing demand of transparency regarding animal experiments.

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The human placenta in toxicology

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The human placenta and placenta-derived cells and structures offer a great potential for toxicology testing but it has also several limitations. The placenta contains a wide spectrum of cell types and tissues, such as trophoblast cells, immune cells, fibroblasts, stem cells, endothelial cells, vessels, glands, membranes and many others. It may be expected that in many cases the relevance of results obtained from human placenta will be higher than those from animal models due to species specificity of metabolism and placental structure. For practical and economic reasons we propose to apply a battery of sequential experiments for analysis of potential toxicants. This should start with using cell lines, followed by testing placenta tissue

explants and isolated placenta cells, and lastly by application of single and dual side *ex vivo* placenta perfusion. With each of these steps, the relative work load increases while the number of feasible repeats decreases. Simultaneously, the predictive power enhances by increasing similarity with *in vivo* human conditions. When toxic effects appear at any step, the subsequent assays may be cancelled. In our laboratories a number of toxicants have been successfully tested by applying this system. The test battery is useful to reduce costs and increase specificity in testing questionable toxicants.

Characterization of lymphoblastoid cell lines as a novel *in vitro* test system to predict immunotoxicity of xenobiotics

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Evaluating immunomodulatory effects of xenobiotics is one of the main priorities in toxicity studies. The aim of our study was to characterize human lymphoblastoid cell lines (LCLs) for the evaluation of immunotoxic effects of xenobiotics. LCLs were derived from peripheral blood lymphocytes B from healthy donors at Tel Aviv University. The cells were immortalized by Epstein-Barr Virus (EBV). Human LCLs retain most of phenotypic properties of lymphocytes B, including the production of antibodies. Moreover, their nuclear DNA remains intact (Morag et al., 2010). LCLs are emerging as a novel tool for studying inter-individual variability in drug response, including adverse drug reactions (Morag et al., 2013).

We evaluated the impact of selected xenobiotics on the proliferation of LCLs and on cytokine release. Four known immunotoxic compounds: tributyltin chloride, cyclosporine A, benzo(a)pyrene and verapamil hydrochloride and three negative controls: urethane, furosemide and mannitol were selected for evaluation (Carfi et al., 2007). Based on proliferation assay, we could separate immunotoxic from non-immunotoxic xenobiotics by using ten cell lines from different donors. The determination of IC₅₀ values on LCLs was in agreement with the data obtained on human peripheral mononuclear cells (PBMCs).

The impact of xenobiotics on the function of LCLs was evaluated. The expression of genes of pro-inflammatory cytokines: TNF α , IFN γ , IL-1 β and IL-6 was determined in resting cells and in cells stimulated with ionomycin/PMA,

anti-IgM or LPS. Four hour incubation of three different LCLs with ionomycin/PMA revealed significant up-regulation of cytokines' gene expression. We have confirmed this data by determining the presence of secreted cytokines in cell culture media. All six LCLs tested released cytokines: TNF α , IFN γ , IL-6, IL-4, IL-2 and IL-10 that were up-regulated by ionomycin/PMA. Among the released cytokines, IL-10 was the most abundant. We were able to modulate ionomycin/PMA induced cytokine release by pre-incubation of LCLs with cyclosporine A also for concentrations tested at which viability of the cells was higher than 80%. While the trends in all tested LCLs were similar, we could observe certain differences, which might be a consequence of inter-individual variability of the donors.

In conclusion, LCLs provide a novel tool for *in vitro* testing of immunotoxic effects of xenobiotics.

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A multi-organ-chip platform for long-term maintenance and substance testing of human tissue co-culture

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Current *in vitro* and animal tests for drug development are failing to emulate the systemic organ complexity of the human body and, therefore, to accurately predict drug toxicity. In this study, we present a smartphone-sized, self-contained multi-organ-chip-platform aiming to support predictive substance testing and disease modeling *in vitro* at relevant throughput. A peristaltic on-chip micro-pump reproducibly operates a microcirculation system interconnecting several tissue culture spaces within a PDMS-embedded micro-fluidic channel system. The layout supports both flexible integration of conventional miniaturized tissue culture formats, such as Transwell[®] inserts, special organotypic matrices and tissue exposed directly to the fluid flow. This multi-organ-chip is capable of maintaining 3D tissues derived from cell lines, primary cells and biopsies

of various human organs. Co-cultures of human artificial liver microtissues and skin biopsies, each a 1/100000 of the biomass of their original human organ counterparts, could successfully be maintained functional over a period of up to 28 days. Crosstalk between the two tissues was observed in 14-day co-cultures directly exposed to fluid flow. Applying the same culture mode, liver microtissues showed sensitivity at different molecular levels to the toxic substance troglitazone. Further experimental results also including the vascularization of the microcirculation channel system with human endothelial cells will be presented, as well as the influence of the vascularization on other organs. Thus, here we provide a potential new tool for systemic substance testing.

Results of a (pre)validation study of the Hen's Egg Test for Micronucleus-Induction (HET-MN)

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Due to the EU chemical legislation REACH and the 7th Amendment to the EU-Cosmetics Directive a high amount of improved *in vitro* tests to examine the genotoxic potential of test chemicals is required. Because of its autonomy and metabolism (ADME) the hen's egg is supposed to be a complex biological system that provides toxicologically relevant similarities to a mammalian organism. The validation of the Hen's Egg Test for Micronucleus-Induction (HET-MN) is taking place presently focussing on supplementing and replacing existing *in vitro* and *in vivo* genotoxicity tests.

The HET-MN was developed at the University of Osnabrueck and firstly transferred to the test naive laboratories of Henkel in 2007 (Wolf et al., 1997, 2002, 2003, 2008; Greywe et al., 2012). During the method transfer to two further laboratories (Federal Institute for Risk Assessment and Harlan Cytotest Cell Research) in 2010 two adequate positive controls and three different test chemicals have been analyzed. In the current phase

of validation, the double blind examination of coded test chemicals, which have not been tested before in the HET-MN, has been completed for the first six out of 20 test chemicals. The intra- and interlaboratory comparison showed a good reproducibility. However the application regime (single dose at day 8 vs. repeated dose at day 8, 9 and 10) seems to affect the test outcome to a relevant extent: While the repeated dose regime is being used to minimize acute toxic effects of test chemicals in order to increase the applicable dose it might mask the genotoxic effect in some cases. Based upon these results the single dose regime will be included in the second part of the HET-MN validation exclusively.

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Using 21st century toxicology to model disease risk

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Adverse outcome pathways provide a significant tool for screening single chemicals against a specific toxicological end point by mapping out and testing against the preceding, causative events and interactions. We have applied these principles to the mapping out of a pathway of events resulting in disease risk (specifically for a number of tobacco related diseases). We propose an approach using these pathways to model the comparative risks of tobacco and nicotine products, which deliver a mixture of substances, in the development of these diseases.

In contrast to a screening tool or a test for an acceptable level of toxicity this approach aims to place substances (single chemicals or mixtures) in a hierarchy of their potential to contribute towards risk of disease development or progression.

In March 2012 the FDA published a draft guidance describing the evidence required to launch a novel modified risk tobacco product in the US market. We propose the use of this package of *in vitro* data to significantly reduce the potential *in vivo* requirements to support a regulatory application for a modified risk tobacco product.

Human embryonic stem cells differentiation reveals toxicity signatures for HDAC inhibitors and mercuric toxicants

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Differentiation of human embryonic stem cells (hESCs) has been aimed to use as a tool for many clinical applications, previously we have used hESC differentiation to show the mechanism of developmental toxicants. In the present study we have attempted to show the toxicity mechanisms of HDAC inhibitors (Valproic acid (VPA), SAHA, Trichostatin A (TSA)) and mercuric toxicants (Methyl mercury (MeHg), Mercury Chloride (HgCl₂), thimerosal) using hESC differentiation in combination with genome wide expression profiling. Gene expression analysis showed that VPA, reduced neural tube (OTX2, DNMT3A) and forebrain (ISL1, and EMX2) related transcripts which have been proved previously with *in-vivo* studies. In addition VPA over expressed axonogenesis related markers such as SLIT1, SLITRK3, and SEMA3A. GO enrichment score analysis reveals dysregulation of neuronal related biological processes such as neurogenesis and neuronal differentiation with all three HDAC inhibitors. The mRNA and miRNA correlation analysis explains many miRNA signatures for VPA toxicity including miR-20a, miR-302, miR-301, miR-155, and its corresponding mRNA targets. Also we could demonstrate a set of neuronal related markers (including transcription factors) that has been dysregulated by all HDAC inhibitors and mercuric toxicants preferably MeHg and thimerosal. Together this multilineage differentiation system attempts to represent

the toxicity of the positive developmental neuronal toxicants with substantiate molecular evidence.

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The alternatives to animal experiments in basic research and education: status and possibilities in EU

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One way to analyze the progress of alternatives to animal experiments in research and education is to estimate the funding, the number of publications and the training of scientists in these areas.

The lecture analyses and summarizes the areas of research and education where courses are available, which EU countries are more advanced than others and grant more funding, what type of investments have been made by Governments and EC, which projects are funded and the area of research, how many countries and people are involved in the projects, what results have been achieved and what more has to be done. Several issues are reported and the limitations and improvements of the state of art of research for what concerns animal use, areas of use and publications.

The web sites, sources of information, courses available have also been included to provide a quick reference for people in training and scientists who want to change the old animal models with new and advanced *in vitro* cells and tissue culture methods and the technologies available, which able to provide new models, similar to complex *in vivo* system, but more specific for the human system and therefore more reliable. A discussion about the accepted and current methods of validation by the regulatory authorities (e.g. EURL-ECVAM) and the limitations of such methods to provide comparable and more advanced human-related methods to validate *in vitro* alternative models to replace animals in research are also discussed. A brief discussion concerning the hurdles found in the acceptance of alternatives has also been made.

An overview of the EU and international legislation has been included with an outlook of recent events in Europe, prompting a change in legislation due to public pressure (e.g. the cosmetics directive etc.). The increase of people awareness and knowledge, both in science and legislation, around animal experiments in our societies, the business behind the industry, and how internet and media information have shed light on animal research to the general public that had never been achieved before, is likely to prompt a profound change in the way scientists, industry and academia have considered animals and their use, so far, in our civil societies. A discussion on how the future of research is evolving and the need for investments to fund the development of ever more advanced and integrated methods and the dissemination of these alternatives to animal experiments, in research, industry and education, will be made, in the

light of recent analysis of the side effects of drugs for humans and the limitations shown by animal models, seen more often as old outpaced standards that in the light of our current scientific knowledge, technologies available and progress are no longer scientifically approvable, nor medically appropriate, and nonetheless ethically acceptable.

Talk based on a review paper in progress based on published data and different sources of information from different EU countries and the EC web site and portals, such as:
http://ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm

<http://ecvam-dbalm.jrc.ec.europa.eu/>

PubMed search with key words to analyse subject and number of publications per area.

EU countries research funding reviews (local web sites from EU Government Authorities)

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3D organotypic cultures of liver cells for metabolism and toxicity testing

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The 3R concept of Russell and Burch (Russell and Burch, 1959) is more than ever gaining political and scientific significance due to the regulatory and legislative requirements and the tremendous scientific progress that not only shows the inadequacy of animal models but also the huge potential of the *in vitro* alternative methods, especially 3D organotypic systems in toxicological studies. From 11th March 2013, there is a complete ban on animal testing for cosmetics in Europe. To cope with legislative requirements and the need for reliable and validated *in vitro* methods, the European commission and Cosmetics Europe initiated a jointly funded research program namely; SEURAT-1 (safety evaluation ultimately replacing animal testing). The main objective of SEURAT-1 is establishing an animal free liver based *in vitro* system with high *in vivo* relevance for application in repeated dose long term toxicity testing. NOTOX is one of the building block projects of SEURAT-1 aiming at development of a systems based strategy for the assessment of human relevant repeated dose toxicity using organotypic cultures.

3D cultivation allows organotypic microtissue formation ensuring cell-cell contacts and contacts to the extracellular matrix. The communication between cells at tight junctions and across extracellular matrix induces and maintains cellular differentiation, functionality and viability. 3D cell cultivation techniques can tremendously improve studies on drug metabolism, drug toxicity or adverse drug effects especially long-term repeated dose effects.

Using a high-throughput 3D cultivation system, we produced 3D organotypic cultures of human hepatic cells using a 96 well plate based hanging drop method using InSphero (Zurich) GravityPlus system. This method allows scaffold-free reorgani-

zation of cells under the force of gravity. We show that the production of 3D organotypic cultures of various hepatic cell lines and primary liver cells is possible. The hepatic cells in these 3D cultures were analyzed for viability and functionality (Mueller et al., 2011). We show that the organotypic cultures maintain high liver-specific function over 3 weeks of culture. The production rates of albumin and glucose as well as CYP2E1 activity were higher in the 3D versus the 2D cultures. The IHC analyses illustrate that the organotypic cultures express liver-specific markers such as albumin, CYP3A4 and CYP2E1 throughout the cultivation period. The effects of several compounds (acetaminophen, valproic acid, chlorpromazine, troglitazone and rosiglitazone) were monitored and compared. Acute toxicity studies reveal that the organotypic cultures are more sensitive to acetaminophen toxicity than the 2D cultures (Gunness et al., 2013). This high-throughput *in vitro* technology is suitable for drug screenings using cell lines as well as primary cells.

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Qualification of neural differentiation protocols and points to consider when transferring standardized protocols between laboratories

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Qualification of neural differentiation protocols and points to consider when transferring standardized protocols between laboratories.

The ESNATS project has made huge strides in developing novel toxicity assays based on human embryonic stem cells as a powerful alternative to animal testing. There is a current need to reduce and replace animal models with alternative *in vitro* methods, to accelerate drug development and testing. The UK Stem Cell Bank (UKSCB) provides quality controlled stocks of human embryonic stem cells and can advise on stem cell culture, characterisation, cryo-preservation, safety testing and standardisation.

As part of the UKSCB involvement in the ESNATS project, the UKSCB was tasked with the qualification of two of ESNATS differentiation protocols, one assay based on multi-lineage differentiation (UKK) and the second based on developmental toxicity during the generation of neuroectodermal cells (UKN1). Both these protocols can be found on the internal ESNATS website.

Any new protocol developed will have been rigorously tested in the lab of origin, but it should also be tested in a different lab to see if the assay is robust, reliable and reproducible when transferred to a new laboratory setting. Inter-operator and inter-laboratory variability is often a problem. The protocol should be carried out precisely as instructed in terms of experimental design, reagents and equipment as much as possible, but this may

not always be feasible as labs may not, for example, have the same PCR machines. Any deviation from the protocol should be noted and adjustments made to accommodate the change(s). Observational user perception can also incorporate bias.

For stem cell culture there are many standardised protocols available, but small differences in how the cells are grown, ie on feeders or matrigel, or how they are passaged; manual cutting or enzymatic, can affect the end results. Starting material should be checked for sterility and mycoplasma contamination, as well as cell identity, karyology and specific characteristic phenotypic markers.

Both protocols were successfully transferred and incorporated into the testing workflow using the protocols provided. There were some differences in the end results, and after investigation it was found that these were potentially due to minor differences in the experimental process, that could have not been foreseen when the protocol was developed. Strong communication between the groups involved was essential for this to be resolved.

Recommendations would be to tighten parameters to optimise standardisation which would help to make protocols more robust. Small details, that may seem inconsequential to the host laboratory, and which may not have been deemed necessary to include in the protocol, may be of enormous benefit to the end protocol user. Even the smallest deviation from a protocol could be the reason for a significant difference in results.

