Abstracts of the 9th World Congress, Prague, 2014

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M. Goldman
The rational use of animals in drug development: contribution of the Innovative Medicines Initiative

R. Mokrý
European strategy for 3Rs and replacement of animal experiments

U. Marx
Human-on-a-chip – a paradigm shift from animal testing

N. Gillett
Industrial perspectives on the 3Rs and animal welfare

R. Kolar
How long must they suffer? Success and failure of our efforts to end the animal tragedy in laboratories

H. Zhengming
Future perspectives for alternatives to animal testing in China

R. Kavlock
Lessons learned from ToxCast and prospects for the future

Humane Science in the 21st Century

2014

9th World Congress on Alternatives and Animal Use in the Life Sciences
24–28 August 2014
Prague, Czech Republic

ALTEx Proceedings
Dear WC9 participants,

We are proud that in 2014 Prague, the capital of the Czech Republic, a major European center of arts and sciences in the heart of Europe, is hosting the 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC9).

WC9 is the first world congress on alternatives held in Europe after EU Member States accepted the “3Rs-Principle” of Russell and Burch as the basis for EU Directive 2010/63/EU “on the protection of animals used for scientific purposes”. Thus today the European scientific community, animal welfare organizations and the general public, represented by the European Parliament and Commission, give the 3Rs the highest priority, since this approach is taking into account both human ethical principles and animal welfare. Today, in the life sciences, the implementation of the 3Rs principle has become an integral part of innovation and research. Advanced experimental study designs embark on the novel concept of disease models and toxic effects based on pathways in cells and tissues of human origin, e.g. the “Human Toxicology Project” and the “human-on-a-chip” project.

The Czech society, hosting the WC9, is built upon humane traditions that are caring about the environment and the welfare of animals. Building upon an increasing global confidence in ethical approaches in science and its significance for modern society, we have chosen our vision and challenge as motto of the 9th World Congress in Prague:

“Humane Science in the 21st Century”.

The global commitment of a new generation of scientists to the 3Rs principles is demonstrated by the scientific program of WC9 with more than 450 oral presentations and 460 posters. The scientific program of WC9 offers state-of-the-art presentations and discussions on advanced cell and tissue culture technologies and testing strategies, on ethical issues of animal experimentation and on the future of the life sciences in the 21st century. We are particularly proud that due to generous sponsoring we have been able to invite 41 young scientists to actively participate in WC9 by sharing their results for discussion in oral presentations and posters.

In addition to the scientific program, we hope that the social program reflecting the cultural history of the city of Prague and picturesque and friendly atmosphere in combination with Czech food, wine and music will further stimulate the exchange of scientific knowledge and expertise.

We wish to thank the very high number of sponsors and exhibitors, since without their support we would not be able to ensure the high scientific standard of WC9, all participants for their contributions, the Scientific Committee, Program Management Team, Local Organizing Team and GUARANT International for their continuous support in planning and organizing WC9 in Prague.

Dagmar Jírová
National Institute of Public Health
Prague, Czech Republic

Horst Spielmann
ACT & EUSAAT
Freie Universität Berlin, Germany

Co-Chairs, 9th World Congress on Alternatives and Animal Use in the Life Sciences
Dear WC9 participants,

ALTEx joins the congress co-chairs Dagmar Jírová and Horst Spielmann in welcoming you to the 9th World Congress on Alternatives and Animal Use in the Life Sciences in the beautiful city of Prague, Czech Republic.

This Abstract book contains short summaries of the 8 keynote lectures and 891 abstracts that were accepted for oral or poster presentations. These are shared over the 10 Congress Themes covering new technologies, predictive toxicology, 3Rs in academia and education, communication, dissemination and data sharing, efficacy and safety testing of drugs and biologicals, human relevance, ethics, refinement and welfare, global cooperation, regulatory acceptance and standardization, and additional sessions. The Themes are subdivided into 84 scientific sessions. The abstracts represent the work of contributing authors from a total of 49 countries from five continents. The number of abstracts submitted for this congress is a staggering 33% higher than for the previous World Congress.

This impressive number of abstracts, sessions and countries, represented by their scientists, demonstrates that the 3Rs Reduction, Refinement and Replacement are gaining further momentum globally in the areas of toxicology, quality assurance, basic research, ethics in the use of animals, regulation and education with input from cutting edge technologies: A clear sign that concern for experimental animals and the desire to improve human relevance of results are continually driving the development of alternative methods forward. They are gaining increasing interest in the scientific community and drawing from new areas. WC9 will help to continue this trend which will hopefully be supported with new funding opportunities in this area, the revision of animal protection laws or toxicity testing procedures and also more stringent ethical standards set by funding bodies and journals on an equally international basis.

ALTEx is honored to publish the WC9 Abstract book in ALTEx Proceedings. The full Abstract book is also available on the website http://www.altex.ch/ALTEx-Proceedings.90.html. We especially thank Robert Vojtech and Iva Zahradníková from Guarant International for their excellent cooperation in producing the Abstract book. We also thank the Doerenkamp-Zbinden Foundation (Switzerland) for funding the production and printing of the Abstract book.

We wish all participants of WC9 a stimulating and successful congress that leads to new projects and international cooperations which you will hopefully report on at WC10 in Seattle in 2017.

With best wishes,

Sonja von Aulock
Editor in chief, ALTEx & ALTEx Proceedings
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<td>Scientific Session II-12a</td>
<td>Skin sensitization</td>
<td>Scientific Session I-3a</td>
<td>Tissue-on-a-chip / Human-on-a-chip</td>
<td>Scientific Session IX-5</td>
<td>Regulatory acceptance of alternatives</td>
<td>Scientific Session III-1</td>
<td>3Rs in academic education, training programs and anticipated needs</td>
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<td>Scientific Session II-5</td>
<td>Discussion session: Application in decision making and testing strategies</td>
<td>Scientific Session II-7</td>
<td>Update from Europe – Alternative testing strategies program</td>
<td>Scientific Session III-3</td>
<td>Innovative teaching and training tools</td>
<td>Scientific Session VII-3a</td>
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### Wednesday, 27 August

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<th>D Karlov + Rokoska + Hercovka</th>
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<td>Scientific Session II-1 2b</td>
<td>Skin sensitization</td>
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<td>Scientific Session IX-3 (Round Table)</td>
<td>Activity updates from international validation centres</td>
<td>Scientific Session VI-1c</td>
<td>Distress evaluation</td>
<td>Scientific Session VI-4a</td>
<td>Absorption, distribution, metabolism and excretion (ADME)</td>
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<td>Björn Ekwall Memorial Award</td>
<td>Lunch</td>
<td>Poster discussion</td>
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<td>Scientific Session IX-8</td>
<td>Towards harmonisation in the application of alternative approaches within chemical regulation and management</td>
<td>Scientific Session IX-6</td>
<td>Breaking down barriers and promoting international cooperation on 3Rs</td>
<td>Scientific Session V-4</td>
<td>Medical devices and biologicals</td>
<td>Scientific Session VI-2</td>
<td>Use of stem cells in screening</td>
<td>Scientific Session VI-4b</td>
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### Thursday, 28 August

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<td>Scientific Session X-1</td>
<td>Scientific Session X-2</td>
<td>Scientific Session IX-9 (Round Table)</td>
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<td>Young Scientists Travel Award Short Presentations</td>
<td>Cosmetics around the world</td>
<td>Establishing criteria for an independent 3R-index: “Access to 3R’s”</td>
<td>Human-on-a-chip – Advancing regulatory science through innovation and worldwide networking for alternative testing</td>
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<td>12:00–13:00</td>
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<td>Satellite Meeting</td>
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<td>Animal welfare and the 3Rs under Directive 2010/63/EU meeting</td>
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<td>WC9 Practical Training Course on Alternative Methods: Skin and Eye In Vitro Toxicity</td>
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Keynote Lectures

Animals: biomechanisms or evolving organisms on the way to a reflexive thought?

M. Vácha
Department of Ethics, Third Faculty of Medicine, Charles University, Prague, Czech Republic
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There are three approaches towards animals in a classical philosophy: (a) an animal as a mere thing or a kind of a biomechanism, (b) in the opposite extreme at least certain animals as equal to humans with no significant difference and (c) central position stating that an animal is occurring somewhere between a thing and human person. Modern evolutionary biology and especially the idea of a convergent evolution are shedding new light on this discussion. This new approach suggests that any general features of organisms that are of great adaptive value would have arisen in certain moment of evolution, and intelligence and reflexive thought are most probably not exceptions. From this perspective, animals, or at least some of them, can be viewed as organisms on the way to the reflexive thought. The philosophical consequences of this hypothesis are discussed in this text.