A novel, flexible method for assessing barrier tissue integrity

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The regulation of paracellular ion transport is a key property of epithelial and endothelial tissues. Measurement of changes in paracellular ion transport and more specifically transepithelial resistance provides a wealth of information about barrier tissue function and importantly is a key indicator of barrier tissue integrity, as disruption or malfunction of the structures involved in regulation of paracellular ion transport often arises due to the effects of pathogens and toxins. The Organic ElectroChemical Transistor (OECT) provides a very sensitive way to detect minute changes in ionic currents in an electrolyte, due to inherent amplification of signal provided by the transistor (Owens and Malliaras, 2010). The OECT is a conducting polymer device fabricated of organic electronic materials which combines key advantages of low cost and easy fabrication which may be designed to suit the application in hand. We show two configurations of the OECT: In the first configuration we integrate Caco-2 cells grown on filters with the OECT and demonstrate the ability to measure dynamic, minute variations in barrier tissue integrity induced by toxic compounds in real time, with unprecedented temporal resolution and extreme sensitivity (Jimison,

et al., 2012). The flexibility of the system enables us to carry out multiple simultaneous measurements in situ in an incubator. Detection of several toxic species including peroxide, ethanol (Tria, et al., 2013) and the pathogenic bacterium *S. typhimurium* was successfully demonstrated, with detection sensitivity greater or equal to existing methods. In the second configuration we grew MDCK cells directly on the devices for simultaneous electronic and optical monitoring of cell integrity, taking advantage of the transparency of the devices. We show real time evolution of electronic barrier tissue properties with concurrent videos of cell layer formation and destruction with toxic species. Current directions include development of methods for measurement of barrier tissue properties in 3D.

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Screening of a large group of medical substances and environmental pollutants in a neural crest stem-cell based functional migration assay

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The migration of neural crest cell (MINC) assay is a peer reviewed human cell-based method (Zimmer et al., 2012) able to detect the effects of chemicals on one of the key events of nervous system development, i.e. neural crest cell migration. Disturbance of the neural crest cell (NCC) migration process by toxicants is known to lead to severe malformations in different species. Several factors, including genetics and exposure to chemicals, can cause neural crest (NC) -related developmental defects.

The MINC uses human embryonic stem cell-derived NCCs to detect interferences of chemicals with the migration process; this event is analyzed by the counting of the cells able to repopulate a cell-free scratch after 48 h of exposure to the chemical.

Analysis of positive and negative controls during initial characterization of the assay suggested a good predictivity of the MINC. In contrast to most other currently available hESC-based developmental neurotoxicity (DNT) test systems, the MINC assay allowed measuring the impairment of a developmental functional process, separately from the effects of the toxicant on the cell toxicity and differentiation.

To further explore the utility of the MINC to pre-screen/prioritize potential DNT toxicants, a wider list of substances has been tested in this study. Twenty-seven compounds, comprising several modern pharmaceutical substances and environmental pollutants, have been selected in order to create a list of test compounds with wide structural heterogeneity. The selected panel contained also known DNT- positive and negative controls and substances showing general developmental toxicity.

For each compound, a reasonable testing concentration range was determined on the basis of pilot experiments and data of cytotoxicity assay in different test systems (human

embryonic kidney-293 (HEK293) cells and CGR8 murine embryonic stem cells). Moreover, physiologically-based pharmacokinetic modeling analysis, clinical monitoring data, and published epidemiological data were used to establish realistic test concentrations.

For each chemical, the MINC assay allowed the determination of the lowest observed adverse effect level (LOAEL) in absence of cytotoxicity; compounds showing a LOAEL inside of the toxicologically-relevant testing concentration range have been classified as DNT positive in our test system, and their concentration-dependent effect has been determined by generation of concentration-response curves.

The results of this testing study showed impairment of the NCC migration after the exposure to 11 different chemicals. Some of these positive compounds, as the environmental pollutants cyproconazole (5 μ M), triadimefon (50 μ M) and PBDE-99 (20 μ M) have already been associated with developmental toxicity in *in vivo* and/or *in vitro* studies. More interestingly, others showed unexpected DNT effects, such as the drugs geldanamycin (16 nM) and IFN β (500 pM).

Thus, this study confirms the sensitivity of the MINC assay in the identification of DNT compounds and it shows its potential use for the prioritization of a wide group of substances belonging to different classes and with unknown effects on the development of the nervous system.

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Zimmer, B., Lee, G., Balmer, N. V., et al. (2012). Evaluation of developmental toxicants and signaling pathways in a functional test based on the migration of human neural crest cells. *Environ Health Perspect* 120, 1116-1122.

Uncertainty of testing methods – what do we (want to) know?

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It is important to stimulate innovation for regulatory testing methods. Scrutinizing the knowledge of (un)certainly of data from actual standard *in vivo* methods could foster the interest in new testing approaches. Since standard *in vivo* data often are used as reference data for model development, improved uncertainty accountability also would support the validation of new *in vitro* and *in silico* methods, as well as the definition of acceptance criteria for the new methods. Hazard and risk estimates, transparent for their uncertainty, could further support the 3Rs, since they may help focus additional information requirements on aspects of highest uncertainty.

Here we provide an overview on the various types of uncertainties in quantitative and qualitative terms and suggest improving this knowledge base. We also reference principle concepts on how to use uncertainty information for improved hazard characterization and development of new testing methods.

Reference

Paparella, M., Daneshian, M., Hornek-Gausterer, R., et al. (2013). Uncertainty of testing methods – what do we (want to) know? *ALTEX* 30, 131-144.



Epigenetically-conserved biomarkers of an undifferentiated human embryonic stem cell phenotype

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Human embryonic stem cells (hESCs) can undergo epigenetic changes *in vitro*, which may compromise their differentiation capacity. More recently, epigenetic differences between induced pluripotent stem cells (iPSCs) and ESCs have been identified, with unclear effects on the behaviour and potency of the cells. A minimal “epigenetic signature” of hESCs would therefore be useful as a simple way of validating new or established lines, and of confirming or disconfirming the integrity of pluripotent stem cells after long term culture *in vitro* or chemical insult.

Variable and conserved patterns of Cytosine-Guanine Island (CGI) methylation were assessed in 4 human embryonic stem cell lines (RCM1, RH1, RH3 and RH4) by hybridisation of methyl-binding domain column-purified genomic DNA to a human CGI array. These CGI methylation data were compared to corresponding patterns derived from somatic tissues (brain, muscle, spleen, blood) and to hESC mRNA expression profiles. Substantial variation was apparent in CGI methylation between hESC lines across the whole genome, notably at the X chromosome where we observed significant differences between different female hESC lines. Comparison with adult

tissues identified limited sets of CGIs with conserved hESC-specific methylation patterns. Gene Ontology analysis of these gene sets showed that expressed genes with associated CGIs that were methylated or unmethylated specifically in hESCs were significantly enriched for transcriptional repressors and activators, respectively. Small interfering RNA targeting in hESCs of three selected candidates induced cell differentiation, as assessed by reduction or loss of mRNA and immunocytochemical signal for OCT4, NANOG and SOX2, upregulation and downregulation of diverse developmental lineage markers and changes in cell morphology. Our study identifies conserved epigenetic biomarkers of hESCs whose expression contributes to the maintenance of the undifferentiated hESC phenotype. These biomarkers will likely prove to be useful in their own right as a simple means of verifying hESC lines, and also increase our understanding of the transcriptional networks controlling pluripotency and the decision-making processes occurring upon the initiation of differentiation.

Enhancing the readout of the embryonic stem cell test with molecular approaches

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After decades of research into the development and implementation of embryonic stem cells in assays for the detection of developmental toxicants, discussion is still ongoing about the actual applicability domains of these assays in terms of biological domain and chemical space covered. A single differentiation route, e.g. into cardiac myocytes or neural cells, may provide an indicator for much broader effects, given that early differentiation events are probably shared by many differentiation routes. In addition, although a single differentiated cell type may be used as readout, embryonic stem cell differentiation assays contain cell types from multiple lineages, that may be instrumental in inducing the differentiation route of focus. We have embarked on studying early molecular events in the embryonic stem cell tests, with the aim to enhance the mechanistic readout of the assay and to enable the identification of specific adverse outcome pathways affected by compound exposure. Whilst focusing on transcriptomics, this information should ideally be integrated with information about e.g. proteomics and metabolomics to provide integral assessment of molecular compound effects and enabling a computational approach towards a mechanistic analysis of findings. Such mechanistic knowledge may provide a more specific basis for the extrapolation of assay results to actual chemical hazard in man. In addition, besides studying the classical cardiac myocyte differentiation embryonic stem cell test (ESTc) (van Dartel et al., 2010), we have developed our own protocols for neural (ESTn) (Theunissen et al., 2010) and osteoblast (ESTo) (de Jong et al., 2012) differentiation lineages. We showed differential sensitivities between these assays

(Theunissen et al., 2013). The combination of various stem cell based and other assays into a battery that might increase comprehensiveness was informative when analysed using an approach of mechanistic validation, considering the detectability of compound-specific effects in mechanism-specific assays (Piersma et al., 2013). We have argued that mechanistic validation in addition provides crucial information for the optimal place of individual tests within a tiered and battery approach, based on biological relevance. It does not require large numbers of compounds tested in a statistical approach, but rather, dedicated testing of well-chosen compounds based on the biological domain of the assay should inform on the mechanistic validity, specificity and sensitivity of the assay. In such a biologically driven approach, potency information should prevail over binary scoring of compound effects. The presentation will review comparative studies of ESTc and ESTn, illustrating the informative value of molecular approaches in assessing compound effects, and addressing the complementarity and redundancy of these two test systems for the detection of developmental toxicants.

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Cell culture-based *in vitro* method for determining the activity of the botulinum toxin

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Botulinum toxin is a bacterial exotoxin. It is produced by the anaerobic spore-forming bacterium *Clostridium botulinum*. It is one of the most potent bacterial toxins known. The lethal dose for humans is about 1 ng/kg body weight. Botulinum toxin is a neurotoxin that inhibits the release of neurotransmitters from the nerve endings, which stimulate muscle contraction. Thus it leads to flaccid paralysis of the affected muscles. Botulinum toxin is used therapeutically in cosmetic medicine to suppress the formation of wrinkles by paralyzing subcutaneous skin muscles. The standard test for its biological activity is the mouse lethality assay. This assay determines the dose at which half of the treated mice die from asphyxia. The aim of the current project is to establish a cell-based assay to determine the activity of botulinum toxin on the basis of the inhibition of a stimulus-dependent release of a reporter enzyme directed into the neurotransmitter storage vesicles of nerve cell lines by botulinum toxin.

Monoclonal cell lines were established from different neuronal cell lines including SH-SY5Y, Neuro-2a and PC-12 that stably express a fusion protein of a reporter enzyme with a target sequence that directs the reporter into neuro-secretory vesicles. In these cell lines, release of the reporter concomitantly with the neurotransmitter was stimulated by depolarization with a high KCl concentration. Culture conditions were optimized to maximally increase stimulus-dependent reporter release and adapt the assay to a 96-well plate format. Under these conditions, reporter release was increased 3- to 10-fold by transmitter release buffer over the respective control. In ongoing experiments it will now be tested, whether the stimulus-dependent reporter release can be inhibited by pre-incubation of the cells with botulinum toxin and whether this inhibition occurs in a concentration range of the toxin, which makes the system suitable as an *in vitro* system for testing botulinum toxin activity in drug preparations.

Neurotoxicity *in vitro*: assessment of the predictivity of neuronal networks coped to microelectrode arrays for identification of neurotoxicants

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A challenging aspect to assure the safety of a product is the assessment of its neurotoxic hazard potential. Currently, only *in vivo* methods are regulatorily accepted and so far, no *in vitro* model has been fully validated. The majority of the test systems are reduced to the analysis of cytotoxicity in immortalized cell lines, without including unique characteristics of the nervous system, such as axonal transport, synaptic transmission or its electrophysiology. Recently, with the advance in technology and the ability to maintain neuronal models for prolonged periods, a test system emerged, combining the use of microelectrode

arrays (MEAs) and *in vitro* culture of 2D neuronal networks (NN). Herein, we report on the in-house validation of the NN MEA assay using a set of 43 compounds with known neurotoxic and non-neurotoxic potential with the aim to use it for screening of compounds under development. The results demonstrate that the method presents an accuracy >75%. In order to reduce the rate of false positives we are currently working on the combination of the electrophysiological assessment with a panel of cytotoxicity assays.



Inter-laboratory validation of the yeast estrogen and yeast androgen screens for identification of endocrine active substances

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Endocrine disruptor compounds (EDCs) are a group of natural or synthetic compounds that have the capacity to interact with the endocrine system of living organisms and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. Due to the impact that this interaction could have on human health, there is an increasing interest in assessing the risk of the exposure to EDCs. Currently, several *in vitro* and *in vivo* assays have been developed and few of them validated and regulatory accepted. For instance, the US EPA developed the Endocrine Disruptor Screening Program, which has been recently implemented. For the program a large number of experimental animals will be still use used even for testing some of the *in vitro* assays. Herein, we performed the inter-laboratory validation of two robust models that addresses agonistic and antagonist effect at the human hormone receptor,

the YES (Yeast Estrogen Screen) and the YAS (Yeast Androgen Screen). Both assays are non-animal alternatives to the estrogen and androgen receptor binding assays proposed in the EDSP and OECD Conceptual Framework. The ring trial is the final experimental part of the validation process at the European Center for Validation of Alternative Methods (ECVAM). A set of 24 compounds with estrogenic, anti-estrogenic, androgenic or anti-androgenic activity were tested in five different laboratories in a blinded fashion. The analysis of the data demonstrates a high reproducibility for both methods among the different participating laboratories. Most importantly, both assays exhibited a good predictivity. In conclusion, the methods have been successfully transferred to other laboratories and they exhibit a high accuracy to identify EDCs that interact with sex-hormone receptors.

Human platelet lysates successfully replace fetal bovine serum in adipose-derived adult stem cell culture

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Fetal bovine serum (FBS) is still the gold standard as a cell culture medium additive due to its high level of growth stimulatory factors. Although supplementation of growth media with FBS is common practice in cell and tissue culture, FBS bears a number of disadvantages and its use has been questioned due to: (1) an ill-defined medium supplement, (2) qualitative and quantitative batch-to-batch variations, and (3) animal welfare concerns regarding the harvest of bovine fetal blood.

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.

Since stem cells technologies are likely to become important for the development of human-based alternatives to animal testing, *in vitro* toxicology, drug safety assessment, and for tissue engineering, the move away from animal-based cell culture

medium supplements is necessary. Novel stem cell-based test systems are continuously established, and their performance under animal-derived component free culture conditions has to be defined in prevalidation and validation studies.

The results presented here demonstrate that human platelet lysates can adequately fulfill this task. Adipose-derived mesenchymal stem cells (ADSC) were expanded in media supplemented with platelet lysates. Proliferation assays by resazurin and WST-8 compared with direct cell counting confirmed the growth promoting effect of platelet lysate, comparable to high FBS. Furthermore, we established culture conditions that ADSC kept their undifferentiated state as proofed by CD73, CD90 and CD105 expression and the lack of negative marker CD45. Preliminary tests whether ADSC can be differentiated towards adipogenic, osteogenic, or chondrogenic phenotypes under platelet lysate supplemented growth conditions were also successful. The data will be shown in a companion presentation.