For further reading see Ayala, 2010; Morris 2003, 2008.

References

The rational use of animals in drug development: contribution of the Innovative Medicines Initiative

M. Goldman
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Animal models are widely used in research and development to assess the efficacy or safety of new pharmaceutical products. However, their limitations in predicting actions of drugs in humans is more and more apparent, therefore there is an urgent need to revisit the use of animals in pharmaceutical research. This presentation will focus on the review of how the Innovative Medicines Initiative (IMI), the largest public-private partnership in life sciences, is reducing, refining and replacing the use of animals in the context of its global mission, namely to boost research and development of new medicines across the European Union.

The Innovative Medicines Initiative is a public-private partnership between the European Commission and the pharmaceutical industries members of the European Federation of Pharmaceutical Industries and Associations (EFPIA). IMI’s key mission is to enhance the competitiveness of the pharmaceutical sector in Europe for the benefit of patients and scientists by supporting open innovation and non-competitive research in the pharmaceutical research and development. IMI was launched in 2008 by the European Union and the EFPIA, with a total budget of 2 billion euro to be spent over a 7-year period, making the IMI the largest PPP in life sciences. EFPIA pharmaceutical companies invest in the IMI in the form of in-kind contributions by committing internal human resources or providing access to data sets and infrastructure and sometimes in the form of direct monetary contributions. This industry investment is matched by funds from the European Union to support the other consortium members, including academic teams, small and medium-sized enterprises (SMEs), patients’ organizations, regulatory agencies, and relevant not-for-profit institutions. IMI fosters open innovation in pharmaceutical R&D and biomedicine via public private collaboration by addressing industrial and societal challenges and by setting a neutral platform for aligning healthcare, research and regulatory priorities.

Use of animals in research and testing is a highly sensitive, yet vital part of the long and complex process of creating new medicines. However, the insoluble problems of species differences and animal to human extrapolation inevitably limit the value of animal studies for the prediction of the action of drugs in humans. A number of major technological developments have recently opened up possibilities for more directly human-based approaches with the further added value of a dynamic two-way interaction between what takes place in the laboratory and in the clinic. This progress is leading to a fundamental re-thinking of the role and use of experimental animals in pharmacological research and biomedicine.

In an era of increasing economic pressure on the healthcare systems and the pharmaceutical industry, public-private partnerships (PPPs) offer unique opportunities to overcome the hurdles which prevent efficient and safe medicines to reach patients suffering from debilitating diseases. With increased attention paid to investigations centred on human beings, human materials or based on in silico models, PPP like IMI contributes to rationalise the use of animals in biomedical research by focusing on validated models directly pertinent to drug action in patients.

European strategy for 3Rs and replacement of animal experiments (example of cosmetics sector)

R. Mokrý
DG Health and Consumers of European Commission, Brussels, Belgium
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In 2010 the European Union adopted Directive 2010/63/EU on the protection of animals used for scientific purposes. The aim of the Directive is to strengthen legislation, and improve the welfare of those animals still needed for research and safety testing, as well as to firmly
anchor the principle of the Three Rs, to Replace, Reduce and Refine the use of animals, in EU legislation. Directive 2010/63/EU took full effect on 1 January 2013. The European Commission strongly supports efforts to find alternative methods to testing on animals. Where this is not possible the number of animals used must be reduced or the testing methods refined so as to cause the least harm to the animals.

In the field of cosmetics, provisions in relation to animal testing were introduced into the Cosmetic Directive 76/768/EEC in 2003. Accordingly, animal testing in the Union was prohibited since 2004 for cosmetic products and since 2009 for cosmetic ingredients (“testing ban”). As from March 2009, it is also prohibited to market in the Union cosmetic products containing ingredients which have been tested on animals (“partial marketing ban”). On 11 March 2013 the European Union put in place a complete ban on the marketing of the remaining cosmetic substances tested on animals. This was the final step in a 10 years process to totally ban animal testing for cosmetic purposes.

The EU has set a good example in demonstrating the safety of cosmetics without new animal tests. This example is one of many other countries are now starting to follow. From China and India to the countries of South East Asia we see encouraging steps towards the banning of animal testing for cosmetics. The European Commission is willing to work with all stakeholders in any country which wishes to follow the European Union’s lead and embrace alternative methods to test the safety of cosmetics.

**Human-on-a-chip – a paradigm shift from animal testing**

*U. Marx*<sup>1,2</sup>

<sup>1</sup>Institute of Biotechnology, Technische Universität Berlin, Berlin, Germany; 2TissUse GmbH, Spreenhagen, Germany

The keynote lecture provides a historical sketch of my efforts since 1990 to replace laboratory animals by emulating systemic human biology. It started with a multi-cartridge hollow fibre bioreactor in the early 1990s combining five human organ equivalents failing to operate, but perfectly replacing ascites mice. Scientific and commercial implementation of a miniaturized artificial human lymph node in 2004 (Giese et al., 2006) reinvigorated my enthusiasm to design a human-on-a-chip platform with a team of equally enthusiastic scientists. The ten-organ-chip visualized by 2009 was too radical to get full funding. Instead, the platform development of an integrated two-organ-chip operated by an on-chip-pump was funded in 2010. Proof of concept combining human liver and skin equivalents at steady culture conditions over 28 days was achieved in 2012 with repeated dose troglitazone testing (Wagner et al., 2013; Schimek et al., 2013). Subsequently, industry interest is resulting in diverse feasibilities, varying organ arrangement and substance exposure on the platform. 2012 also marked the launch of an amazing top-down US initiative on that topic. Highlights of this programme are given. Hot spots for human-on-a-chip development in other parts of the world are summarized. The keynote finally emphasises scientific aspects of further development (Marx et al., 2012; Giese C., Marx U., 2014), ethical repercussions and economic feasibility of the human-on-a-chip.

**References**


**Industrial perspectives on the 3Rs and animal welfare**

*N. Gillett*

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Contract Research Organizations (CRO) can creatively partner with their pharmaceutical, biotech, and chemical customers to implement and devise new applications of the 3Rs in research and in animal welfare. CROs run many discovery and safety studies, including in vitro technologies and a wide range of animal based models, so they are in a unique position to accelerate innovation and develop new approaches to the assessment of new drugs, devices, and chemicals. This presentation will cover the progress across all aspects of animal use in applying the principles of 3Rs and improved animal welfare. We will focus on the pharmaceutical industry and the trend towards integrated toxicology testing strategies to maximize the information obtained using different platforms, including in vitro technologies. In addition, progress on the use of 3Rs in animal production will be discussed. Finally, the challenge of validating and applying viable in vitro alternatives and identification of current gaps for other alternatives to animal use will be addressed.

**How long must they suffer? Success and failure of our efforts to end the animal tragedy in laboratories**

*R. Kolar*

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This World Congress is the first outside of the “old” Western hemisphere and thus can be regarded a historical milestone. It reflects the evolution and outreach of scientific and ethical principles regarding animal experiments. In the last decades, many initiatives to advance the 3Rs have come into existence. Even the idea to replace animals as a whole is no longer seen as the unrealistic vision of sentimental animal-lovers but has become an official doctrine of the European Union as well as – for toxicology – of the US government.

However, too many intentions and conventions have proved to be no more than lip services. Most major political initiatives to avoid or reduce animal experiments have either failed dramatically, like the EU-REACH regulation; or they have been watered down in the political decision-making process, losing much of their potential effectiveness, like the revised EU Directive on animal experimentation.

The core ethical problem persists: The question how to decide for which purposes it could be regarded ethically acceptable to deliberately inflict pain, suffering or distress on sentient beings has been extensively investigated, and solutions have been proposed by academia, authorities and different stakeholders. However, the reality in the laboratories reveals that almost always the decision at the end of an ethical review process still is at the cost of the animals.

The presentation gives a closer look at these inconsistencies, puts them into the context of our society’s general views on animals, and highlights the most urgent needs for action.
Future perspectives for alternatives to animal testing in China

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The alternatives to animal testing (AAT) is a concrete manifestation of the principle of 3Rs in scientific research and product safety evaluation, and also, is a scientific problems of great concern for us in the field of life science and biological technology. With the rapid development of AAT technology and acceptance of animal welfare concept internationally, it is a driver for alternative approaches to animal testing in China.