Human platelet lysates promote the differentiation potential of adipose-derived adult stem cell cultures

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Adipose tissue, derived from liposuction, is a rich source for adult human mesenchymal stem cells. The generation of this type of cell is – in contrast to human embryonic stem cells – ethically acceptable, that paved the way for a vast amount of research concerning their potential use in regenerative medicine. However, any future clinical application of adult stem cells is impeded by the use of FBS as an animal-derived growth supplement in expansion culture media, due to the possibility of introducing xenogenic molecules into human stem cells. This fact called for the search and the development of alternatives to FBS that are animal-derived component-free and safe for any therapeutic application. In addition, human stem cell cultures gained importance as innovative human-based alternative to animal testing, *in vitro* toxicology, drug testing and safety assessment. Thus, animal-derived component-free culture protocols are also mandatory for a successful application of human stem cell-based testing systems under fully humanized test conditions.

A number of studies have proven human platelet lysates (PL) as a suitable substitute in cell expansion media, including adult human stem cells. Recently, we succeeded in using PL as an alternative for FBS in the cell culture of a number of continuous human and animal cell lines. PL were prepared as cell-free extracts from activated thrombocytes after thrombapheresis.

In the present study, adipose-derived stem cells (ADSC) were used as adult stem cell model. The minimal criteria defining multipotent mesenchymal stromal cells are (1) the capacity to adhere to plastic culture surfaces, (2) the expression of specific surface antigens (e.g. CD73, CD90, CD105) as markers of the undifferentiated state, and (3) the potential of the cells to differentiate into the adipogenic, chondrogenic and osteogenic lineage. ADSC were maintained under PL and then switched to the respective differentiation media. Differentiation endpoints were assessed according to established protocols: (1) lipid droplets in adipocytes were stained by Oil Red O, (2) proteoglycans in chondrogenic spheroids were detected by Toluidine Blue, and fine structure of spheroids was monitored by scanning electron microscopy, and (3) calcium deposits in differentiated osteoblasts were stained with silver nitrate (von Kossa staining). There were no differences between FBS- and PL-grown ADSC, indicative for retention of the differentiation potential of ADSC under serum-free, animal-derived component-free culture conditions in PL-supplemented culture media. The degree of osteogenic differentiation was even more pronounced under PL compared to FBS.



Evaluation of two new recombinant Factor C based assays as alternatives for Limulus blood based endotoxin detection methods

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Two new tests for the quantitative determination of endotoxin are commercially available. EndoZyme[®] is a homogeneous test format whereas EndoLISA[®] is a solid phase based method using a lipopolysaccharide-selective, precoated microplate for sample preparation. Both methods use recombinant Factor C for the detection reaction. Factor C is the first lipopolysaccharide activated enzyme of the Limulus blood coagulation cascade. In this study, we examined these test systems regarding to sensitivity, specificity and on real life samples in comparison to

Limulus Amoebocyte Lysate (LAL) test. Both new assays cover a sensitivity range of four orders of magnitude and possess good specificities for lipopolysaccharides from different sources. Comparison of the test systems using real life samples also show comparable results. In conclusion, recombinant test systems are good alternatives for the replacement of the Limulus blood based endotoxin detection systems, thus protecting the population of the endangered horse shoe crabs.



Humane education and ethical science: Campaigning for replacement in Iran

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Campaigning against animal experiments and for alternatives in Iran has grown significantly in recent years. The Iranian Anti-Vivisection Association (IAVA) has been promoting replacement in education and training and in science. Three InterNICHE outreach visits have supported this activity, and presentations and Multimedia Exhibitions of alternatives have been held at major congresses and seminars across the country. For many teachers and students these events have provided their first exposure to alternatives, and feedback has been positive. Much activity has focused on the veterinary field, but other faculties are increasingly involved. Discussions at national level for veterinary education and nearly 80 Azad medical universities and hospitals aim to encourage a shift to alternatives. The influential University of Tehran is introducing alternatives as part of curricular reform, and a small workshop on software alternatives in physiology and pharmacology was organised here, with a national level workshop to be held soon.

Negotiations on using ethically sourced animal cadavers within anatomy teaching continue, and a small-scale body donation program promoted by IAVA at clinics has provided ethically sourced dog cadavers for specific replacement. The country's isolation and the imposed sanctions present an obstacle to the import of alternatives, but duplication of freeware provided by InterNICHE, and an InterNICHE library of alternatives, help ensure access to and availability of selected products, some of which have already brought about replacement. IAVA and InterNICHE plan to organise a national platination conference, to train teachers in a range of alternatives, and to promote the Farsi translation of a book questioning animal use in research and testing. The movement for humane education and animal protection in Iran is young and small, but it is animal rights, anti-vivisection and vegan oriented, reflecting the motivation of its members and the opportunity within education to facilitate change.

How many animals have been really used for REACH purposes: appraisal after the second deadline

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Regulation 1907/2006 for the Registration, Evaluation, Authorisation and restriction of chemicals (REACH) was implemented with the very agreeable aim: “to ensure a high level of protection of human health and the environment, including the promotion of alternative methods for assessment of hazards of substances”.

From the very beginning, the discussion about how many animals would have been sacrificed for that purposes was very alive with many different broadcasts, ranging from less than 4 million (van der Jagt et al., 2004) up to more than 54 million (Rovida and Hartung, 2009), if the current guidance for testing is actually executed.

Now the deadline for the two most demanding REACH Annexes for Phase in substances have past: Annex X on 1st December 2010 for substances manufactured or imported above 1000t/y and Annex IX on 1st June 2013 for substances manufactured or imported above 100t/y. All the submitted dossiers for registered substances are public available on the website of ECHA (<http://echa.europa.eu>).

Compared to expectations, those dossiers contain much less testing proposals than estimated. Official data for latest deadline are not yet available, but those for 2010 demonstrate the over exploitation of existing data and read-across from similar substances. Even though the validity of this approach may be questionable from both scientific and legal point of view, the final result in term of animals used for the experiments is positive.

The negative aspect of this first account concerns the low efforts that has been spent by registering companies to implement real alternative methods, i.e. *in vitro* techniques. According to ECHA reports (ECHA, 2011), beyond some tests in the areas of skin, eye irritation and genotoxicity, no *in vitro* tests have been performed for REACH purposes. According to most registrants, the main reason is the fear that the *in vitro* test will not be accepted and in the end, ECHA will request to repeat

the test *in vivo* with the idea that performing a screening test *in vivo* instead of the full test, as required by REACH, is easily justified.

Unfortunately, the ECHA database does not have an effective tool for queries and therefore analyzing the type of tests that have been applied in the dossiers is impossible. This opportunity is offered in the Global Portal to Information on Chemical Substances of the OECD (www.echemportal.org). This approach has been already pioneered in the field of Reproductive toxicity, demonstrating that after the 2010 deadline, many dossiers contain screening studies *in vivo* performed for REACH purposes (Rovida et al., 2011). In this presentation, new data extended to all endpoints of the registration dossier will be analysed and presented.

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Dialogue with EFSA to avoid useless animal testing in the area of genotoxicity

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When speaking about alternative to animal testing, the most common objection is: “That is required by regulators”. But is that really true?

Recent experience during a process for the re-authorisation of the dye Quinoline Yellow for feed purposes demonstrates that prejudice of regulators against *in vitro* methods cannot be used as an excuse.

Actually the respective event concerned the field of food, when EFSA asked an *in vivo* Comet assay by oral administration for the assessment of Patent Blue V as a food additive (6th June 2011). The reason for the request was a positive result found in a previous AMES test which raised concern about the possible genotoxic effect. In fact, the recent EFSA guidance on genotoxicity testing strategies (EFSA, 2011) asks for confirmation *in vivo* whenever there is a concern resulting from an *in vitro* test.

In order to comply with this call, Fiorio Colori, which is the only Company, which manufactures this colorant in the European Union, commissioned the test *in vivo* while simultaneously repeating the ambiguous outcome of the AMES test. As expected, both studies returned a negative result, demonstrating that probably the previous uncertain result was due to the presence of a toxic impurity. By the way, this reminds of the importance of a highly controlled manufacturing process for all food additives. As a conclusion, the confirmation test *in vivo* was useless, but had costs of about 20,000 € and caused the killing of 64 rats.

Following this experience, when a similar request arrived from EFSA on another dye, Quinoline Yellow, to be used as feed additive, Fiorio reacted and explained to EFSA the value

of repeating again a doubtful *in vitro* tests before immediately doing the assay *in vivo*. The presented argumentations included a statement extracted from the EFSA guideline saying that: “In the case of inconclusive, contradictory or equivocal results from *in vitro* testing, it may be appropriate to conduct further testing *in vitro*, either by repetition of a test already conducted, perhaps under different conditions, or by conduct of a different *in vitro* test, to try to resolve the situation”. In fact, the positive *in vitro* results derived from a study that was not performed according to basic GLP requirements and most importantly, the purity of the tested colorant was not defined. This was enough to convince EFSA to modify the request and ask the applicant to submit an *in vitro* mammalian cell micronucleus test (OECD TG 487). Indeed, the result of that test was negative.

The lesson learnt from this experience is that regulators are not deaf to the needs of the industry, but on the opposite they ask for a dialogue, with the awareness that who manufactures a substance may have the best knowledge of it. The second very important consideration is about the importance of the purity of the substances that are currently in use in our daily life. Control of the manufacturing process of the chemical substances is of utmost importance to preserve the health of consumers.

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Acute toxicity of alcohols determined by alternative methods

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Acute oral toxicity testing is required worldwide for classification and labelling of chemicals and preparations. In recent years, attempts have been made to develop and validate alternative methods for acute toxicity prediction in order to reduce the numbers of animals or totally replace the animal experiments with *in vitro* assays. It has long been appreciated that there is a significant correlation between acute systemic toxicity *in vivo* (expressed as LD₅₀ values) and the impairment of cell viability *in vitro* (cytotoxicity), which reflects the adverse effects of toxic substances on universal processes of cellular physiology and molecular biology, intrinsic to all eukaryotic cells. In April 2013, EURL ECVAM finalized the validation study to assess the predictive capacity of the 3T3 NRU *in vitro* cytotoxicity test method to identify substances not requiring classification for acute oral toxicity, based on a cut-off of LD₅₀ >2000 mg/kg b.w.

The aim of this study was to estimate the acute toxicity values of selected saturated alcohols using the 3T3 NRU test and Tubifex tubifex ecotoxicity test, and correlation of the results with values LC₅₀ (*Pimephales promelas*), IGC50 (*Tetrahymena pyriformis*) and LD₅₀ (rat, mouse) available in literature and public databases (e.g. ESIS; RightAnswer®).

The 3T3 NRU cytotoxicity test was performed according to the ICCVAM Report (2006) and OECD GD 126 (2010). The concentration which led to a 50% reduction in cell growth (IC₅₀) was calculated from the concentration-response curve and the LD₅₀ value was estimated using formula: $\log LD_{50} \text{ (mg/kg)} = 0.372 \log IC_{50} \text{ (}\mu\text{g/ml)} + 2.024$. *Tubifex tubifex* toxicity test is based on inhibition of movement of *T. tubifex* population exposed for three minutes to an aqueous solution of the test chemical. The acute toxicity index, EC₅₀ (Tt) is characterised as the effective concentration which causes inhibition of movement of 50% of the *T. tubifex* in a population (Tichy et al., 2007).

Statistical evaluation, using Origin software, revealed strong correlation between the 3T3 NRU (IC₅₀) and *T. tubifex* (EC₅₀) tests (correlation coefficient $r=0.97$) and also close correlation between 3T3 NRU (IC₅₀) and LC₅₀ for *Pimephales promelas*, IGC50 for *Tetrahymena pyriformis* and reference LD₅₀ values from i.v. acute toxicity test in mice. The available historical acute oral LD₅₀ values for rats showed extreme variability dis-

allowing proper statistical evaluation. However, if mean LD₅₀ values were employed, the correlation between 3T3 NRU (IC₅₀) and LD₅₀ in rats was considerably strong ($r=0.93$).

The study results confirmed good predictive capacity of the 3T3 NRU test for acute oral toxicity in a group of alcohols including their correct categorization and thus extending the applicability domain of 3T3 NRU test. However, results obtained by the 3T3 NRU test method should always be used in combination with other toxicological data (e.g. from QSAR, read-across, toxicokinetic data) to build confidence in the classification decision. The 3T3 NRU assay should be included in a Weight of Evidence approach or as a component of an Integrated Testing Strategy.

Acknowledgment

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Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of human embryonic stem cells

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Embryonic development is partially recapitulated under *in vitro* conditions by differentiating murine (Gaspar et al., 2012) and human embryonic stem cells (hESCs) (Meganathan et al., 2012). In this context, it is repeatedly described that Thalidomide is a developmental toxicant acting in a species-dependent manner and therefore 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 develop the UKK a developmental embryotoxicity test system (Krug et al., 2013). We validated the UKK test system model to identify potential thalidomide embryotoxicity/teratogenicity effects at pharmacological dose(s) (Meganathan et al., 2012). Transcriptomic analysis of undifferentiated and 3 to 21 days differentiated hESCs demonstrated that at day 14 of differentiation all germ layer specific cell types were formed (Meganathan et al., 2012). Accordingly, proteome analysis showed loss of POU5F1 regulatory proteins PKM2 and RBM14 and an over expression of proteins involved in neuronal development (such as PAK2, PAFAH1B2 and PAFAH1B3) after 14 days of differentiation (Meganathan et al., 2012). Applying the –Omics technologies we were able to demonstrate differential expression of limb, heart and embryonic development related transcription factors and biological processes (Meganathan et al., 2012).

In addition, this study resulted into identification of RANBP1 which was inhibited by Thalidomide and normally participate in the nucleocytoplasmic trafficking of proteins (Meganathan et al., 2012). Interestingly, also an inhibition of glutathione transferases (GSTA1, GSTA2) has been observed that specifically protect the cell from secondary oxidative stress (Meganathan et al., 2012). 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.

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A dynamic multi-organ-chip containing enterocytes and hepatocytes for the absorption of orally administered drugs prediction and substance toxicity testing

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Human-on-a-chip systems are a new and interesting way of substance testing, and are already widely used. Basic concept for human-on-a-chip systems is co-cultivating different types of cells, representing human organs, in closed environment. Combining enterocytes, hepatocytes and target-organ cells allows the examination of drug Adsorption, Distribution, Metabolism and Toxicity of oral administrative drugs *in vitro*.

Good bioavailability makes the absorption of drugs via the oral route a subject of intense and continuous investigation in the pharmaceutical industry.

The Caco-2 cell line represents absorptive polarized intestinal epithelial cells that are used as a confluent monolayer

on a cell culture insert filter for *in vitro* tests. HepaRG cell line is terminally differentiated hepatic cells derived from a human hepatic progenitor cell line that is most commonly used for *in vitro* toxicity assays because it retains many characteristics of primary human hepatocytes and provides reproducible results in a metabolically complete and scalable system.

The aim of the present investigation is development of a multi-organ-chip, containing Caco2 monolayer and HepaRG spheroids as a new tool for the absorption of orally administered drugs prediction and substance toxicity testing.



Ethical review of projects involving non-human primates funded under the European Union's 7th Research Framework Programme

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In the European Union (EU), year-by-year approximately 10,000 non-human primates (“primates”) are submitted to scientific procedures for a variety of different purposes. However, their cognitive capacities and complex behaviours mean that there are serious animal welfare and ethical concerns about this use. Directive 2010/63/EU on the protection of animals used for scientific purposes confirms the special status of primates and restricts the purposes for which they may be used. In the EU 7th Research Framework Programme (FP7), a specific ethical review process for applications for funding of projects involving primates has been implemented.