In 1997, 3Rs principle was firstly written into government documents. Several opinions about the development of laboratory animal science, which was issued by Ministry of Science and Technology, the research on alternative methods was started in a lot of institutes. In 2002, the project on research of laboratory animal welfare guideline and technical specifications in accordance with global standard, was supported by Ministry of Science and Technology. In 2006, the Ministry of Science and Technology published the “Guidance suggestions for the care and use of laboratory animal”. It is emphasized in Chinese Pharmacopoeia (2010 Edition) that the chemical methods, physical methods or cytological methods should be used, instead of animal test, for biological product quality verification to minimize animal use. In 2012, “To encourage the development of AAT, and Ensuring animal welfare” was written in “Twelfth Five-Year Plan” (2010-2015) developed by Ministry of Science and Technology.

In China, approach to AAT mainly conducted in regulatory testing for cosmetics and pharmaceuticals in the past three years, for example, research on the methodology of AAT (such as alternative methods to pyrogen test); to organize academic and activities training courses by cooperation with scientific groups worldwide, to enable technical personnel to understand and master the AAT methods approved by OECD (BOCP, HET-CAM, ST3 NRU phototoxicity test, EPIISKIN®, etc.); to compile academic books (e.g., Laboratory Animal and Animal Experimentation, Alternative Laboratory Animal Methods Principles and Application, Toxicology Alternatives). The all mentioned above showed that the importance and position of AAT in the test technology has been received and approved by government and scientists. There are signs that some significant changes in attitude to AAT in regulatory testing are taking place. At present, the preliminary results obtained by scientific groups play an important role in promoting the development of AAT in China.

However, compared with the rapid development of AAT methodology in other countries, the research on AAT started later in China, and is still in early days for us. The AAT method is at the stage in laboratory research level, and have not be validated by formal procedures. Now, the research on AAT is mainly conducted in the field of cosmetics toxicity testing in China, others rarely involved. The research and validation system for AAT has not been established, and the relevant laws and regulations beneficial to promote the development of AAT still need to strengthen and improve.

Looking to the future, the Chinese government is trying to establish guideline and technical standards for AAT according to OECD guideline, hoping to promote the research on AAT in cosmetics toxicology and drug testing, and also to promote the validation, regulatory acceptance and application of AAT for cosmetic safety assessment and drug safety evaluation in China. Therefore, the academic activities for AAT need to be strengthened to make technical staff to know the latest research progress of alternative methods. The training course should be performed regularly to make technical staff to master the AAT which has been approved by OECD, and latest technology in this field. In the meanwhile, we also hope to strengthen the close collaboration with international organizations and scientific groups, and promote the development of AAT in China.

Lessons learned from ToxCast and prospects for the future

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More than a decade ago, the US Environmental Protection Agency began exploring how the field of computational toxicology, which blends advances in modern molecular biology and chemistry with information sciences, could help improve the fundamental manner in which the safety of chemicals could be evaluated. In 2007 it launched the ToxCast program to rigorously evaluate the use of high throughput screening assays to elucidate the range of biological activities that large numbers of chemicals could interact with to cause disease. Since that time it has screened nearly 2000 chemicals across hundreds of biological pathways and developed a number of approaches to characterize the potential hazards, and indeed risks of chemicals. This presentation will cover some of the lessons learned in that program, including the critical need to build supporting infrastructure (tools and databases), the need to build robust data workflows, the value of building large scale collaborations, of factoring in exposure aspects as well as potency in assessing priorities, to be transparent in data collection, processing and release, of working closely with scientists in the regulatory arena to facilitate translation of the effort into practical applications, and lastly, that as in any research program, unexpected findings happen and you need to be prepared to critically assess all aspects of the outputs. The last few years has witnessed an evolution in viewpoints of this transformative approach, with much attention now focused on how and when the approach can be utilized in the chemical safety arena. Current examples include the USEPAs endocrine screening program and OECDs efforts in codifying adverse outcome pathways. But clearly much more work needs to be done, and additional challenges lie ahead, such as more robust incorporation of metabolic competency in in vitro assays, accounting for the complexity of emergent properties of tissues and organs, and considering how the effects of cumulative exposures can be evaluated. However, we have made great strides in the last decade at transforming toxicology. This is an abstract of a proposed presentation and does not necessarily represent EPA policy.
Theme I – New Technologies

Coordinators
Mardas Daneshian, University of Konstanz, Germany
Horst Spielmann, FU Berlin, Germany

Session I-1: Virtual tissue models

Co-chairs
Jan Hengstler, IFADo, Germany
Thomas B. Knudsen, EPA, USA

Session I-1: Oral presentations

I-1.729

A model of early ovarian development as a future tool in toxicity testing

K. H. Watanabe

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As alternative methods are developed to reduce the number of animals used in toxicity testing, mathematical/computational models will become more valuable as tools to predict effects of chemical exposure. The aim of this research is to formulate a mathematical model of normal early ovarian development in mice, from conception to the primordial follicle stage. To create the model, we reviewed the published literature (Edson et al., 2009; Liu et al., 2010; Loffler and Koopman, 2002; Tevosian, 2013; Ungewitter and Yao, 2013) to produce a graphical conceptual model of ovarian development at the molecular level. Then, we formulate a mathematical representation of the conceptual model and use computational methods to solve the model equations and simulate ovarian development. This presentation will provide an overview of model development and strategies for how it can be used to reduce the number of animals used in toxicity testing. A foreseeable application of this model is as a tool to predict how chemical exposure at different stages of development can affect the developing ovary.

References

I-1.743

Novel computational approaches for high content image analyses (HCA) of organoid 3D neurosphere cultures in vitro

E. Fritsche1, M. Schumck1, T. Temme2, M. Barenys1, T. Glasmachers2 and A. Mosig2

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Neurospheres are three dimensional (3D) cell clusters consisting of neural progenitor cells (NPCs). NPCs provide the cellular basis for the developing brain and persist in the hippocampus up to old age. There, they are responsible for adult learning and memory. Safety and efficacy testing with 3D neurospheres by employing high content image analyses (HCA) require advanced scanning and evaluation processes due to defocusing of the sphere core and sphere-specific endpoints. Therefore, for this mixed neuron/glia culture we developed algorithms for radial migration, quantification of neuronal differentiation, neurite outgrowth and distance-dependent density distributions of neurons. For quantification of neuronal differentiation the new algorithm reaches an average detection power (DP) of 80-85% (versus manual evaluation) and a false positive rate (FP) of 10-15% improving results of the commercially available “Neuronal Profiling” bio-application (Thermo Scientific; DP: 50%, FP: 40%). This is due to the application domain of the “Neuronal Profiling”, which was initially designed for pure neuronal cultures.

In conclusion, HCA of neurospheres is a promising technique for medium throughput screening to be used in safety and efficacy testing in the future.

I-1.816

High-throughput PBPK and microdosimetry: cell-level exposures in a virtual tissue context

J. Wambaugh

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Toxicokinetic (TK) models can determine whether chemical exposures produce potentially hazardous tissue concentrations. Tissue microdosimetry TK models relate whole-body chemical exposures to cell-scale concentrations. As a proof of concept, we approximated the micro-anatomic architecture of the hepatic lobule with a discrete topology by a graphical model that can be connected to a chemical-specific physiologically-based TK (PBTK) model. The development of traditional PBTK models is time and resource intensive. Successful methods have been developed for pharmaceutical compounds to determine TK from limited in vitro measurements and chemical
structure-derived property predictions. These high throughput (HT) TK methods provide a more rapid and less resource-intensive alternative to traditional TK model development. We have augmented these in vitro data with chemical structure-based descriptors and mechanistic tissue partitioning models to construct HtPBPK models for over three hundred environmental and pharmaceutical chemicals. When evaluated with human in vivo data for 74 chemicals we find that we can generally predict when HtPBPK models will perform well, and when more complicated effects (e.g., transporters) impact HtPBPK assumptions. For those chemicals where the assumptions that allow HtPBPK models are appropriate, virtual tissue simulation of quantitative chemical-specific effects is possible.

This abstract does not necessarily reflect Agency policy.

I-1-837
Multiscale modeling and simulation of embryogenesis for in silico predictive toxicology
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Translating big data from alternative and HTS platforms into hazard identification and risk assessment is an important need for predictive toxicology for developmental toxicity. Understanding how chemical disruption of molecular function in the human embryo propagates to higher levels of biological organization ultimately requires systems biology and computer simulation to recapitulate spatio-temporal dynamics of cellular networks. Virtual Tissue Models (VTMs) can provide this level of detail for discrete morphogenetic events, based on simulating connectivity between scales of biological organization. The efficacy of VTMs to integrate data with embryological information on an anatomical plane offers a novel platform for predictive toxicology (Swat et al., 2012; Andarsari et al., 2012). However, developing biologically accurate simulations is challenging. Using arsenic toxicity we illustrate how we build such models and the insights that they can provide. Arsenic, an environmental toxicant, inhibits angiogenic intersegmental blood vessel (ISV) sprouting in Zebrafish by decreasing directed migration speed and perturbing directional path-finding (McCullum et al., 2011; Shirinifard et al., 2013). Using literature mining and pathway analysis we developed a biological model of ISV growth. We then experimentally determined effects of arsenic on components of our biological model and constructed an in silico model that explored mechanisms of arsenic toxicity. Our simulations combine spatiotemporal VEGFα and VEGFR2 expression with ISV growth dynamics and reproduced both control and arsenic perturbed ISV growth behaviors. Our simulations and experiments combined showed that: 1) A VEGFα gradient created by local uptake can support ISV sprouting and extension; 2) Slow initial growth with rapid depletion of local VEGFα causes ISV collapse or failure to initiate; and that 3) Arsenic inhibition of ISV growth is due to down regulation of both VEGFα and VEGFR2. Model driven experimental design simultaneously increases understanding of mechanisms of toxicity while decreasing the number of animal experiments needed.

References

I-1-889
Virtual liver approaches
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Currently, transcriptomics of cultivated primary human hepatocytes and also virtual liver models are used to predict hepatotoxicity of chemicals. In cultivated hepatocytes genome-wide, concentration and time-dependent data are meanwhile publicly available for more than 150 chemicals. Conclusions of the biostatistical analyses are: (i) hepatocytes show a “stereotypical” gene expression response to toxic concentrations of numerous chemicals that comprises mostly proliferation and inflammation genes, (ii) more specific alterations induced only by subgroups of compounds are observed for target genes involved in lipid or energy metabolism, cytoskeletal organization, stress response and DNA repair, (iii) although these “alert genes” help to identify toxic mechanisms and can support the chemical read across approach, they are not yet sufficient to reliably predict hepatotoxicity in humans. A complementary approach to hepatocyte in vitro systems is the “virtual liver”. The virtual liver represents a spatio-temporal model of liver tissue with integrated metabolic and signaling response principles. Such simulations can be used to test or generate hypothesis. Examples of hypothesis originally generated by virtual liver approaches and later validated in vivo are: (i) The key role of sinusoidal endothelial cells (LSEC) for hepatotoxicity. Once hepatotoxic compounds destroy a critical fraction of LSEC the liver switches from a “perfect regeneration mode” to a “scar formation mode” which upon repeated insult finally leads to fibrosis and cirrhosis. (ii) Flow direction changes of detoxifying enzymes upon toxic exposure. For example CCl4 or paracetamol cause a switch of ammonia metabolizing enzymes (e.g., glutamate dehydrogenase) from ammonia production to ammonia consumption. This stress response protects the exposed organism from an excessive increase in ammonia blood concentrations. Such compen-
soratory mechanisms would be difficult to identify by in vitro systems alone. The examples illustrate advantages of the complementary use of in vitro systems and "virtual liver" approaches.

I-1-890

Use of transcriptomics approaches in human cell-based DNT testing

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Transcriptome analysis is a powerful tool to study gene expression changes in cultured cells exposed to toxicants. Such toxicogenomics data have been obtained for different model systems of developmental neurotoxicity (DNT), either as additional information or as qualitative endpoint. For the use of transcriptome data in toxicant prediction, classification or potency comparison, the experiments need to be standardized, and the ways of data exploration, interpretation and visualization will still benefit from further improvements. To address some of these challenges, we used neurally-differentiating human embryonic stem cells, and we studied the time-dependence of the transcriptome response concerning duration of exposure to toxicants and maturity of the culture system under investigation. In a next step, we investigated the design principles of concentration-dependent transcriptome deviations. We found that short exposures to compounds yielded information on potential pathways of toxicity, while long exposure mainly described phenotypic alterations of the cells exposed to the toxicants. These phenotypic changes were suitable for compound classification and prediction. Moreover, they allowed a relative potency ranking. The studies showed that it will be beneficial in the future to group transcriptome alterations of individual genes into superordinate biological processes, in order to condense the information and to facilitate the interpretation of studies as well as the visualization of the results.

Session I-1: Poster presentations

I-1-416

Performance standards for human epidermis model Keraskin-VM for skin irritation alternatives

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A skin irritation alternative method was developed using reconstructed human epidermis (Keraskin™-VM), and reported comparable to the EpiDerm™ Skin irritation test. Validation of Keraskin™-VM model has been undertaken following OECD TG439 performance standards for reconstructed human epidermis (Rhe) tests. Three laboratories in compliance with OECD GLP were participated in the validation. The method was transferred to two naive laboratories by a lead laboratory. The method transferability was evaluated into two phases; informative phase using 5% sodium lauryl sulfate as a (+) control, lactic acid, and butyl methacrylate, and blind phase using 3 irritant and 3 non-irritant chemicals listed in OECD TG439. After confirmation of successful transferring to the two laboratories, proficiency test was proceeded at the three laboratories. All 6 chemicals were correctly classified (accuracy, 100%). Within-laboratory reproducibility was assessed using 20 reference chemicals for 3 runs at three laboratories. Two laboratories resulted in 85%, and one laboratory in 90%. Between laboratory reproducibility was evaluated using the same 20 reference chemicals for 3 runs at three laboratories, resulting in 95%. Further results on reproducibility will be discussed. Keraskin™-VM model is believed comparable to the 4 Rhe model adopted by OECD.

For further reading see Jung et al. (2014) and OECD (2013).

References


I-1-657

Application of zebra fish and Neoderm®-ME, a new 3D pigmented skin model for the evaluation of anti-melanogenic effects of hexapeptoids; PAL-10 and PAL-12

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Peptoids are a class of peptidomimetics whose side chains are appended to the nitrogen atom of the peptide backbone, instead of α-carbons. It was reported that peptoid is more durable against the degradation by proteases and there is larger chance for making derivatives and chemical library than peptide. Peptoids are actively used in drug discovery, but rarely used in the discovery of cosmetic ingredients. In this study, we examined if PAL-10 and PAL-12, new peptoids, can inhibit melanogenesis by using zebra fish and Neoderm®-ME, developed by Tegoscience. After treating PAL-10 and PAL-12 at different concentrations on B16 cells (mouse melanocyte), Neoderm®-ME and zebra fishes, anti-melanogenic effects were compared with arbutin, a positive control. PAL-10 and PAL-12 significantly manifested anti-melanogenic effects in a dose-dependent manner in all three in vitro models as determined by image, histology and melanin contents. We also assessed skin irritation potential of PAL-10 and PAL-12 in Neoderm®-ED (a 3D human full thickness skin model) according to OECD TG436. There was no skin irritation caused by PAL-10 and PAL-12 at concentrations higher than those where anti-melanogenic effects were produced. We can conclude that PAL-10 and PAL-12 can be used as new cosmetic ingredient with strong whitening efficacy.
Evaluation of the Multi-ImmunoTox Assay (MITA) composed of 3 human cytokine reporter cell lines by examining the immunological effects of drugs

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We established a luciferase reporter assay system, MITA, to evaluate the effects on key predictive in vitro components of the human immune system (Kimura et al., 2014). The system is composed of 3 stable reporter cell lines transfected with 3 luciferase genes, SLG, SLO, and SLR, under the control of 4 cytokine promoters, IL-2, IFN-g, IL-1β, and IL-8, and the G3PDH promoter. We first compared the effects of dexamethasone, cyclosporine, and tacrolimus on these cell lines stimulated with PMA and ionomycin, or lipopolysaccharides, with those on the mRNA expression by the mother cell lines and human whole blood cells after stimulation. The results demonstrated that MITA correctly reflected the change of mRNA of the mother cell lines and whole blood cells. Next, we evaluated other immunosuppressive drugs, off-label immunosuppressive drugs, and non-immunomodulatory drugs. Although MITA did not detect immunosuppressive effects of either alkylating agents or anti-metabolites, it could demonstrate those of the off-label immunosuppressive drugs, sulfasalazine, chloroquine, minocycline, and nicotinamide. Compared with the published effects of the drugs, these data suggest that MITA can provide a novel high-throughput approach for detecting the immunological effects of chemicals, other than those that induce immunosuppressive effects, through their inhibitory action on cell division.