The present study aimed at gaining a better understanding of the use of primates in FP7-funded projects, to determine how project proposals are assessed from an ethical point of view, and to consider whether any changes are required to the ethical review process for the next research framework programme, Horizon 2020, taking into account relevant requirements of Directive 2010/63/EU.

Internet searches returned a total of 41 FP7-funded projects involving the use of primates. Of these, 31 were considered to involve invasive experiments and 10 non-invasive behavioural procedures or the use of primate tissues. For 6 further projects, it was difficult to ascertain whether primates were used, and if so, in which types of procedures. These altogether 47 projects mainly fall into three research areas: neuroscience, infectious disease research, and evolutionary biology.

The findings of the study showed that detailed information on the projects available in the public domain was limited and not

easily accessible. It was not possible to tell whether all relevant projects were identified, or to be certain of the types and severity of the primate experiments, although in some projects the level of harm was considered to be “severe”. Information was also scarce regarding the numbers of animals used, the sourcing of animals, their housing and care, application of the 3Rs within projects, or the fate of animals.

In addition to these Internet searches, project grant holders and the relevant Commission official were consulted about their experiences with the FP7 ethics review process. The overall view of the respondents was that the FP7 ethics review process was necessary, meaningful, and beneficial. However, the survey also revealed a number of concerns in regard to its efficiency, relating e.g. to transparency and communication issues, the interplay between national project authorisation and EU project evaluation, application of EU standards to primate experiments performed outside the EU, and, in some instances, unwillingness to accept the ethics review process as an integral part of project evaluation. Overall an ethics follow-up during performance of the projects and also upon their completion was recognised as a valuable tool in ensuring that ethical and animal welfare requirements were adequately addressed.

The ethics review process for EU funding under the Framework Programmes needs to ensure that for projects involving primates the specific requirements of Directive 2010/63/EU have been properly addressed with the aim of limiting and ultimately replacing the numbers of primates used and of limiting the harms inflicted upon the animals.

A human liver 3D cell model for substance testing

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Introduction

Cell culture monolayer models have widely been used to analyze physiological effects of test substances. With respect to the biological function organ characteristics are limited and less pronounced compared to the *in vivo* situation. Cells grown in a three-dimensional (3D) matrix demonstrated physiological characteristics that are closer to *In vivo*. The liver is the central organ for nutrition, metabolism, protein synthesis and detoxification. Acute liver failure is mainly caused by drug-induced toxicity. To overcome this, predictive human hepatic *in vitro* models are needed for substance testing. Here we report on an established liver 3D model to follow effects induced by compounds.

Aim: The aim of the study was to proof the concept of the established 3D liver model.

Methods

HepaRG cells were seeded into polystyrene scaffolds and cultured for one week. To differentiate, cells were treated with 1% DMSO for 28 days followed by substance treatment. HepaRG cell morphology and seeding were investigated using haematoxylin staining, formazan formation, microscopy. Cell proliferation (MTT assay) and cytotoxicity (LDH assay) were

determined over time. Liver parameters: CYP450 expression (qRT-PCR) and activity, Albumin secretion (ELISA). The effect of Acetaminophen (APAP) on the metabolic activity, ROS (dihydrorhodamine 123), mitochondrial membrane potential (JC-1 assay), Glutathione (GSH assay), and hepatotoxicity was determined.

Results

The 3D liver model has clear benefits above the corresponding 2D liver cell model and better represents the *in vivo* situation. For instance, APAP-treatment led to a significant increase of reactive oxygen species (ROS) and LDH release, breakdown of the mitochondrial membrane potential and to a decrease of reduced and total GSH, respectively. The pretreatment of the liver cell with 5 mM NAC prevented the hepatotoxic effects of APAP by reverting the above mentioned parameters.

Discussion

The 3D liver cell model is a robust and accurate system for *in vitro* test applications to study the liver function in drug metabolism, hepatotoxicity. It may contribute to a reduction of animal use.



Construction of an impedance monitoring system for cell seeding and drug screening in a 3D cell culture model

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Introduction

Three-dimensional (3D) cell culture models are upcoming in life science, pharmacology and tissue engineering. Therefore, different measurement systems are needed for cell monitoring and testing of substances for pharmacological and toxicological studies in a 3D liver cell culture model. The aim of our study was to construct a label free and non-destructive measurement system that allow us to monitor cellular changes of *in vitro* cell culture models during cell seeding and drug treatment.

Material and Methods

To construct a 3D liver cell model, HepaRG cells were seeded on porous plasma activated Polystyrene (PS) scaffolds to supply more physiological (3D) culture conditions. To measure the cell seeding behaviour during cell proliferation, cells were seeded at different concentrations into scaffolds for 48 h.

For drug testing, 1×10^6 HepaRG cells were seeded into the scaffolds and after 28 days of differentiation time, the 3D liver cell models were treated for 24 h with different concentrations of Acetaminophen (APAP) followed by impedance spectroscopy (IS). To measure impedance, we constructed two different electrodes, parallel-plate like electrodes made of brass and a needle-like structured coplanar electrode. Parallel-plate like electrodes with a few millimeters in diameter as well as needle-like structured electrodes with sub-millimeter electrode gaps are used to measure the electrical properties of biological samples in the frequency range between 3Hz and 40GHz. Measurements were done with a commercial impedance and network analyzer (Agilent 4294A and E5061B). In addition, biochemical assays were performed: Cytostaining within the scaffolds, MTT and LDH assay (drug effects).

Results and Discussion

Using the example of human hepatic 3D cell models we showed that certain frequency domains in the impedance or reflection coefficient spectra can be assigned to specific biological functions, which enables the monitoring of the cell seeding in scaffolds. The needle-like electrodes better reflected the cellular changes of the hepatic 3D cell model than the parallel plate-like electrodes. Because this electrode as representing a coplanar waveguide structure, turned out to be inappropriate, due to the non-invasive contact with cell carrier, which resulted in a strong variation of the impedance signal. In contrast, the needle-like electrodes sense mainly the small volume between the electrode tips with low invasive contact. The impedance measurement using the needle-like electrode at 73 MHz was in linear range. The reflection coefficient S11 correlated with the increasing cell number which was determined using MTT assay.

The drug monitoring demonstrated some intracellular dose-dependent APAP effects on the 3D liver cell model, indicating hepatic cell damage. The results obtained from the label-free method of the IS reflection coefficient S11 analysis were comparable with the biochemical MTT assay, which has the disadvantage of being destructive.

Conclusion

Applications of IS as label free measurement system in the 3D cell culture model are a new challenge for the accurate monitoring of drugs/diets or chemicals. In addition, this non-destructive measurement system reduces costs, time and contributes to reduction of the numbers of animal experiments in these fields.

Factors influencing regulatory acceptance and use of 3R models & strategies to improve this process: The case of the EOGRTS

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The acceptance and use of 3R models for regulatory purposes proves to be a very challenging process. Although many 3R models are available to refine, reduce or replace the conventional animal model, regulatory acceptance and use of these models falls behind. This begs the question: Which factors influence the acceptance and use of 3R methods for regulatory purposes and which possibilities are available to augment this process? The authors target this question in two ways.

Firstly, the case study of the Extended One Generation Reproduction Toxicity Study (EOGRTS) is used. About 70% of the toxicological tests in Europe are done to meet reprotoxic endpoints. The heavy reliance on animal testing in this area encounters serious ethical, scientific and economical objections which led to a series of 3R initiatives. The EOGRTS is one of these initiatives. It aims at replacing the two generation test while reducing the animal use by about 40%. In 2011 the EOGRTS was formally incorporated in the OECD guidelines. However despite this formal incorporation (FI) the actual regulatory acceptance (ARA) and use of the EOGRTS by industry (UI) are

still under heavy discussion within Europe. The authors offer an overview of the drivers and barriers that are identified at these 3 sub stages of regulatory acceptance and use. The study was conducted through a combination of literature research and a series of 20 expert interviews.

Secondly, this study analyses the findings by means of the multilevel perspective on technology transition. This model distinguishes 3 levels at which developments are found that influence acceptance and use of 3R models. The micro level where innovations are developed and validated, the meso level of the existing regulatory regime and the macro level covering the societal context. To get a 3R model like the EOGRTS accepted for regulatory purposes, developments at all three levels have to be taken into account. Recognition and understanding of this mechanism will provide regulatory authorities and industry stakeholders with a basis for practical discussions on how to further integrate scientifically sound alternatives into regulatory testing.



Mind the gap- developing and implementing integrated testing strategies into nanotoxicology

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Nanotechnology is currently one of the fastest-expanding areas, and little is known about the risks for humans and their environment. It is therefore natural that authorities (e.g. the European Food Safety Agency EFSA) are increasingly demanding a risk assessment for these materials. Given the diversity of materials and possible uses, and using the classical means of toxicity testing, this would require large numbers of experimental animals. In 2007, with the NRC report, it was stated that classical toxicology as reached a pivotal turning point. As a consequence, a paradigm change was announced, which takes into account the successful development of animal-free methods. These methods are to be combined into integrated testing strategies (ITS), using the *in vivo* experiment only as a very last resort. Currently, little is known about suitable ITS in the field of nanotoxicology.

The largest funding body in Switzerland, the Swiss National Science Foundation (SNSF) has launched a National Research Programme (NRP 64) in order to better understand the risks and opportunities of nanomaterials. In the course of this programme, approaches are developed which are suitable for nanotoxicity testing. We have identified the fields and areas where the development of ITS as “proof of concept” would be the most promising to convince the responsible authorities in Switzerland to allow for ITS as an alternative to *in vivo* toxicity testing and therefore either help to reduce animal use, or, ideally, avoid it altogether. With the assistance of the project leaders of NRP 64, we are currently identifying the *in vitro* methods that appear most suitable to be included into such testing strategies.

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New perspectives for realistic and efficient *in vitro* screening for inhalable drugs

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Inhalation therapy is widely used for treating lung diseases, but it also has significant potential for systemic drug delivery. The currently used standard *in vitro* drug screening technology is based on cells completely engulfed in cell medium (submerged culture conditions). This is physiologically unrealistic and hence expected to be less predictive for inhalation therapy, where the drug is deposited in aerosolized form from air directly onto the pulmonary epithelial cells. Several systems for direct aerosol-to-cell delivery of drugs of airborne substances have been introduced, but high requirements with respect to drug efficiency, dose accuracy and ease-of-handling have prevented wide-spread use for efficacy testing until now.

Here, we introduce an innovative Air-Liquid Interface Cell Exposure system (ALICE-CLOUD), which uses the principles of cloud dynamics for fast, efficient and physiologically realistic droplet-to-cell drug delivery (ALICE-CLOUD). We describe the design of the ALICE-CLOUD, its aerosol-to-cell transport performance and its successful application to determine the efficacy of a potentially new drug for inhalation therapy.

During a typical ALICE-CLOUD exposure experiment (duration: 3.5 min) 83.6% of the invested liquid drug (200 μ l) is deposited in aerosolized form onto a standard multi-well plate equipped with transwell inserts for culturing cells under physiologically realistic air-liquid interface (ALI) conditions. For a 6-well plate (cell covered area: 20.2% for 6-well inserts) this corresponds to a drug-to-cell delivery efficiency of 16.9%. The insert-to-insert variability and repeatability of the cell-delivered drug dose is 12.2% (95% confidence level) and 18.6% (95% CL) respectively.

As a proof-of-concept study for functional drug screening with the ALICE-CLOUD we demonstrate efficacy testing of a novel substance for inhalation therapy. Here we investigate the FDA-approved proteasome inhibitor Bortezomib – a FDA-approved proteasome inhibitor for systemic treatment of multiple myeloma – for its anti-inflammatory efficacy (IL-8 induction). An A549 reporter cell line (human epithelial-like lung cell) containing a luciferase gene under the control of the interleukin-8 (IL-8) promoter is used for monitoring anti-inflammatory effects in living cells. Upon treatment of cells with proinflammatory stimuli such as tumor necrosis factor α (TNF α) the IL-8 promoter is activated and expression of luciferase is increased, which was quantified by bioluminescence intensity. Co-treatment with Bortezomib reduced the IL-8 response depending on the applied dose. Comparative analysis with the standard submerged cell culture technique shows that the therapeutic efficacy is independent of the exposure type (ALICE-CLOUD or submerged), if the cell-delivered Bortezomib dose (not the nominal dose in the medium) is considered. This suggests that Bortezomib – and possibly also other representatives of this class of biopharmaceutics (proteasome inhibitors) is a promising candidate for inhalation therapy.

The ALICE-CLOUD is a compact aerosol-to-cell drug delivery system, which combines ease-of-handling with high drug delivery rate (ca. 0.4 μ L/cm²/min), substance-to-cell delivery efficiency (ca. 16.9%) and dosimetric accuracy (18.6%). For the first time, the ALICE-CLOUD offers the possibility of using physiologically realistic ALI cell cultures not only for *in vitro* testing of abundantly available inhalable substances (e.g. for toxicity testing), but also for expensive liquid materials such as novel drugs for inhalation therapy.



Novel laser-based identification of cancer cells and monitoring of cell-agent interactions

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Luckily increasing effort is put into evaluating alternative non-animal methods for studying diseases and testing products to save animals' lives.

Raman spectroscopy is an innovative, purely light-based and non-contact method for cell identification and observation of cell-agent interactions. As Raman works within physiological environment and does not require chemical staining or antibody-based markers the examined cells remain entirely vital and undisturbed. Raman can also look into the depth of tissue-engineered products, where common microscopic evaluation fails. Thus, Raman spectroscopy is an ideal tool for preservative identification and quality control, and may serve as biosensor to follow cellular differentiation or to monitor cell reactions to chemicals.

In this work, we use Raman spectroscopy to discriminate cancer cell lines, and to observe cell-agent interactions. Two breast cancer cell lines, one positive for HER2 and one negative were exposed to the anti-cancer drug Herceptin that will only interact with the HER2 positive cells. Raman was used to discriminate the two cell lines and to visualize their interaction with the drug. The results were compared to the standard techniques such as immune-histochemistry or in-situ-hybridization.

Secondly, Tissue-engineered neuronal models can be established from neuronal cells, neuronal stem cells (ENT) or from glioblastoma cells (GB). For our studies neuronal cells and glioblastoma cells were cultured separately on a semi-permeable membrane. After several days of culturing, the tissue was fixed and cut into 10µm slices. The embedding medium was removed and the slices were rehydrated for Raman measurements. ENT is composed of neuronal cells and/or neuronal stem cells. Glioblastoma cells were cultured in a further model. Analysis of spectra of neuronal and glioblastoma cells shows significant differences as visualized in the PCA plot. The two cell populations cluster in clearly distinct regions/areas. Training of a "Support Vector Machine" (SVM) enabled to identify the GB cells within a mixed population of unknown composition.

Raman spectroscopy is an exciting new tool for non-destructive cell analysis and drug reaction survey. The technology provides valuable information about the metabolome of individual cells and enables discrimination of cell types, differentiation states and fate of cells or tissue. The purely laser light-based method is reliable and efficient for cell and tissue characterization or quality control, especially when standard methods lack the ability for safe identification and monitoring. Thus, Raman spectroscopy can enrich nearly all fields of cancer and stem cell research and will greatly support future medical diagnosis and therapy.

The DNT-EST: a predictive embryonic stem cell test for developmental neurotoxicity testing *in vitro*

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Increasing public concern about the potential adversity of chemicals, environmental pollutants and pharmaceuticals has spurred renewed efforts to assess deleterious effects on the developing nervous system. Developmental neurotoxicity (DNT) testing has thus become an important component in toxicological testing strategies. In response to the growing needs for toxicity testing establishing alternative methods enabling a higher throughput became a main goal in DNT test development.