For further reading see Kimura et al. (2014).

Reference

Evaluation of zebrafish embryo as alternative model to predict hepatotoxicity

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Hepatotoxicity is one of the causes of drug attrition. Zebrafish assays show promise to assess hepatotoxicity, though subjective phenotypic scoring and time are main drawbacks. In the ZETOX project, liver toxicity in zebrafish embryo is assessed using gene expression as biomarker approach, complementary to phenotypic analysis with the purpose to contribute to mechanistic understanding and improved human hepatotoxicity prediction. Liver morphological effects of 5 hepatotoxic (acetaminophen, amiodarone, coumarin, methapyrilene, myclobutanil) and 2 negative compounds (saccharin, biotin) were assessed in zebrafish embryo at 5 days to define sublethal concentrations for gene expression experiments. Analytical methods were optimized to analyze the stability and internal concentration of the chemicals. Detection of hepatocyte markers (CP, CYP3A65, GC, TF) were accomplished by in situ hybridization for coumarin and myclobutanil and confirmed by real-time qPCR. Experiments showed decreased expression of all markers. Next, other liver-specific biomarkers (FABP10a, NR1H4) and general apoptosis or stress-induced markers (CASP3A, CYP3A19, TP53, ZGC165022) were screened using real-time qPCR for the 7 compounds. Differential gene expression in relation to observed hepatotoxic effects will be discussed in view of optimization of this whole-organism-based method for mammalian hepatotoxicity.

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Developmental effects of ToxCast™ chemicals on alternative animal models: C. elegans and zebrafish

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Alternative animal models share many advantages with in vitro assays, with the added advantage of exhibiting complex whole organism responses to chemical exposures. Over 1000 unique compounds in the ToxCast Phase I and II libraries (Kavlock et al., 2012) were screened using a high-throughput C. elegans larval growth and development assay (Boyd et al., 2009). Changes in the size of individual nematodes were measured using COPAS Biosort flow cytometry after 48-h chemical exposures. Isotonic regressions were used to analyze the response over seven concentrations (0.5-200 μM), providing efficacy and potency estimates, expressed as a change in response between control and highest concentration (Δ) and the chemical concentration at which half of Δ is reached (CΔ/2). More than 50% of the compounds caused a significant decrease in nematode growth; the most toxic 5% of these compounds included several organic pollutants (e.g., DDT, PFOS), which have been banned from use due to their toxicity and bioaccumulation. Comparisons of compound activities in the C. elegans growth assay were made to those from zebrafish embryonic develop-
ment studies (Padilla et al., 2012; Truong et al., 2014), as well as rodent data available in the US EPA’s ToxRefDB (Knudsen et al., 2009). These comparisons showed good agreement between C. elegans and zebrafish data and moderate agreement with rodents.

References


I-2-511

The throughput-compatible embryonic stem cell test (EST)

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The EST is an ECVAM validated cell-based assay to evaluate embryotoxicity of substances based on cytotoxicity and cardiomyocyte-differentiation endpoints. The later uses the formation of embryoid bodies (EBs) from mouse embryonic stem cells (ESC) by the hanging-drop (HD) method. The traditional process of EB production and handling technique is prone to generate EBs with non-uniformity and shows a labor-intensive process. The method for EB production was adapted to a novel top-loadable, throughput-compatible HD-plate in a 96-well format to enhance the efficiency of the EST-proceeding. ESC cultured for 5 days in the HD-plate generated EBs with size uniformity at days 3 and 5 (diameters: 319 µm ±3.0% and 466 µm ±5.2%, respectively). At day 5 direct-transfer of EBs to a 96-well adherence plate by adding excess of medium into the HD-wells, the EBs adhered and differentiated with a final cardiomyocyte differentiation efficiency of contracting EBs of 88% ±13% at day10. The differentiation efficiency and the embryotoxic classification of substances from the ECVAM test panel were in-line with published data.

We adapted the traditional manual HD method to develop a technically more sophisticated, throughput compatible process with significant time saving (80%). The EST provides an efficient tool to investigate alternative endpoint complementing the conventional embryotoxic substance characterization.

I-2-622

The usage of *Daphnia magna* for screening of muscarinic cholinoreceptors antagonists

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In the experiments on the rats we have developed methodological approach to the evaluation of the selectivity of muscarinic cholinergic receptors (M-ChR) antagonists action in the whole organism conditions. According the results obtained during investigation the protective effect of M cholinoitlyes substances from the eCV AM test panel were in-line with published data - different M cholinolytes between the ratios of eD50 of M antagonists in the tests with arecoline and organophosphates (DDVP, DFP, etc.) depends on M subtype ChR occupation. The efficiency of antagonists in inhibition of tremor reaction caused by M-ChR agonist arecoline administration associates with interaction of M2 subtype of ChR. It was established by the method of linear regression, that there was a high degree of correlation (r=0.99) for different M cholinoitlyes between the ratios of ED50 of M antagonists in the tests with arecoline and organophosphates and the ratios of dissociation constants of antagonists complexes with M-ChR from the homogenates of rat’s cerebral cortex and heart containing M1 and M2 ChR subtypes, respectively. Thus, the ratio of ED50 arecoline/DDVP serve as a measure of the selectivity of drugs action. On the experiments to *Daphnia magna* the effects of some non selective, mainly M1, and M2, ChR antagonists on the toxicity of DDVP and arecoline were studied. It was shown that a ratio of the average effective concentrations (EC50) of M antagonists in the tests with arecoline and organophosphates also may be used as a measure of the selectivity of M-ChR antagonists action.

I-2-644

The Tox21 “1500 genes” high throughput transcriptomics project

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The Tox21 consortium aims to use in vitro and alternative in vivo systems to: 1) identify patterns of compound-induced biological responses, 2) prioritize compounds for more extensive toxicological evaluation, and 3) develop predictive models for biological responses in humans. In support of this effort, we initiated a project to identify a targeted subset of ~1500 human genes to evaluate how the transcriptome in different biological systems responds to chemical exposures. The transcriptome is a reflection of all genes expressed at a particular point in time in response to a particular environment and is altered in response to biological, physical, and chemical treatments in a dose- and time-dependent manner. The goal is to ultimately generate a similar list for rat, mouse, zebrafish, and *C. elegans*. This set of ~1500 sentinel genes will be implemented using a high-throughput, cost effective gene expression technology and will have the following attributes: (1) it captures maximal expression variability and pathway coverage, especially for pathways hypothesized to be involved in adverse health effects, and (2) the results can be extrapolated to the transcriptome. The status of this effort and the initial experiments to demonstrate the validity of the approach will be presented.
A new approach to the automatic segmentation and evaluation of pigmentation lesion by using active contour model and speeded up robust features

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Digital image processing techniques have wide applications in different scientific fields including the medicine. In this paper, we propose an automatic method for segmenting the skin lesions and extracting features that are associated to them. This is achieved by combining the Speeded-Up Robust Features (SURF) and Active Contour Model (ACM). In the suggested method at first the area of the skin lesion disorder is segmented from the image and then some features like the mean, variance, RGB and HSV parameters are extracted. The work has been tested on 20 images. Comparing the segmentation results by use of Otsu thresholding method, ACM and SURF show the superiority of ACM method over the two others. The proposed method for skin lesion segmentation which is a combination of SURF and ACM gives the best results. To assess the practical robustness of our method, we have used it for segmentation of different types of skin lesion images. Result of applying the proposed method on 20 images shows the high performance, speed and accuracy of it. We believe that this work is applied the most powerful and newest methods of image processing for segmenting the skin lesions.

Physiological oxygen concentrations in a sandwich culture on gas-permeable membranes remarkably enhance rat hepatocytes functions and genes expression

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Oxygen supply is a critical issue in the optimization of in vitro hepatocyte microenvironments. It is essential to match the oxygen consumption rate (OCR) and provide the proper oxygen tension at the cellular level. To achieve these goals, a sandwich culture based on oxygen-permeable membranes was used to investigate the effects of physiological oxygen concentrations on the efficient functioning of hepatocytes in vitro. Rat hepatocytes were cultured using the sandwich culture method with Matrigel on the PDMS (polydimethylsiloxane) membranes under various oxygen concentrations [20%-O2 (+), 10%-O2 (+) and 5%-O2 (+)]. In parallel, TCPS (Tissue culture treated-polystyrene) plates with PDMS membrane inserts were used as the control groups.

The results indicated that the hepatocytes cultured under 10%-O2 (+) exhibited extended survival, normoxic OCR, improved maintenance of metabolic activities and functional polarization. Additionally, the expression levels of various drug-metabolism genes, as examined by PCR arrays, were closest to those of freshly isolated hepatocytes. This study shows that it is important to meet the cellular oxygen de-
mand of hepatocytes at in vivo-like physiological levels, and it also reports on an oxygen permeable membrane system to provide a simple method for in vitro studies.

For further reading see Matsui et al. (2010, 2012); Sakai et al. (2012).

References

I-2.371
A feasibility study into the biodistribution of test compounds in zebrafish embryos
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The embryonic zebrafish is a promising early screening model bridging the gap between simple cell-based assays and studies with higher animals. An important obstacle for the model is the determination of internal exposure which might deviate substantially from the exposure concentration in the water. This may result in false negative or false positive outcomes. Only analyzing the concentration in whole-organism homogenates does not consider the complexity of the zebrafish as a whole organism approach. In this feasibility study we addressed this issue by examining the biodistribution of test compounds in zebrafish. Hereto, zebrafish embryos and larvae were treated with radioactive compounds and biodistribution was semi-quantified by autoradiography of serial microscopic sections. Total body burden of larval and embryonic zebrafish largely depends on logP for hydrophobic compounds. Bioaccumulation of compounds may result in an overestimation of effects. In this study differences in biodistribution were observed in both embryos and larvae. In embryos up to 80% of test compounds stuck to the chorion, whereas in larvae up to 75% of test compounds remained in the gastrointestinal tract. This limited bioavailability may result in an underestimation of effects. By testing biodistribution of more compounds, the predictability of the zebrafish model will further increase.

I-2.477 *
Cellular Ellman’s method adaptation using SH-SYSY is a possibility for neurochemical multiparametric evaluation
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Introduction: Acetylcholinesterase is a membrane-bound enzyme, with its active site on the cell outside. It is an important enzyme studied in neurotoxicology and neuropathology, since it is involved in neuron system development and neurophysiological processes.

Aim: Developing a modified Ellman’s method for acetylcholinesterase activity assay without cell damage, allowing its use in in vitro neurotoxicological test batteries, reducing costs, experimental times, procedures, and contributing to future replacement of animal testing in neurotoxicology.

Method: Ellman’s method was modified for 96-well plate using neuroblastoma cell line (SH-SYY). Cellular conditions (density and viability by XTT) and interferences on the method (PBS, media components) and on cell viability (DTNB, acetylthiocholine, reaction products) were tested in kinetic analysis. Anticholinesterasic chemicals (quinidine and aldicarb) were tested.

Results: Media components and PBS interfere in Ellman’s method. Washing attached cells twice with PBS and analyzing the test in PBS, result in minimum interference. 5 mM of Quinidine sulphate (drug) decrease in 47% the activity of AChE in real time, with no cell damage. Aldicarb (pesticide) during 30 min incubation presented higher viability IC50 than enzyme IC50 (1.962 mM and 0.608 mM, respectively).

Conclusion: Non-damaging Ellman’s method adaptation is a promising method for multiparametric neurochemical evaluation.

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I-2.491
A data analysis pipeline for Tox21 phase II quantitative high throughput screening assays and its application to toxicity profiling of flame retardants
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The U.S. Tox21 program (Tice et al., 2013) is currently screening a 10K compound library against toxicity pathways using a quantitative high throughput screening (qHTS) approach. Robust compound activity profiling is an essential step towards compound toxicity prioritization and predictive human disease model construction. However, the existence of non-reproducible signal artifacts (e.g., signals caused by compound activity carry-over or noise) and compound-dependent assay interference (e.g., compounds that are auto-fluorescent or cytotoxic) complicates compound activity interpretation. We have developed a data analysis pipeline that addresses these issues and found that signal reproducibility greatly improved after removing the non-reproducible artifacts while compound active rate was reduced after removing compounds flagged as having compound-dependent assay interference. We demonstrated the in vitro activity profiling results using 49 brominated or organophosphate flame retardants (FRs) present
in the Tox21 10K compound library. For the FRs evaluated, some of the outcomes were consistent with known in vitro health effects and new compound-pathway interactions were identified. To facilitate data exploration, a web portal (http://spark.rstudio.com/moggces/profil-
ing/) was designed to host the compound signal/activity profiling data analyzed by the pipeline. The pipeline and the web portal are useful tools for compound signal/activity profiling in qHTS assays and for data exploration.

Reference

I-2-582
Automation-compatible EST assay
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The Embryonic-Stem-Cell Test (EST) is a cell-based assay to evaluate embryotoxicity of substances and belongs to the portfolio of ECVAM validated in vitro assays. The current EST-process is a manual and time consuming process due to manual transfer of EB’s into a receiver plate. In this study the EST assay was modified to enable an automation-compatible EST-process.

EBs were formed for 5 days in a 96-well hanging drop plate to achieve EB formation and induce cardiomyocyte differentiation. At day 5 EBs were directly transferred into an adhesive 96-well plate by placing the HD-plate on top of a receiver. Adhered EB’s were monitored for cardiomyocyte differentiation at day 10. ESCs aggregated in the hanging drop and formed round shaped EBs of uniform size within 5 days. Size analysis of EBs resulted in diameters of 319 µm ± 3.0% at day 3 and 466 µm ± 5.2% at day 5, respectively with a contraction efficiency of 68% ± 13% at day 10. Compounds selected from the ECVAM test-validation panel resulted in similar classifications as with the original EST protocol.

We adapted the validated EST method towards an automation-compatible process leading to significant time savings (up to 80%) to further foster the use of the EST-assay.

I-2-668
Evaluation method in Bhas 42 cell transformation assay selecting transformed foci using hydrogen peroxide, H₂O₂
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Bhas 42 cells, which were established by the transfection of the v-Ha-ras oncogene into the BALB/c 3T3 A31-1-1 cells, form a contact-inhibited monolayer. By exposure to carcinogens, however, aberrant colonies (transformed foci) which are capable of invading the non-transformed contact-inhibited monolayer are induced. In the cell transformation assay (CTA), transformed foci are counted by observation to evaluate carcinogenic potential of chemicals. We developed a method to select transformed foci using H₂O₂ (Japan Patent Application No. 2009-206686, European Patent Application No. 09169 631.0). By exposure to H₂O₂, non-transformed cells die but transformed cells are alive. At the end of culturing in CTA, Bhas 42 cells plated in 96-well plate were exposed to H₂O₂ and survival cells in each well were evaluated by measuring optical density (OD). Comparison of results between observation and H₂O₂ methods showed that evaluation using cut-off value of OD was more comparable with the observation method than that using OD values. By combination of H₂O₂ and 96-well plate methods, it made it possible to evaluate carcinogenic potential of chemicals without observation of transformed foci in the Bhas 42 CTA.

I-2-741
Using Skin-PAMPA for transdermal patch testing
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Using skin as absorption site presents interesting features that have facilitated the progression of transdermal drug delivery in the past decades. Efforts in drug research have been devoted to find a quick and reproducible model for predicting the skin penetration of molecules.

The parallel artificial membrane permeability assay (PAMPA) has been recently extended by this group for the prediction of transdermal penetration (Skin PAMPA™) (Sinkó et al., 2012). This commercially available system has been modified to make it suitable for transdermal patch testing.

Four API’s (nicotine, fentanyl, ketoprofen and rivastigmin) have been investigated, each applied in 1-3 marketed transdermal patches. The permeation vs. time profile demonstrated linear release profile in every case, though the cumulative permeated amount was about 30% higher than expected that can be caused by the edge effect reported by Hadgraft and co-workers (Hadgraft et al., 1991). In vitro/in vivo correlation of permeation profiles were performed based on manufacturers’ data and resulted in acceptable correlation.