Recently, we developed a new *in vitro* assay using mouse embryonic stem cells (mESC) to predict adverse effects of chemicals and other compounds on neural development – the so-called DNT-EST (Visan et al., 2012). After treatment of neurally differentiating stem cells for 48 h or 72 h at two key developmental stages, endpoints for neural differentiation, viability and proliferation were assessed. As a reference we treated undifferentiated stem cells 2 days after plating for 48 h or 72 h in parallel to the differentiating stem cells, also measuring viability and proliferation.

Here, we show that chemical testing of a training set comprising nine substances (six substances of known developmental toxicity and three without specific developmental neurotoxicity) enabled a mathematical prediction model to be formulated that can discriminate positive from negative DNT compounds with an *in vivo/in vitro* concordance of 100%. Thus, the mESC model introduced might represent a useful tool for assessing adverse health effects of exogenous agents that affect brain development.

Reference

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Pre-validation of the *ex vivo* model PCLS for the prediction of acute inhalation toxicity

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Introduction

In acute inhalation toxicity studies, animals inhale substances at given concentrations. Without additional information, it is difficult to estimate the appropriate stating concentration for *in vivo* inhalation studies. In the context of REACH and the principle of 3Rs, there is an increasing public demand for alternative methods. The goal of this BMBF-funded project was the standardization and pre-validation of precision cut lung slices (PCLS) as a suitable *ex vivo* alternative approach to reduce animal numbers of inhalation toxicology. The project was conducted in three independent laboratories (Fraunhofer ITEM, BASF SE, RWTH Aachen). BfR is currently providing support in biostatistics.

Methods

In all participating laboratories, PCLS were prepared freshly and exposed to 5 concentrations of 20 industrial chemicals in serum-free DMEM under standard submerged culture conditions for 1 hour. After 23 hours post-incubation with serum-free DMEM, toxicity was assessed by the release of lactate dehydrogenase (LDH assay) and by mitochondrial activity (WST-1 assay). In addition, protein content and pro-inflammatory cytokine IL-1 α were measured by BCA assay and ELISA, respectively. For

all endpoints a sigmoid dose-response model was fitted to the data and EC50 values were calculated. For each endpoint test acceptance criteria were established.

Results

This study shows the final results for all 20 chemicals. More than 900 dose-response curves have been fitted and analysed. Log₁₀[EC50 (μ M)] obtained for all assay endpoints showed best inter-laboratory consistency for the data obtained by WST-1 and BCA assays. While WST-1 and LDH indicated toxic effects for majority of the substances, none of the substances induced a significant increase in IL-1 α . The reproducibility within the participating laboratories appeared to have acceptably low between-lab variations for WST-1 and BCA assay. The results show (i) that the test protocol used in this study is adequately transferable for practical use and (ii) that the tissue model using the WST-1 endpoint is reliable.

Conclusion

The standardization and reproducibility of PCLS as an acute toxicity model was successfully established with a small training data set and verified by 20 different chemicals. Binary prediction model showed promising results.

Alterations of canalicular and basolateral transporters by cyclosporin A in human HepaRG[®] cells

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Many drugs are able to induce cholestasis in humans; however, the mechanisms remain poorly understood. We have used the human HepaRG[®] hepatocytes to investigate the effects of cyclosporine A (CsA) on the influx and efflux of bile acids and the mechanisms involved in the observed changes. In addition, a comparison has been made with tacrolimus, another immunosuppressive agent reported to be more potent than CsA and to cause only rare cases of cholestasis.

The earliest effect was observed on efflux activity. Indeed as early as 15min after addition of 10 μ M CsA efflux of taurocholate was inhibited with a maximum after 2h. The inhibition was totally reversible after 4h. However, it became irreversible with higher CsA concentrations (> 50 μ M). At low concentrations the involvement of the protein kinase C-p38 Ca⁺⁺-dependent pathway in the occurrence of cholestatic effects was demonstrated by the use of specific inhibitors and phosphorylation of p38 that was evidenced after a 5min treatment. At high concentrations (>50 μ M) pericanalicular distribution of F-actin was altered and related to constriction of bile canaliculi as shown by time-lapse analysis.

Later on influx of taurocholate was also found to be inhibited and therefore could be the consequence of early reduction of its efflux. Indeed, at the low concentrations (<10 μ M) CsA also reversibly inhibited Na⁺-dependent influx of taurocholate, with a maximum after 2h and a complete reversion after 4h. No reversion was observed with concentrations >50 μ M.

Comparatively, tacrolimus had no cholestatic or cytotoxic effect at low concentration (<10 μ M) whereas a lower inhibition than with CsA was detected à 50 μ M (45% versus 88%, respectively).

In summary, our results bring new information on the mechanisms involved in the induction of intra-hepatic cholestasis by CsA and the absence of effects of tacrolimus, at low concentrations, agrees with clinical data.



EPAA calls for a “stem cells in safety testing” forum to keep fluent communication

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The European Partnership for Alternative Approaches to Animal Testing, one of the leading European PPP promoting alternative approaches has been focusing a large part of its Science activities on Stem Cells. Indeed, in October 2011 the EPAA partners concluded that improved communication between different groups working in stem cell research was highly needed to facilitate incorporation of stem cells in future safety testing strategies. A “Communication Sub-Group” (CSG) of the Science Stem Cell team was created to establish a permanent non-commercial “International Stem Cell Forum”. This Subgroup aims identifying research groups using stem cells for safety testing, exploring stem cells types used and their applications, while providing opportunities for knowledge sharing and exchange. Ultimate goal is establishing a dialogue platform incorporating most groups for a unified approach for safety testing.

A workshop with research groups, industry and regulators was held in April 2013. Presentations showed that both human embryonic stem cells and induced pluripotent stem cells (iPS cells) are being studied by regulators, academia, and industry (including SMEs). Work developed involves, i) development of *in vitro* toxicity assays linked to key events in cellular toxicity pathways, ii) development and optimization of protocols to improve yield, functionality and scalability for production of hepatocyte-like cells, followed by test functionality and scale up, iii) development and optimization of protocols for iPS cells and differentiation in eg hepatocytes, cardiomyocytes, renal, brain, pancreatic, from patients or healthy subjects, to be used for preclinical testing iv) development and optimization of me-

dium to high throughput screens for early predictive toxicology to reduce risk in early clinical development which can be scaled up, automated and integrated into screening platforms. Experience of using different cell types, protocols, cell origins was acknowledged and need for early, continuous regulatory input recognized. Regulators, strongly supportive, highlighted requirements needed for regulatory acceptance of those approaches eg i) methods robustness demonstration and inter-laboratory technical reliability ii) direct usability for human risk assessment at least equally efficiently to current (animal-based) approaches iii) high human translatability iv) robust understanding of false positives and negatives v) use under scientifically sound human risk assessment.

In view of diverse experiences, knowledge, approaches, techniques, CSG representatives called for reinforced collaboration through a dedicated platform, identifying the need for an “established stakeholder” on stem cells. The platform will focus on safety testing rather than more generic “toxicology” or pharmacology. Hence, a permanent “Stem Cells in Safety Testing Forum” was created. Issues identified as priorities for the Forum were:

- i) assay protocols standardization
- ii) choice of test compounds,
- iii) maturity of cell phenotype,
- iv) defining criteria for novel models validation and acceptance
- v) epigenetics.

A follow-up workshop is being planned for the end of the year

Next generation sequencing (NGS) approaches to detect fixed point mutations in human airway epithelia

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Background

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 bronchial epithelium containing basal cells, epithelial cells with cilia in motion and goblet cells producing surfactant.

Objectives

This system is analyzed 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.

Methods: the tumour initiator MNNG producing point mutations as a consequence of O6-alkyl-guanine adducts and TPA as tumour promoter are used to characterize the test system. The treatments were conducted as harsh as possible in order to increase the probability to gain fixed point mutations. Analysis of TEER, LDH and histone phosphorylation was conducted to prove survival.

Next Generation Sequencing (NGS) is performed using the Ion Torrent PGM platform. We analyzed hotspot mutation sites in 40 well known cancer associated genes and sequenced a total region of 22.027 bp with a sequencing depth of 500-2000x. Amplicon libraries were prepared in a highly multiplexed PCR reaction using the Ion AmpliSeq™ Cancer Hotspot Panel v2 primer pool (Life Technologies) and the Ion Ampliseq Library 2.0 kit (Life Technologies). Libraries were bar-coded and sequenced on an Ion Torrent PGM system with a read length of 200 nt using the Ion OneTouch™ 200 Template Kit v2 (Life

Technologies) and the Ion PGM™ Sequencing 200 Kit v2 (Life Technologies). Sequencing runs were designed to give an estimated read depth of 2000 for each amplicon. On average we generated ~700.000 reads per treated sample and ~330.000 reads per control sample with a mean length of ~130bp. Read Quality is calculated as phred scaled probability of incorrect base call ($-10 \log_{10}$ Probability {base call is wrong}) while mapping quality is calculated as phred scaled probability that the mapping position is wrong ($-10 \log_{10}$ Probability {mapping position is wrong}).

Results

Within the analyzed amplicons no statistically significant sequence nucleotide variation (SNV) could be identified. The question is whether detection of SNVs could be expected taking into account that we analyzed 22 kb compared to 3.3 gb of the human reference genome hg19. Further mutagenisation and selection of potentially altered cells in prolonged cell culture could increase the probability to find significant SNVs.

We found a decreased mean mapping quality comparing treated and untreated epithelia. This reflects higher randomly distributed SNVs due to treatment with carcinogens. The mean read quality might negatively influence this effect.

Deeper bioinformatics and statistical analysis of single reads might be reasonable to follow specific MNNG induced SNVs of known positions (i.e. G=>A). That would allow to find out whether specific SNV-changes are due to MNNG treatment, that cannot be recovered by routinely used calculation-algorithms like the „Variant-Caller“.



Willi Halle Memorial Lecture: Today Willi Halle would endorse the concept "Toxicity Testing in the 21st Century"

H. Spielmann

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Willi Halle, one of the pioneers of *in vitro* toxicology, passed away at the age of 86 after a long period of suffering on May 26, 2013, in Berlin (Germany). During the "cold war" he worked "in isolation" at the "Inst. For Drug Research" of the East German Academy of Science but he was not allowed to contact scientists from the West for about 40 years. He always said that his real life as scientist started after the Berlin Wall fell in 1989, when he had reached retirement age.

Willi Halle's major contribution to the field of *in vitro* toxicology is his vision that cytotoxicity will be the basis of modern toxicology and allow predicting systemic toxicity from cytotoxicity data. In 1987 he published an article in the journal "Pharmazie" entitled "Prediction of LD-50 values from cell culture data" (original "Vorhersage von LD-50 Werten mit der Zellkultur"). To promote this concept he established the "Register der Zytotoxizität" ("Registry of Cytotoxicity" = RC) again in German in 1987 and in 2003 in English (ATLA, 2003) "The Registry of Cytotoxicity: Toxicity Testing in Cell Cultures to Predict Acute Toxicity (LD50) and to Reduce Testing in Animals".

Willi Halle's RC is a major fundament of the OECD Guidance Document (GD) No. 129 "On using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests" published in 2010.

The RC is also the basis of the "ECVAM EURL Recommendation dated April 2013 "on the 3T3 Neutral Red Uptake Cytotoxicity Assay for Acute Oral Toxicity Testing".

In East Germany Willi Halle was founder and president of the "Society for Cell and Tissue Culture" (Gesellschaft für Zell- und Gewebezüchtung) even before a similar society had

been established in West Germany. Immediately after the Berlin Wall fell in 1989, Willi visited ZEBET in West Berlin and Bjorn Ekwall in Sweden. Bjorn and Willi were good friends and co-authors of publications on "basic cytotoxicity".

Upon Bjorn's initiative in 1999 Willi Halle became honorary member of the Scandinavian Society of Cellular Toxicology SCCT and in 2001 he was awarded the honorary membership of MEGAT/EUSAAT.

Due to Willi's concept of predicting systemic toxicity from cytotoxicity data, he wholeheartedly supported the concept and vision "Toxicity Testing in the 21st Century" published in 2007 by the US Academy of Sciences.

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REACH – Who is responsible for ensuring that animal testing is conducted only as a last resort?

G. Stoddart

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The Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)¹ is the largest animal testing programme in the world and it has been estimated that between 13 and 54 million animals could be used to test the safety of an estimated 30,000 chemicals for REACH between 2009 and 2018². However, the REACH Regulation contains many mechanisms to reduce the numbers of animals used to fulfil information requirements. Although the legal text contains explicit requirements for animal tests, it also requires that vertebrate testing be conducted only as a “last resort”. The effectiveness of this provision in ensuring the use of non-animal methods wherever possible requires both commitment from registrants and effective monitoring in dossier evaluation by the European Chemicals Agency (ECHA)³.

ECHA is responsible for “policing” the REACH regulation, and in particular evaluating dossier compliance with REACH requirements. ECHA has made it clear that it believes the responsibility for maintaining the “last resort” principles lies with the registrant and the member states, a position which the PETA International Science Consortium (PISC) refutes. PISC strongly believes that ECHA is the only organisation that can practically take on this responsibility and must play a critical role in ensuring animals are used only as a last resort.

Mounting evidence indicates that registrants are not using animal tests only as a last resort. The Article 117 report⁴ on “The Use of Alternatives to Testing on Animals for the REACH Regulation” indicates that tens of thousands of animals may have been used in avoidable tests⁵. Worryingly, ECHA has not adequately investigated these apparent breaches⁶. Further, the agency charged with policing the REACH regulation appears to be requesting animal tests that are in breach of the last resort principles. A number of appeal cases^{8,9} strongly suggest that ECHA is taking an extremely conservative approach to the use of read-across, a mechanism by which the largest numbers of animal tests can potentially be avoided (Bishop et al., 2012)¹⁰.

Another concern is ECHA’s apparent misinterpretation of the REACH Regulation. For example, in its response to an appeal of an ECHA decision by the appellant Honeywell⁷ ECHA claimed that Article 25 is limited to the part of the REACH Regulation entitled “data sharing and avoidance of unnecessary testing”⁷; a claim rejected by the Board of Appeal. This decision puts the onus of ensuring that animal testing is only conducted as a last resort very much on ECHA (in addition to the registrant and Member State Competent Authorities).

PISC submitted a complaint to the European Ombudsman in 2012 to determine if ECHA is avoiding its responsibilities and not adequately investigating breaches of the last resort principles¹¹. In addition to discussing the outcome of this investigation (if available) various appeal cases will be discussed. PISC believes this evidence indicates that ECHA should conduct an internal review and make the necessary changes to meet its obligations under the law, and above all take responsibility for ensuring animals really are used only as a last resort.

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Development of a microfluidic biochip for chronic monitoring of 3D neural tissues derived from human embryonic stem cells

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“Cell biochips” containing engineered tissue interconnected by a microfluidic network, allows the control of microfluidic flows for dynamic cultures, by continuous feeding of nutrients to cultured cells and waste removal. Thus, these types of systems can enhance functionality of cells by mimicking the tissue architecture complexities when compared to *in vitro* analysis but at the same time present a more rapid and simple process when compared to *in vivo* testing procedures.

Embryonic or adult stem cells have demonstrated the potential to self-renew and differentiate into a wide range of tissues including neurons, hepatocytes, cardiomyocytes, and cells of the intestinal lineage, depending on the culture conditions. The precise microenvironments required for optimal expansion or differentiation of stem cells are only beginning to emerge now, and the controlled differentiation of embryonic stem cells based on tissue engineering remains a relatively unexplored field.