Skin PAMPA system appears to be a useful tool for transdermal patch comparisons, though standard protocol needed to be modified. Results can be used for patch comparison and for ranking, therefore Skin PAMPA can provide valuable information for transdermal patch development.

References
Session I-3a: Tissue-on-a-chip / Human-on-a-chip

Co-chairs
Suzanne Fitzpatrick, Johns Hopkins University, USA
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Session I-3a: Oral presentations

I-3a-316

Human beating heart on a chip for cardiotoxicity testing as an example case of the Dutch organ-on-chip initiative

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A novel Dutch initiative in the form of a precompetitive R&D Institute (hDMT) integrates state-of-the-art stem cell technologies with tailored physics/chemistry/engineering expertise to create miniature human organ and disease tissues-on-a-chip, closely mimicking human (patho)physiology, enabling "(pre-)Clinical Trials on Chips" and development of personalized drug treatment, and reducing the need for animal models (van de Stolpe and den Toonder, 2013)

To illustrate the potential, the Cytostretch cardiotoxicity model will be presented. Cardiotoxicity, often as cardiac arrhythmias, is a frequent cause for drug withdrawal from market and late-stage clinical drug failure. A stretchable Micro-Electrode Array has been developed, consisting of a thin stretchable and patterned membrane with embedded electrodes (Cytostretch), enabling alignment of plated human cardiomyocytes in a chosen direction and cardiomyocyte stretching with heartbeat frequency (Dambrot et al., 2011; Braam et al., 2013; Saeed et al., 2014). This allows mimicking the beating human heart during various levels of exercise (with associated increases in cardiac load and stretch), while monitoring cardiomyocyte electrical activity to detect arrhythmias (e.g., QT elongation) caused by drug compounds. The genetic background of a patient may codetermine toxicity, thus next generation models will include iPSC-derived cardiomyocytes with known human ion channel variations. This approach is expected to rescue cardiotoxic drugs, by identifying patients at risk with a companion diagnostic assay.

References

I-3a-584

In vitro microphysiological systems: advancing regulatory science through innovation

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Advances in bioengineering and material sciences, microfabrication, and microfluidics technologies enabled the development of microphysiological systems that mimic the functional units of an organ. These advances have made it possible to initiate the engineering of cellular environments and/or functional units of lung, heart, blood vessels, muscles, bones, liver, nervous system (including eye), gut, and kidney. These microsystems reflect human physiologically relevant parameters, including proper cell-to-cell, cell-to-matrix, biochemical and mechanical signaling, but lack the complex architecture of tissues. The National Center for Advancing Translational Science (NCATS) / US Food and Drug Administration (FDA) / Defense Advanced Research Projects Agency (DARPA) partnership for the development of in vitro microphysiological systems is a groundbreaking example of the types of partnerships that are needed to bring innovative new technologies into the regulatory paradigm. NCATS, FDA and DARPA are collaborating to develop a chip to screen for safe and effective drugs which is far more swift and efficient than current methods. NCATS will provide science-based solutions to reduce costs and the time required to develop new drugs and diagnostics. FDA will determine how this new technology can be utilized to assess drug safety. DARPA and NIH will facilitate collaborations between researchers and FDA to advance program goals.

I-3a-694

A dynamic human-on-chip platform for neurotoxicity studies

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Homunculus™ is a multi-organ, platform designed for use in the pharmaceutical industry, personalized medicine etc. Homunculus platform consists of a flexible, microfluidic control unit and a cell chip with multiple, separate cell compartments, each the size of a standard well from a 96-well plate.

Using HepaRG and NSC-iTERT cells in Homunculus neurotoxicity evaluation model was developed. Suitable for hepatic and neural cells
media was selected, and functional activity of cells was confirmed by whole-transcript expression profiling.

Liver spheroids and neural cells were cultivated up to 14 days with daily media exchange. Culture medium were perfused in closed circuit, imitating blood circulation and daily exposure to the drug was applied. Viability and functional activity of cells could be monitored and different end-point analysis method was established to evaluate neurotoxicity of the drug.

Using a Human-on-chip approach, Homunculus gives researchers the unique opportunity to investigate human cell models responses in vitro. It combines the advantages of the cell line and animal model neurotoxicity studies.

In this presentation, I will describe work we have been carrying out in the Biomimetic Microsystems platform at the Wyss Institute for Biologically Inspired Engineering at Harvard. The goal of this platform is to engineer human “Organs-on-Chips”: microfluidic devices lined by living human cells created with microchip fabrication techniques that recapitulate organ-level functions as a way to replace animal testing for drug development and to create in vitro human disease models. These biomimetic devices provide a window on human physiology as they enable real-time, high-resolution microscopic imaging as well as analysis of biochemical, genetic and metabolic activities of living cells when they are positioned within the context of functional tissue and organ units. I will review recent advances we have made in development of multiple organ chips, including human lung, gut, kidney and bone marrow chips, as well as on-chip models of human diseases, including pulmonary edema and inflammatory bowel disease. In addition, I will describe our ongoing efforts to develop more than 10 different organ chips, to integrate them into a “human body on chips”, and to engineer an automated instrument for real-time analysis of cellular responses to pharmaceuticals, toxins and other chemicals.

Developments towards physiologic in vitro models in Switzerland


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The next step towards more biomimetic in vitro models is the design of multi-organ devices which allow communication among different tissue types. The Swiss competence center TEDD (Tissue Engineering for Drug Development and Substance Testing) presents an overview of novel “body on a chip” concepts currently under development in Switzerland. The majority of those concepts employ microtissue engineered approaches in combination with microfluidics. The microfluidic chips contain dedicated micro-chambers to immobilize and/or grow microtissues or cells in an in-vivo-like environment, in which the shear stress, the continuous transport of drug, nutrients and oxygen and/or the mechanical stress induced by the breathing motion are reproduced. The models which are evaluated currently comprise liver and lung tumor devices to evaluate the impact of prodrug-activation on tumor growth, lung on a chip as disease models and for nanoparticle toxicology testing and a platform technology to monitor label-free chronic toxicity effects.

Human organs on chips as replacements for animal testing

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In this presentation, I will describe work we have been carrying out in the Biomimetic Microsystems platform at the Wyss Institute for Biologically Inspired Engineering at Harvard. The goal of this platform is to engineer human “Organs-on-Chips”: microfluidic devices lined by living human cells created with microchip fabrication techniques that recapitulate organ-level functions as a way to replace animal testing for drug development and to create in vitro human disease models. These biomimetic devices provide a window on human physiology as they enable real-time, high-resolution microscopic imaging as well as analysis of biochemical, genetic and metabolic activities of living cells when they are positioned within the context of functional tissue and organ units. I will review recent advances we have made in development of multiple organ chips, including human lung, gut, kidney and bone marrow chips, as well as on-chip models of human diseases, including pulmonary edema and inflammatory bowel disease. In addition, I will describe our ongoing efforts to develop more than 10
Session I-3b: Oral presentation

**I-3-008**

**µ3DVasc: a novel microfluidic bioreactor setup for the reconstruction of vascularized 3D tissues in vitro**

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The development of three-dimensional (3D) organotypic cell culture models has been emerging as a novel method, which may implement the 3R principle (Huh et al., 2011; Ghaemmaghami et al., 2012). As the nutrition of almost all tissues is ensured via a network of blood vessels, a disposable microfluidic 3D vascular microstructure (µ3DVasc) has been fabricated to provide a more physiologically relevant in vitro model of vascularized tissues. The curvilinear and porous channel, which provides space for culturing human microvascular endothelium, is produced by the combination of swift heavy ion and microthermoforming technology (SMART) and is connected to a microfluidic circuit (Giselbrecht et al., 2006). Adjoined to the artificial vasculature is a second microfluidic compartment, which can be colonized with hydrogel supported 3D structures of different tissues and serves to remove the lymph fluid. Furthermore, the porosity of the microstructure supports the supply of 3D tissues with nutrients and gases and allows for analysis of transendothelial transport of immune cells or drugs (Hebeiss et al., 2012). To study the reconstruction of the Blood-Brain-Barrier we have developed a 3D culture of human pericytes and astrocytes in collaboration with the Wyss Institute at Harvard. This unique model mimics the anatomic situation in vivo and enables the investigation of potential interactions at crucial tissue-tissue interfaces.