We have developed a small-volume *in vitro* system in which 3D neural tissues derived from embryonic stem cells are placed within up to four micro-chambers connected by micro-channels.

Multi-electrode arrays (M.E.A.) were designed onto the porous membranes to record and stimulate electrophysiological activities from 3D neural tissues. A dedicated perfusion system based on air pressure was used to allow the circulation of the culture medium to the different micro-organs through a microfluidic system.

This human biochip will enable the determination of toxicological profiles of new drug candidates. In addition, this chip should provide insight long-term exposure to pharmacological compounds, a capability which has not previously been demonstrated using previous *in vitro* systems. This system will thus be a more predictive tool in experimental pharmaceutical screening for efficacy and toxicity.

Acknowledgment

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Verification of systems biology research in the age of collaborative competition

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This work resulted from a scientific collaboration between Philip Morris International (PMI) and IBM's Thomas J. Watson Research Center on a project funded by PMI

The success of systems biology in academic and industrial settings hinges on the proper handling and analysis of the volumes of high-throughput data currently being generated. Research entities, such as companies and academic consortia, often conduct large, multi-year scientific studies that entail the collection and study of thousands of individual experiments, regularly over many physical sites and with internal and outsourced components. To extract maximum value, it is critical to verify the accuracy and reproducibility of data and computational methods before the initiation of such large, multi-year studies. However, systematic and well-established verification procedures do not exist for many of the automated collection and analysis workflows in systems biology, which could lead to inaccurate conclusions.

Industrial Methodology for PROcess VERification in Research (IMPROVER) was designed as a methodology to validate industrial research processes related to systems biology by deconstructing an industrial research workflow into individual components, termed building blocks that can be independently verified. As a first initiative of the IMPROVER project, in 2012, the Diagnostic Signature Challenge (DSC) was designed with the goal to assess and verify computational approaches that classify clinical samples based on transcriptomics data from 4 disease areas (Psoriasis, Multiple Sclerosis, Chronic Obstructive Pulmonary Disease and Lung Cancer).

The second initiative, the Species Translation Challenge (STC) was designed in 2013 to address whether or not biological events observed in rodents are “translatable” to humans.

In the next phase we will provide the community with network models of molecular events contributing to the onset of early Chronic Obstructive Pulmonary Disease (COPD). These models of key biological processes include access to underlying scientific literature citations that have been expertly curated to provide mechanistic substantiation for each molecular relationship present in the network model. We will sponsor two challenges using innovative crowdsourcing approaches to facilitate biomarker discovery for COPD while leveraging the computational approaches developed in the first challenge and the translational aspects developed during the second challenge.

Biological network perturbations play a fundamental role in today's systems-based biology, pharmacology, and toxicology. These network models may consist of qualitative causal relationships between biological entities to represent current scientific knowledge. The purpose of the Network Verification Challenge is to engage the scientific community in the review of the relationships between molecular entities and to make improvements on the represented biology covering fundamental processes involved in respiratory disease.

At completion, IMPROVER expects to provide an accelerated mechanism for the dissemination

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The ADAPT principles for regulatory authorities

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Animal protection organisations can have a unique perspective on the regulatory hurdles to the acceptance of alternative methods because they are observer stakeholders in many aspects of the regulatory process. The European Coalition to End Animal Experiments (ECEAE) is an umbrella organisation of 27 animal protection groups across Europe that campaigns on animal testing issues. The ECEAE is a key stakeholder at the European Chemicals Agency (ECHA), which decides on new animal tests and we have witnessed first-hand some of the barriers to the use of alternative methods put in place by the regulatory system.

Here we present our recommendations for considerations for regulatory authorities (RAs) to facilitate the effective implementation of alternative methods. The acronym ADAPT (Assessment, Decision, Acceptance, Policing, Transparency) can be used to identify those areas for which clear policies are needed.

Assessment—Does the RA have a proactive mandate to assess new methods and approaches for their regulatory regime? Lack of clarity about who has responsibility to consider new approaches, particularly if they are not like-for-like replacement methods can prevent them progressing beyond validation.

Decision—who takes responsibility for ultimately deciding whether an alternative method is suitable for the RA? Even assuming a method is validated according to international principles, it is not always clear the point at which a country or region can (or should) accept it. The approach in the EU is to wait for

OECD acceptance before deciding about suitability for the EU. This is unnecessarily laborious, we believe, particularly if the method is a European one.

Acceptance—assuming the RA wants to accept the method, are there legal hurdles in the way to final acceptance? Does legislation have to be revised and, if so, could it be more than one? The precise wording in the Annexes to REACH is being used as a reason why the Extended One Generation Reproductive Toxicity Study cannot yet be used. The inability of some validated methods to (sub)classify (according to CLP or non-EU regulatory schemes such as the EPA, which does not accept GHS) also prevents their use for regulatory purposes.

Policing—do all relevant RAs have appropriate mechanisms in place to monitor the use of alternatives in regulatory submissions and will appropriate action be taken if animal tests are done unnecessarily? RAs in our experience claim to consider animal welfare and alternatives but rarely have monitoring procedures in place. ECHA for example found 107 cases of “pre-emptive” testing in their report on REACH but said they did not have the capacity or mandate to investigate further.

Transparency—does the RA promptly inform its customers that they will now accept an alternative method, do they make it clear that it is no longer acceptable to conduct the animal test? Typically messages about suitable alternatives when they have passed all these hurdles are slow to be made or are hidden in reports. RAs need to be aware that they have a responsibility to promote alternatives that they will now accept.

Review of REACH from an animal protection perspective

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The European Coalition to End Animal Experiments (ECEAE) has been the leading animal protection organisation monitoring the implementation of the REACH legislation. We have been a key stakeholder; actively commenting on proposals to test on animals and following, wherever possible, the decisions on these made by the European Chemicals Agency, ECHA. However, the ECEAE has experienced many problems related to animal testing and we are disappointed to see that the Commission's REACH review (published in March 2013) appears not to have gone far enough to acknowledge these issues.

In this presentation we summarise our key findings during the first four years of the REACH process. We have criticisms of the Agency, the Commission, Member States and the industry in upholding one of the central goals of the legislation, "the promotion of alternative methods" with the requirement that "animal testing is a last resort".

The Agency has taken a narrow and we believe unlawful view of its role in terms of preventing unnecessary animal testing and is failing to reject testing proposals in all but very limited circumstances. They are failing to promote alternative methods and are taking an increasingly conservative tick-box approach in requesting additional animal testing.

The Commission is funding alternative methods through its support of ECVAM, Framework projects (such as ALXR8, SEURAT and OSIRIS) and the EPAA, with a budget that appears to far exceed that of overall national budgets. However,

the Commission has failed to take swift and decisive action to promote the use of alternatives (even those it has developed and validated) to ensure that they are used as soon as they are validated, such as the Extended One Generation Reproductive Toxicity Study or the Reconstituted Human Epidermis skin irritation models.

Member States on the whole have failed to make an investment in promoting alternative methods for REACH or in providing expertise to assist the Agency in its decision making. The total reported investment in 2010 in alternatives was just over 8 million Euros across all 27 European countries (see Article 117(1) report). Member States have shown little interest in enforcing REACH and Directive 2010/63, which states that an animal test must not be conducted if a scientifically appropriate alternative method is available.

It is early days but the Industry appears to have avoided animal testing to a maximum degree for the first deadline. Only 2% of the tests used in 2010 registrations were newly-performed animal tests (see Article 117(3) report). However, 37% of these new animal tests were apparently unnecessary – either conducted pre-emptively – without submitting a testing proposal – or in the presence of accepted alternatives. Industry groups need to invest in greater promotion of alternative approaches and ensure that for the 2013 and 2018 deadlines smaller companies are aware of their obligation to use alternatives to avoid animal testing wherever possible.



A “low toxicity profile” can waive the 90-day repeated dose test for REACH

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Demonstrating redundancy of a particular animal test within a package of tests conducted for regulatory purposes is an important way to reduce animal testing. It has been used most frequently in the pharmaceutical sector, where some acute toxicity and quality control tests have been made obsolete. The chemicals sector is less familiar with this approach but the demands of REACH will encourage greater efficiency in testing. In 2011 the UK Competent Authority suggested to the European Chemicals Agency that substances showing low toxicity in the 28-day repeated dose test would also show low toxicity in the 90-day repeated dose test. Through an analysis of the NONS database they showed that for all 17 substances with a NOEL equal to or greater than 1,000 mg/kg bw/day, the 90-day test also gave a NOEL of equal to or greater than 1,000 mg/kg bw/day.

In an attempt to increase the dataset upon which this hypothesis was formed, the BUAV conducted an analysis using the ECHA CHEM database in 2012. We found 182 substances with experimental results for both the 28 and 90 day in rodents via the oral route. Out of these only 26 had a NOEL reported to be equal to or greater than 1,000 mg/kg bw/day. Two substances were reported to be skin sensitizers and five were reported to be eye or skin irritants. For the remaining 19 substances, the NOEL in the 90 day was also close to or greater than 1,000 mg/kg bw/day. The pattern actually held for the five substances

reported to be skin or eye irritants so irritation is probably not indicative of systemic toxicity and can be ignored.

The results of both datasets now provide strong evidence that if a substance fulfils the following criteria then the conduct of the 90-day test is not useful and therefore should not be performed: A 28-day result for the substance in the rat from an OECD equivalent study from 1981 onwards, rated with a Klimisch score of 1 or 2, which indicates a NOEL of equal to or greater than 1,000 mg/kg bw/day AND the substance does not meet the criteria for classification as a skin sensitizer, for acute toxicity or for genotoxicity. This profile must be demonstrated with appropriate test data.

Substances with this so-called “low toxicity profile in a high quality dataset” are currently of low prevalence among industrial chemicals (10-15%). However, non-conduct of the 90-day test for 15% of the 2013 deadline substances would spare nearly 50,000 animals and save industry 50 million euros. It is important therefore that the industry evaluates whether their substances can fit this profile and therefore whether they can use the weight of evidence (Annex XI) approach to waive the 90-day test. In the meantime it would be helpful if other bodies including ECHA could explore other databases to see if the “low toxicity in a high quality dataset” rule continues to apply.

The harm:benefit assessment under the new Directive 2010/63 – will any projects be rejected?

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Directive 2010/63/EC is an update of the rules regarding the use of animals in experiments from the previous Directive (86/609/EEC) and came into force across 27 Member States in January 2013. One of the main changes is the requirement that all projects have to receive approval by the competent authority, which is to be dependent on a positive harm:benefit analysis (HBA). The aim of the HBA is ...“to assess whether the harm to the animals in terms of suffering, pain and distress is justified by the expected outcome taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment” (Article 38 (2) (d)).

Project evaluation is new for many countries and is only done in a rudimentary basis by others. Given this limited expertise it is not clear how the HBA will be performed in practice. For example, the expert working group set up by the European Commission to provide guidance on this issue struggled to find any experiments that could fail the HBA. There is a concern that the HBA will focus on the 3Rs and not the fundamental question of whether the project is justified according to Article 38.

Here we offer considerations that should be made for the HBA and ask the question, “will any projects fail?”

What is the purpose of the HBA? – Is it to consider the 3Rs and to assess if the harms can be reduced (reduction and refinement) or the experiment replaced? Or is it to reject outright the conduct of some of the experiments in the project

on the grounds that 1. The harms cannot be reduced below a satisfactory level 2. The benefits are not great enough? Should the pass/fail element be conducted after the 3Rs aspect or at the same time?

What criteria should be applied to the HBA? – can the criteria be defined, quantified and relative weights be applied? If criteria cannot be defined or quantified then is the HBA purely subjective? If it is subjective, how can consistency and balance within and between authorities be ensured? Are there some harms that are independent of benefit (i.e. will never be justified); are there some benefits that will always be justified? Should the whole project be assessed or the individual experiments within it?

Who should conduct the HBA? – Should different types/ numbers of people evaluate the 3Rs aspect and the pass/fail element? What are the pros and cons for single expert versus committee decisions? Should those with only utilitarian beliefs (the philosophical basis of the HBA) be involved? Would other perspectives help give balance, be redundant or skew the results? If the fundamental view of the committee is that most harms are justified, will any projects be rejected?

What could be learned from human ethics committees? – What types of people are involved in these committees? Are there harms that will never be justified? Do they assess benefit or just ensure the harms are never above a defined threshold? How are decisions made, by consensus or vote?



Humanizing toxicity testing in the 21st century: who should be responsible for introduction of human biology-based tests into regulatory process?

K Tsaioun

Safer Medicines Trust, Orleans, USA

Every organization involved in safety testing of new chemicals agrees that the current “tried and true” system of toxicity testing, is far from being predictive of human outcomes. Though current paradigm has been “tried” for decades, it cannot be called “true” to human outcomes, when it misses 94% of human toxicities. So what are we as industry, charities and foundations are doing about it? We will review the roles of all stakeholders in validation and adoption of alternative methods: industry, regulatory authorities, alternative technology inventors (academic and industry) and independent, government and non-profit organizations. Paths to validation and acceptance of alternative methods in different industries will be discussed. Positive and negative influence of government initiatives will be discussed using examples of cosmetic industry 2013 ban on use of animal testing and REACH directive. A new paradigm for faster validation and industry adoption of alternative methods will be presented with

a consortium of independent non-profit organizations managing the process. Safer Medicines Trust is a non-profit organization with headquarters in the UK whose mission is to ensure patient safety via including human biology-based methods into drugs’ regulatory approval process. Safer Medicines is facilitating the dialog between academia, industry and regulatory authorities by forming cross-organizations consortia that conduct activities aimed at validation and qualification of new human biology-based technologies in order to speed up the process of their adoption in the industries, their acceptance by regulatory agencies and improving human subjects’ safety in clinical trials and in the market.

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Cerium oxide nanoparticles air exposure: a comparison study using a human 3D airway model, A549 and BEAS-2B cell lines

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Human 3D airway models are fully differentiated and functional models of the respiratory epithelium (including metabolic activity, mucus production and cilia beating) and therefore may be of added value in the safety evaluation of nanoparticles entering the airways. They are cultured at an air-liquid interface (ALI), allowing relevant exposure via air. It is anticipated that these models may predict a more realistic exposure of inhaled compounds when compared to the use of cell lines. To investigate the respiratory effects of nanoparticles, we exposed the MucilAir human 3D bronchial model (Epithelix Sàrl) and cell lines BEAS-2B and A549 to nano- and micro-sized cerium oxide at ALI conditions *in vitro* cell exposure models.

The test atmosphere was generated by aerosolizing the CeO₂ material. The actual concentration of the test material in the buffer chamber was measured by gravimetric analysis and particle size was measured by a scanning mobility particle sizer and an aerodynamic particle sizer. Cells were exposed to CeO₂ (0, 50, 224, 1000 mg/m³) for 1 hour. After a further 24 hour culture, samples were collected and subsequently analyzed for markers of oxidative stress (hemeoxygenase-1 (HO-1) protein levels), inflammation (different cytokines using multiplex analyses), cytotoxicity (LDH release), genotoxicity (comet assay) and RNA expression (Illumina beadchip (humanHT-12v4)).

Comparison of cell models showed that MucilAir cells were less affected by the air stream compared to the A549 or BEAS-2B cells (based on IL-8 and LDH). MucilAir cells showed a slight HO-1 response for both nano-CeO₂ and micro-CeO₂ exposure, whereas the cell lines did not. In contrast, BEAS-2B

cells showed an inflammatory response (IL-8) upon nano-CeO₂ exposure, whereas MucilAir did not. Both cell lines showed a dose response in the comet assay upon nano-CeO₂ exposure, in contrast to MucilAir. Differences between MucilAir and the cell lines were confirmed by gene expression analysis (PCA analyses).

Comparison of the nano-CeO₂ and micro-CeO₂ exposures showed that prominent effects were found for nano-CeO₂ exposure only. Nano-CeO₂ exposure resulted in a dose response for IL-8 in BEAS-2B and genotoxicity in both BEAS-2B and A549 cells. In addition, distinct gene expression pathway profiles were suppressed (a.o. p53 signaling pathway, cholesterol biosynthesis) or induced (a.o. glutathione metabolism) upon nano-CeO₂ exposure for MucilAir.

In summary, our results show that the MucilAir human 3D airway models is more resistant to air stream and nano-CeO₂ compared to the cell lines, most likely due to its protective morphology (a.o. cilia, mucus layer), which may be a more relevant representation of *in vivo* exposure, compared to the use of cell lines. On the gene expression level however, clear changes were observed for MucilAir after nano-CeO₂ exposure, indicating that the exposure affected the cells, although not resulting in an inflammatory or genotoxicity response under the conditions tested. Due to its morphology, the use of human 3D airway models might predict a more realistic response where cell lines might overestimate the effect of nanoparticles.



Pharmacology and carcinogenicity. Predictivity as a possibility to reduce the number of carcinogenicity studies

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Reduction of animal experimentation might be possible when the new ICH-initiative on S1 Carcinogenicity Testing will be successful. The analysis of a large dataset of carcinogenicity studies and chronic studies of human pharmaceuticals has revealed that the absence of preneoplastic findings (in combination with the absence of genotoxicity and hormonal action) is highly predictable for a negative outcome (i.e. absence of tumors) of a carcinogenicity study, provided that both studies were conducted at a similar exposure.

In addition, analysis of the pharmacological properties of these compounds provided useful information about a class-specific pattern of the correlation between the pharmacology and the carcinogenicity outcome, i.e. tissue-specific tumors.

Examples of important positive classes are prolactin-enhancing agents, β 2-agonists and PPAR-gamma agonists.

Another class of positive compounds is related to liver pathology. Specifically, compounds with metabolism-inducing properties comprise a large part of this class, often inducing liver hypertrophy, which in mice leads to liver tumors, while in rats thyroid follicle cell tumors or Leydig cell tumors are more common.

This level of predictivity at both extremes of carcinogenicity are leading to the initiative at ICH level to study whether the outcome of life-time rodent studies can be predicted on the basis of pathology seen in the chronic toxicity studies, with the idea that in the future such studies are no longer needed.

In analogy with the ICH S6 guidelines in which growth factors and immunosuppressants are exempted from the need to conduct 2-yr carcinogenicity studies due to the inherent risk associated with them, and the reasonably predictable outcome, the ICH S1 Expert Working Group proposes to establish a Category 1, comprising products with high human risk, which should no longer require carcinogenicity testing after a try-out period of around 5 years.

Additionally, Category 3 compounds with a predictable negative outcome in rodents (either negative on the basis of 6 months rat data or positive on the basis of pharmacology) will then be exempted from the need for carcinogenicity studies.

Category 2 is the remaining category, and the upcoming few years will mainly be used to define the borders of this category both with respect to category 1 and 3.

In the try-out period, companies are expected to write a carcinogenicity assessment document at the end of Phase 2 of drug development, placing their product in a Category on the basis of a prediction, before proceeding to perform the actual carcinogenicity studies.

Genomic biomarkers are expected to help to predict pharmacological mechanisms leading to organ-specific tumors, and will help to broaden the Category 3 with the development of new pharmacological classes of compounds.

Are structural analogs to bisphenol A a safe alternative?

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Bisphenol A (BPA) is a chemical often integrated in impact-resistant plastics and surface coatings in canned foods as well as screw-on caps and cashier receipts. BPA is either intentionally added or appear as a consequence of recycling in materials with food contact, which leaves consumers at risk of exposure. An incredibly number of animal experiments has been used for elucidation of the adverse effects of BPA and they have shown that BPA has endocrine disrupting effects and is suspected to be a contributing factor in disorders such as overweight, diabetes, cardiovascular diseases, and behavioral changes in children. Therefore, developing alternatives to BPA is important and structural analogues are now replacing BPA and found in human tissues.

The aim of this study was to characterize the toxicological profile of BPA and five analogs, BPB, BPE, BPF, BPS, and 4-cumyl phenol with focus on general toxicity and endocrine disrupting potential. The investigation was conducted by performing a comprehensive Quantitative Structure Activity

Relationships (QSAR) modeling with respect to a series of adverse human effects, including acute oral toxicity, local irritation, cardiotoxicity, endocrine disruption, reproductive toxicity, genotoxicity, and cancer. Their metabolism via three key cytochrome P450 enzymes and the pregnane X receptor was also evaluated.

The toxicological profile of the six bisphenols were supplemented with and compared to data obtained from experimental cell-based assays covering interferences with the androgen, estrogen, PPAR γ , and aryl hydrocarbon receptors and interferences with steroidal sex hormone synthesis.

Important quantitative and qualitative differences and similarities in potency and efficacy on various endpoints appeared, and these results will be presented. This alternative approach involving *in vitro* and *in silico* methods forms a unique basis for deciding on the next step to be taken in risk assessment of these compounds.



“Skin on a Chip” – Perfused long term culture of skin tissue

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Substantial progress has been achieved over the last decades in the development of skin equivalents to model the skin as an organ. Their static culture still limits the emulation of essential physiological properties crucial for toxicity testing and compound screening. Therefore, we developed a dynamically perfused chip-based bioreactor platform which supports two different culture modes: i) tissue exposed to the fluid flow, and ii) tissue shielded from the underlying fluid flow by standard

Transwell® cultures. Further, this system is capable of applying variable mechanical shear stress and extending culture periods. This leads to improvements of culture conditions for integrated human artificial skin models as well as *ex vivo* skin organ cultures. Thus, we provide a potential new tool for systemic substance testing.

Studies on the mode of action of Multi-Walled Carbon Nanotubes onto human bronchial epithelial cells

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With a production rate of several hundred tons per year, Multi-Walled Carbon Nanotubes (MWCNTs) account for the largest part in the increasing production of nanomaterials. Serious concerns have been raised about the safety of MWCNTs due to their asbestos-like structure. As the most likely route of human exposure to MWCNTs is inhalation, the influence of MWCNTs on the alveolar epithelial cell line A549 has already been widely discussed within the scope of toxicity screenings and is associated with the induction of ROS and other cytotoxic events. In contrast, only few publications exist, which discuss the effects of MWCNTs on cells of the bronchial system. Because of MWCNT's mode of deposition, investigation of the impact of MWCNTs in this area is of great importance. Therefore this project presents the results of several *in vitro* cytotoxicity assays and gene expression analysis carried out with custom made MWCNTs (length 8.57 μm , diameter 0.085 μm) on the human bronchial epithelial cell line Calu-3.

For separation of agglomerated MWCNTs into single ones, MWCNTs were suspended in Dulbecco's cell culture medium using a sonotrode twice (90% duty cycle, 100% amplitude) for five minutes. Calu-3 cells were incubated for 24 or 48 h with MWCNTs at a concentration of 5, 10, or 20 $\mu\text{g}/\text{cm}^2$. When evaluating cytotoxicity assays, the shifting influence of MWCNTs on several absorption spectra should always be considered. Therefore luminescence based cytotoxicity assays were carried out in addition to colorimetric and fluorimetric assays. In regard to reduction of interfering effects, every assay was adapted to the use of MWCNTs by filtering the assay before

analysis. The entirety of cell viability assays showed only a few slight decreases. After an exposure to 5 $\mu\text{g}/\text{cm}^2$ MWCNTs, an increase in dead cell proteases in cell supernatants was measured, while an exposure to 20 $\mu\text{g}/\text{cm}^2$ MWCNTs led to a decrease in ATP.

The induction of oxidative stress and the activation of the NF κ B signaling pathway are generally considered to be important for the formation of fiber-mediated inflammation. Our studies on oxidative stress showed no MWCNT-dependent increase of ROS. Pathway-specific real time PCR arrays (Human NF κ B Signaling Pathway, Human Oxidative Stress and Antioxidant Defense, Human Stress & Toxicity PathwayFinder, SABioscience/Qiagen) were carried out to determine MWCNT's influence on gene expression levels. Our studies identified the regulation of a few genes which are thought to be involved in the regulation of oxidative stress. Additionally, MWCNTs changed the expression of several genes which are involved in DNA repair or regulation of immune response and down regulated the expression of the tumor suppressor gene EGR1. Due to their tight growth in monolayers, Calu-3 cells form an epithelial-like structure, which can be used for studies on transepithelial resistance. In the present study, no effect of MWCNTs was detected on the strength of the transepithelial resistance. It can be concluded that, unlike A549 cells, Calu-3 cells are relatively insensitive to exposure to MWCNTs. In order to establish a predictive toxicity screening for MWCNTs, the choice of a sensitive cell model is essential.



Phase I biotransformation of testosterone by human skin and reconstructed skin tissues

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Reconstructed human epidermis (RHE) is validated for *in vitro* testing for e.g. skin corrosion, irritation, and percutaneous absorption. However, biotransformation capacity is poorly characterized although biotransformation can influence uptake, pharmacological effects and toxicity of compounds making contact with the skin. For a more detailed insight into phase I biotransformation we investigated the standard compound testosterone. As keratinocytes and fibroblasts can contribute differently, studies were performed in excised human skin, reconstructed human epidermis (RHE) and reconstructed full thickness human skin (RHS) as well as in human keratinocytes

and fibroblasts. Testosterone biotransformation by RHE and especially by RHS exceeded biotransformation in human skin, yet, the metabolite profile was close. For studies of cutaneous biotransformation fresh skin has to be used because of a decline in phase-I-enzyme activity in cryoconserved skin. RHE and RHS appear adequate test matrices for investigation of phase I biotransformation of xenobiotics as well as the investigation of biotransformation-related endpoints, in e.g. sensitization and genotoxicity.

Topical application of cationic membrane-active peptides: enzymatic degradation by human skin *ex vivo* and the effect on skin penetration

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Therapeutic active peptides play an increasing role as novel, more sophisticated approaches in the development of drugs, but are limited for topical treatment due to their hydrophilic nature and high molecular weight. Cell penetrating peptides (CPPs) have the ability to overcome the skin barrier owing to the unique characteristics to translocate themselves and other molecules through membranes. Furthermore, CPPs have been tested as carriers to enhance delivery, e.g. of small molecule drugs, nucleic acids, peptides and proteins. To evaluate the potential of cationic membrane-active peptides for topical use, penetration of melittin (main component of honey bee venom), into human skin *ex vivo* was compared to two nontoxic CPPs, low molecular weight protamine (LMWP) and penetratin. In addition, cutaneous metabolism of CPPs and its influence on skin penetration was studied. The stratum corneum prevents penetration into viable epidermis over 6 hours but the peptides

gain access to the viable skin within 24 hours. To evaluate CPP cleavage, fluorescence labeled penetratin and LMWP were exposed to human skin homogenate or trypsin solution and the amount of intact peptide was quantified by RP-HPLC in combination with fluorescence detection. Trypsin is the key enzyme for biodegradation of both peptides. Importantly, enzyme inhibition by phenylmethylsulfonyl fluoride and phenanthroline reduced the degree of penetratin and LMWP penetration into the deeper skin layers whereas the overall quantity of intact peptide was significantly enhanced. In conclusion, the penetration of peptides into viable human skin is a slow process. Skin enzymes cleave CPPs and the products rapidly penetrate into the deeper skin layers. However, when using CPPs as a component of a drug delivery system, peptide integrity is not required over the entire time of exposure.



Increased cutaneous absorption reflects impaired barrier function of reconstructed skin models mimicking keratinisation disorders

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Congenital keratinisation disorders are rare but severe and disfiguring and can represent a significant psychosocial burden. Current therapy is limited to gaining relief of symptoms and includes the application of moisturizing creams and frequent baths. In severe cases retinoids are applied topically or even systemically. Nowadays much is known about the molecular aetiologies of these disorders, however, human-based skin models mimicking keratinisation disorders on the genetic and biochemical level are still urgently needed for preclinical drug development in order to reduce and refine the use of animals with or without typical clinical features. The aim of the present study was to assess a recently established 3D model of congenital ichthyosis, representing severe epidermal barrier function defects, for skin penetration and permeation. We have generated disease models by knockdown of either transglutaminase 1 (TGM1) and epidermal lipoxygenase-3 (ALOXE3) in foreskin keratinocytes and by using keratinocytes and fibroblasts from patients with congenital ichthyosis. The reconstructed skin was

evaluated for permeation of the OECD standard substances caffeine and testosterone. Since nanoparticles are of continuously increasing interest as drug carriers in topical dermatotherapy, the penetration enhancement by solid lipid nanoparticles and core-multishell nanotransporters was followed with the model dye Nile red loaded to the nanoparticles. The results prove the characteristic barrier disturbance as demonstrated by increased permeation of testosterone and caffeine in the knockdown constructs and constructs from lesional cells compared to control models. Particularly, enhanced penetration of Nile red incorporated into nanocarriers was evident in disease models. Thus, the *in vitro* skin disease models reflect disease typical differences in barrier permeability and function and pave the way to personalised disease models. Furthermore, our findings indicate that nanocarriers may be useful in new, topical approaches for the currently very limited treatment of congenital ichthyosis.

Evaluation of the sub-classification of dermal corrosives *in vitro* using the epiCS[®] (CellSystems) reconstructed human skin model

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Skin corrosion is defined as the production of irreversible damage of the skin tissue by the Globally Harmonized System for the classification and labelling of chemical substances and mixtures (GHS). The CellSystems' test method using the epidermal skin equivalent epiCS[®] was validated in 2009 by ESAC (ECVAM advisory board) with a battery of chemicals which were classified either as corrosive (Category 2, Cat.2) or non corrosive (NC, no Category). Our model was able to discriminate corrosives from non corrosives with a very good prediction. The specificity, sensitivity and accuracy of the 12 proficiency chemicals recommended by the OECD Guideline for the testing of chemicals No. 431 were 84.7%, 100%, and 92.4%, respectively.

The purpose of this study was to assess whether our epiCS[®] skin model can reproducibly discriminate between the sub-categories of corrosive chemicals with the classifications 1B/C (less severe corrosive) and 1A (severe corrosive). Here we present data describing the sub-classification (NC, 1B/C, 1A) of about 80 chemicals including liquids, solids, acids and bases (organic and inorganic).

Method: Duplicate epiCS[®] tissues were apically exposed to test and control substances for 3 min at RT and 60 min (37°C) with 25 mg (solids) or 50 μ l (liquids). After a washing step the tissues were exposed to 1 mg/ml MTT for 3 hours. For the assessment of the relative viability the optical density of the extracted formazan dye was quantified by absorption at 550 nm. The classification is based on the following prediction model: A chemical is classified 1A corrosive, if the viability is <50% at 3 min. A chemical is classified as 1B/C corrosive, if the viability is \geq 50% at 3 min and <15% at 1 hr. Non corrosive (NC) classification is achieved if the viability is \geq 50% at 3 min and \geq 15% at 1 hr.

The results of this study demonstrate that the epiCS[®] skin model was able to correctly identify most non corrosive chemicals and that this model is able to distinguish between the 1B/C and 1A chemicals in most cases. In some cases a chemical was over predicted and a non corrosive chemical was classified as 1B/C. We conclude that the robustness of the assay and its predictive capacity is very good and the overall results presented here using epiCS[®] tissues allow to subclassify chemicals into 1A, 1B/C, and NC.



Non-testing strategies – a tiered approach

R. A. Wess

Computational methods are sometimes considered a black box able to deliver automatically endpoint values plus reports including sufficient methodological information (QMRF, QPRF) just on the basis of a chemical structure information. However this may be achieved a tiered approach is more realistic as experienced on the basis of actual REACH registration work done in a consultancy. Briefly the following options should be checked:

1. Not assessing on an endpoint (waiving) of the submission item
 - e.g. biodegradation or persistence of inorganic substances
2. Not testing on an endpoint and assessing on the basis of evidence from available tests with the submission item for a different endpoint
 - Route-to-route extrapolation (ITS, EPM), or (more) chronic instead of (more) acute data;
 - e.g. 90 day Toxicity covering 28 day toxicity; sediment simulation study covering absence of hydrolysis
3. Not using target chemical test data but from
 - a. A test surrogate (identical species, analogue bioavailability)
 - e.g. Pre-drug case (metabolite); Actual exposure case
 - b. An analogue material (analogue species, identical bioavailability)
 - e.g. Point-to-point (read across); Trend analysis (QSAR, QSPR)
4. Not using mixture / multi-constituent / UVCB test data but from its constituents
 - Combined action (CA) or Independent action (IA) model

21st century safety science and non animal approaches at Unilever

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Unilever's Safety & Environmental Assurance Centre is an organisation that constantly seeks to bring new science to the safety and environmental impact assessment of the ingredients in Unilever products. The toxicology and ecotoxicology that underpins those assessments is rapidly evolving. In 2007 a landmark in the evolution was reached with the publication of the US National Academy of Sciences report "Toxicity Testing in the 21st Century [TT21C]: a Vision and a Strategy". The academy identified the need for a fundamental change in the way safety assessments are carried out, envisioning "a not-so-distant future in which virtually all routine testing would be conducted in human cells or lines *in vitro*".

Since the publication of this framework, others in the US, Europe and China have endorsed the approach as a priority for the research needed to implement the vision. For example, a large-scale effort is being developed in the context of the OECD "Adverse Outcome Pathway" (AOP) work programme. This also has particular relevance in environmental toxicity assessments, where new thinking is emerging with respect to Source to Outcome Pathway (S2OP) risk assessments. Unilever and our scientific partners are progressing the science in this area to put in place the tools and novel thinking needed to implement TT21C/AOP- based consumer and environmental risk assessments. In so doing, we aim to ultimately remove our dependence on apical endpoint studies in animals and bring novel science to the decisions we make on the safe use of chemicals within consumer products.

During our research in this area since 2004, we have increasingly found that a major contribution to success has been interdisciplinary teams working and learning together, applying novel modelling and informatics techniques, with specific risk assessment questions at the heart of their research. Several key disciplines have become increasingly important to our work including mechanistic chemistry, exposure science, mathematical modelling (and associated biological inputs) and the use of clinical data. We are currently exploring two specific case studies (www.tt21c.org)

(1) Skin Allergy Risk Assessment

The approach to risk assessment we are currently exploring for skin allergy is based on the AOP for skin sensitization initiated by covalent binding to proteins that was recently reviewed by the OECD.

(2) Systemic Toxicity Risk Assessment (DNA Damage)

We have chosen to explore (in collaboration with the Hamner Institutes in the US) DNA damage responses mediated by the p53 network. Whilst we acknowledge that one pathway will not solve all of the issues around assuring safety for systemically available materials, this case study has allowed us to explore how the various aspects of a TT21C-based risk assessment can be used together.

These case study pathways (as well as others relating to both human health and environmental safety) are allowing us to begin to understand how the various aspects of a pathways-based non-animal risk assessment may ultimately be brought together to allow decision making about consumer and environmental safety.



Comparison of standard and fetal-calf-serum-free cell culture media by impedance measurement

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Although considerable progress is made in the development of synthetic media for culturing cells, fetal calf serum (FCS) is still routinely used as the standard supplement for cell cultures. FCS is extracted from the blood of fetal calves thus raising strong ethical concerns. An abundance of scientific literature shows that fetuses are capable of experiencing pain and distress (see e.g. van der Valk et al., 2004). From the animal welfare point of view the use of FCS therefore is not acceptable and researchers should be obliged to exploit all possibilities to grow their cells in media free of FCS (Falkner et al., 2006). Consequently, in particular the use of FCS in standard protocols should be critically examined and replaced wherever possible. We investigated whether the culturing of the L929 permanent mouse fibroblast cell line is possible using commercially available synthetic media, not containing FCS. Standard protocols for this cell line such as in various INVITTOX protocols require addition of 10% FCS into the cell culture medium. We used bioimpedance as an indicator of cell viability.

BioChips were seeded with 50,000 cells in 300 μ l DMEM (Sigma Aldrich, D6429) supplemented with 10% FCS and pre-incubated (37°C, 5% CO₂) for 24 h. Then they were transferred to the IMOLA-system (Weiss et al., 2013) and bioimpedance

was recorded for 50 h. For the first 22 h all BioChips were supplied with DMEM + FCS (see above). Then IMOLA5 was switched to the FCS-free Panserin 401 medium (IMOLA4 was switched to a flask containing DMEM + FCS again). After 48 h 0.2% SDS (Sigma Aldrich, L4509) was added as a positive control.

The first results indicate that the L929 cell line can easily be cultured with fetal-calf-serum-free cell culture media. However more experiments are necessary to allow statistical analysis of the data.

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Monitoring of multilayer development of human 3D cornea constructs by trans-epithelial impedance measurement

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Measurement of transepithelial electrical resistance (TEER) is a widely accepted method to monitor living epithelial cells *in vitro* (Wegener et al., 2004; Shi and Theng, 2005). In the presented work we used a modified IMOLA-IVD system (Wiest, 2006) to monitor the multilayer development of human cornea epithelial (HCE) constructs at the air/liquid interface by means of transepithelial impedance (real and imaginary part). This is a useful method in the field of toxicology to study the effect of chemicals towards 3D-constructs of living cells. The method is highly sensitive, label-free, non-invasive, reproducible and delivers additional information without the use of further histological analysis.

0.3 ml of HCE cells (2E5 cells per ml) were seeded in cell culture inserts (Millipore, PITP 01250) and cultivated in DMEM/F12 media with 10% fetal calf serum, 1% L-glutamine, 1% Na-pyruvate and 1% streptomycin. The cells were confluent after 3 days. On day 6 the media level was reduced to allow an air/liquid cultivation. The medium was then replaced daily and the impedance was measured for 24 days. On day 23 0.2% SDS were added as a positive control. To measure the impedance, the inserts were transferred to a modified 12 well plate with gold electrodes on the bottom and in the cover. On day 6 the real part of the impedance was 200% of the blank control value and it

raised to 300% on day 23 (n=5). The corresponding imaginary values were 300% (day 6) and 700% (day 23). After addition of SDS the values reached the 100% of the blank controls within one hour. In a second experiment the effect of 0.01% SDS was investigated.

The presented system is an adequate tool to monitor long-term and non-invasive the development of the 3-D-architecture of human corneal cells *in vitro* and to detect with these constructs very low doses of substances which are irritating on the human eye.

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Elucidation of perturbed pathways by using stem cell derived neural progenitors

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Since the developing nervous system can be targeted by pharmaceuticals taken by pregnant women, an early prediction of human prenatal toxicity already during the phase of drug development is of high importance. This requires the establishment of human cellular models allowing an understanding of toxicological mechanisms leading to developmental toxicity.

In the presented study, we have used pluripotent stem cells as a model for the detection of chemical effects on differentiating neural progenitor cells. Differentiating stem cells were exposed for 10 days to a subcytotoxic concentration of 4 different substances. Transcriptomics analysis allowed a first elucidation of pathways involved in the development of adverse effect of developmental toxicants. As already demonstrated in previous studies (Krug et al., 2013; Vojnits et al. 2012; Nerini-Molteni et al., 2012), the toxicity of Valproic acid (VPA) and methylmercury (MeHg) could be profiled based on transcriptomic readouts. In this study, we further assessed the toxicological mechanisms of toxicants belonging to the same chemical categories: Trichostatin A (TSA) and SAHA, which are deacetylase inhibitors, like Valproic acid, and Thimerosal and Mercury (II) chloride which are similar to MeHg and are well-known to interact with the embryonic development *in vivo*.

In conclusion, the proposed cellular model mimics the embryonic neural development and as such can be a valuable tool for the determination of biomarkers. In combination with newly identified biomarker the cellular model might be a powerful tool for the detection of human developmental toxicants in the phase of early drug development.

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Development and characterization of a bioengineered conjunctiva model on the basis of immortalized cells

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Until today none of the existing *ex vivo* or *in vitro* methods has proved fully satisfactory to completely replace the Draize Rabbit Eye Irritation Test. Although damages to the cornea are the most influential drivers of eye irritation for all classes within the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), also conjunctival damages gain more importance particularly as a driver of irritation, especially for category 2A and 2B classification. However, none of the already validated *in vitro* methods for eye irritation testing sufficiently addresses the conjunctival involvement. Therefore it was our aim to develop a bioengineered conjunctiva model for application in eye irritation studies.

The 3D conjunctiva equivalent has been developed from immortalized cells originally derived from human corneas. The 3D model comprises a multi-layered epithelium and a subjacent stromal matrix of collagen-embedded fibroblasts. We established standard operating procedures for the construction and cultivation of the bioengineered models and for its use in an eye irritation toxicity assay under serum-free conditions.

The conjunctiva model was characterized by H&E-staining, immunohistology as well as barrier function analysis by means of transepithelial electrical resistance studies (TER). In addition, a modified MTT method for the determination of tissue viability in cryosections was applied to the conjunctiva models to assess the tissue damage induced by topically applied chemicals. By using this method we can clearly distinguish the reference substances by means of their depths of injury.

Therefore, the 3D conjunctiva model represents a promising non-animal *in vitro* alternative and offers a well-defined and standardized system for assessing the conjunctival effects in an eye irritation reaction.

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***In vitro* non-melanoma skin cancer model for toxicity testing and pre-clinical drug evaluation**

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Actinic keratosis, a non-melanoma skin cancer (NMSC), is the most prevalent carcinoma in situ in man. Especially in immunocompromised patients, untreated lesions can develop into invasive cutaneous squamous cell carcinoma. Since a prediction of transformation to the malignant disease is not possible, elimination of the lesions by field pharmacotherapy in the pre-malignant actinic keratosis stage is essential (Stockfleth, 2008). Yet, current therapy is limited due to low complete clearance rates. Treatment may be improved by developing novel drugs (Schwanke, 2010) and by using nanoparticles for improved bioavailability (Korting, 2010).

The evaluation of effective and safe options for disease control under avoidance of animal studies requires a reliable *in vitro* NMSC model. Adapting a recently introduced approach (Hoeller Obrigkeit, 2009), we constructed an *in vitro* model for actinic keratosis. Haematoxylin and eosin staining shows a multilayered viable epidermis. Epidermal differentiation is confirmed by immunofluorescence staining of marker proteins keratin-14, involucrin and collagen IV. Fluorescence labeled SCC-12 cell nests are only found within the epidermis.

To evaluate the efficacy of drugs applied onto the NMSC model, we quantified the caspase-cleaved fragment of keratin-18 and total keratin-18 being used to estimate apoptosis in patients (Kramer, 2006). For start, we compared the novel purine

analogue OxBu to current first-line NMSC therapy 5-fluorouracil. The post-treatment apoptosis part of cell death following OxBu treatment (0.58) exceeded apoptosis following 5-fluorouracil treatment (0.49) and solvent controls (<0.41).

To assess the barrier properties in the NMSC model, we traced indocarbocyanine covalently bound to dendritic-core multishell nanotransporters (CMS NT) and Nile red loaded CMS NT. Penetration into viable epidermis and dermis of nanotransporters and the cargo Nile red were enhanced compared to the control model.

In conclusion, our NMSC model offers a human-cell based approach to pre-clinical drug evaluation and toxicity testing. *In vitro* organotypic disease models may overcome limited availability of diseased skin and low predictive value of animal-generated pharmacological data for humans.

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Scientific Programme – draft April 2013

1. New technologies

- Virtual tissue models
- HTS
- BIG data
- Novel computational biology tools
- Tissue on a chip
- 3D models
- Bioreactors
- Bioimaging
- Monitoring, telemetry
- Advanced human-based diagnostic techniques
- New readout technologies

2. Predictive Toxicology and safety assessment

- Pathway approaches in toxicology (AOP)
- Systems biology (-omics)
- Update on Tox21
- Update on SEURAT-I
- Exposure
- Topical toxicity
- Repeated dose toxicity
- Skin sensitization
- Endocrine disruption
- Genotoxicity / Carcinogenicity
- Reproductive and developmental toxicology
- Inhalation toxicity
- Ecotoxicology
- Computational modeling and chem-informatics (e.g. QSAR, mechanistic chemistry)
- Risk assessment (e.g. chemicals, drugs, biocides, food, cosmetics, medical devices, nano materials, mixtures, biologicals etc.)
- Application in decision making and testing strategies

3. 3Rs in academia and education

- Information requirements on researchers (e.g. Directive 63/2010)
- Training programs and anticipated needs
- 3Rs in academic education
- Funding programs (e.g. Horizon 2020)
- Funding agencies
- Innovative teaching and training tools
- Benefits of 3Rs – case studies

4. Communication, dissemination and data sharing

- Scientific reporting standards
- Retrospective analysis / non-technical summaries (63/2010)
- Information systems and databases
- Intellectual property and data ownership

5. Efficacy and safety testing of drugs and biologicals

- Pathway-based assays
- Alternative disease models
- Screening strategies
- Potency of human and veterinarian vaccines

6. Human relevance (in vivo, in vitro)

- Stem cells (screening)
- Disease models
- Human biomarkers
- IVIVE
- ADME
- Challenges in translational biology
- Pros and cons of animal models (plenary)

7. Ethics

- Use of materials of human origin
- Testing in human volunteers
- Distress evaluation
- Benefit evaluation
- Transgenics

8. Refinement and welfare

- NHPs
- Primates
- Welfare approaches
- Humane principles in experimental techniques
- Avoidance of severe suffering

9. Global cooperation, regulatory acceptance, standardization

- Regulatory acceptance of alternatives
- Progressing validation
- Update from validation centers
- Good cell culture practice
- Challenges to 3Rs
- Barriers for international harmonization and mutual acceptance
- Legal constraints and uncertainties
- Trade barriers
- Animal welfare implementation across the world
- Update from international societies

The abstracts will be published in an international scientific journal focused on 3Rs with impact factor.

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Full Participation	€ 530	€ 580	€ 635
Full Participation, Students / young scientific*	€ 380	€ 430	€ 475
Accompanying Person	€ 300	€ 350	€ 390

*Students/young scientists under 30 are eligible when presenting an official confirmation of enrolment at a scientific institution.

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Important Dates

Registration Opens	October 2013
Abstract Submission Opens	October 2013
Abstract Submission Deadline	April 2014
Early Registration Deadline	May 2014
9 th World Congress on Alternatives & Animal Use in the Life Sciences	24–28 August 2014

Social Programme

Sunday, 24 August 2014

Get-Together Party at Hotel Hilton Prague included in the registration fee

Monday, 25 August 2014

Welcome Reception at Public Spa included in the registration fee

Wednesday, 27 August 2014

Gala Dinner at Municipal House

For more information on the scientific programme, abstract submission, registration, hotel reservation and social programme please visit our website: www.wc9prague.org.