**References**


**I-3-037**

**Serial linkage of biochips toward an organ-on-chip approach**

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The development of drugs is a costly and time-consuming process. Although high throughput methods based on cell cultures are available, they only give a hint on the real effect of a future drug. Traditional two-dimensional cell cultures are not able to reproduce/emu late the complex interactions of different types of tissue like it exists in the human body. Such interactions include the absorption, distribution, metabolism and elimination (ADME) of a drug (Esch et al., 2011; Sung et al., 2010).

Recently new approaches reforming the cell culture techniques were published. Most of them are based on several microchambers that are cultivated with different cells. Microfluidic channels are used to link these chambers and to ensure exchange of metabolites. With such devices, e.g., primary cells can be cultivated for a longer time period and with a smaller loss of functionality than in former single-type 2D cell-cultures (Sung and Shuler, 2010; Huh et al., 2011).

A lab-free long-term monitoring of organ-on-chip systems would affirm the benefits of such systems. Electrochemical sensors are well suited for this task, but need to be carefully integrated.

As proof-of-concept we serially linked two electrochemical cell-monitoring systems (IMOLA-IVD, Weiss et al., 2013) and showed that the exchange of metabolites can be monitored without any cross-talk in a label-free, long-term and multi-parametric manner.

References

131-101
Cell-based rapid and quantitative toxicity and efficacy monitoring in consecutive before and after the compound metabolism within liver for two ginger compounds at physiological concentration
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Objectives: Animal test is often used for compound toxicity and efficacy evaluation although the results obtained are different from clinical test and show total metabolism (Damia and D’Incalci, 2009). We have developed an original human cell-based assay device, High-Precision Surface Plasmon Resonance (HP-SPR), and method (Ona and Shibata, 2010; Ona, 2013). Here we examined the applicability of HP-SPR to elucidate toxicity and efficacy including metabolism of two ginger compounds as examples working against liver before and after their metabolism.

Methods: Human liver cell Hep G2 was used for two ginger compounds of 6-shogaol (SHOG) and 6-gingerol (GING). The 2D cultured cells were self-attached to an HP-SPR sensor chip surface and covered by collagen to obtain in vivo like cell status (Ona, 2013) and monitored.

Results: In SHOG (heated GING), the efficacy of liver activation was observed at 100 nM. This activation was twice higher in after metabolism. Apoptotic effect as toxicity was observed at 1000 nM to inhibit liver activation before the metabolism. However, after the metabolism, its effect was reverted and detoxification was processed. In GING, 100 nM showed apoptotic effect as toxicity before and after the metabolism, and no effect was observed below 100 nM. The different mode of action was successfully monitored between two ginger compounds.

References

131-141
A multi-organ-chip co-culture of human liver equivalents and neurospheres for long-term substance testing
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Current in vitro and animal tests for drug development are failing to emulate the 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 (MOC) platform capable of co-cultivating up to three organ equivalents inside a combined media circuit. A peristaltic on-chip micro-pump reproducibly operates a PDMS-embedded microcirculation system, emulating the systemic arrangement of organs within the human body. It could be shown, that the multi-organ-chip is capable of supporting long-term co-cultures of human artificial liver microsomes and neurospheres. Cultures were successfully maintained functional over a period of up to 14 days. Liver cell polarity was restored as shown by the expression of specific transporters, tight junctions and the formation of rudimentary bile canalicular-like structures. Vitality of the cells was assessed by TUNEL/Ki 67 staining and was markedly increased compared to static controls. Neurospheres derived from the Ntera-2 cell line were strongly positive for neuronal markers MAP2 and β-Tubulin III after 14 days of culture in the MOC as assessed by immunohistology and qPCR. Chronic exposure of the cultures to 2,5-hexanedione over 14 days revealed a dose-dependent toxicity on MOC co-cultures.

131-219
3D-printing: a new dimension for the 3R’s
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3D printing is a collective term for innovative techniques that have rapidly developed in the last few years. Some talk about a new industrial revolution. Even though the technique is still in the process of development, there are wide ranging potential applications. These
include medical and biomedical applications such as medical devices, pharmaceuticals, medical research, and the development of new in vitro tests. Together with other innovative techniques such as organ-on-a-chip, tissue engineering, synthetic and biological stem cells, 3D printing has great potential to contribute to the 3Rs – replacing, reducing and refining animal experiments. For instance, printed organs-on-a-chip (of human origin) can be used in toxicity and efficacy testing of medicines. This would provide the data needed and save on the use of laboratory animals. However, perhaps the greatest impact of innovations would be in avoiding and preventing animal experiments as a whole. When more knowledge about the physiology of the human body is available, animal experiments will finally no longer be necessary. Thus, advances in innovative technologies have to be used to the fullest extent to make reduction and prevention of the use of animal experiments possible.

I-3-265

Long-term culture of dermal units in multi-organ-chips

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Tests for drug development requires an almost perfect fit with the human (patho-)physiological microenvironment. The majority of currently commercially available skin equivalents are based on static culture systems emulating human epidermis only, or combining epidermis and dermis in so-called full thickness skin equivalents. None of the existing systems contain important elements, such as vasculature, skin appendices, or an immune system. Therefore, current in vitro and animal tests are failing to accurately predict drug toxicity.

Here, we are aiming to model a microvasculature driven cutaneous homeostasis of normal, diseased skin and hair follicle biopisies, as well as their bioengineered equivalents in our perfused, self-contained and endothelialized multi-organ-chip (MOC) system. Our MOC platform uses a miniaturized circulatory network with an integrated micro-pump to provide pulsatile circulation of microliter-volume of medium to support milligrams of human tissue constructs.

In comparison to cultures utilizing conventional static conditions, dermal units cultivated in our perfused MOC system showed remarkable consistency of the cutaneous structure and vitality rendering the MOC system a useful tool for long-term culture.

The current status of the development and remaining hurdles will be further discussed.

I-3-303

Synthesis and characterization of tunable agarose-gelatin cryogel for hepatotoxicity evaluation

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Risk assessment of chemicals in India at present relies on either animal models or 2D monolayer culture systems. Ethical concerns and inaccurate results are driving researchers to develop new in vitro models based on advanced cell culture techniques. To this end, 3D models provide a better alternative due to their tissue complexities. Progressive advancement has been attained in 3D cultures but efforts are warranted to develop appropriate and cost-affordable models for better screening of chemicals given the endemic limitations in the scenario in India. 3D culture as a mainstream approach for toxicity testing requires the development of standard protocols and quantitative analytical methods.

In this study, we developed a 3D based high-content screening model with hybrid Agarose-Gelatin (AG) cryogel for identifying hepatotoxic effects of drugs. SEM and FTIR were adopted which showed well-interconnected porous structure. The Cyclic Swelling Kinetics and Swelling Ratio of the gel were also performed. Biocompatibility studies with HepG2 cell line revealed good cell attachment and proliferation. Preliminary cytotoxicity assay with Paracetamol revealed advantageous of our 3D model over the already available 3D hepatotoxicity testing models.

I-3-389

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
Automated long-term operation of multi-organ-chips

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In order to simplify the handling of complex Multi-Organ-Chips (Wagner et al., 2013) – TissUse, TU Berlin and partners have developed a robotic platform. This technique offers a new approach to the monitoring and handling of intricate Lab-on-Chip devices. Our prototype is capable to maintain up to 10 MOCs. This offers a novel possibility to substance testing or the monitoring of Organogenesis. Each operation can be individually programmed and adjusted to the user’s needs. For example OECD guidelines for acute toxicity testing can be performed as well as the monitoring of organoid development over time (Materne et al., 2013; Atac et al., 2013). The platform features functions such as automatic media supply, sampling and storage, temperature control, fluorescence and microscopic monitoring, PIV and O2-measurement. To display the functionality we performed toxicity tests with different organoid types.

References
Atac, B. et al. (2013). Lab Chip 13, 3555-3561.
innovate. Various in **vitro** tests have been developed to predict the in **vivo** situation. However, no existing assay reflects the biological complexity. Instead, a battery of assays – addressing several key events of the adverse outcome pathway – is required.

To integrate the biologically relevant crosstalk of keratinocytes and dendritic cells, we established a co-culture of human epidermis with primary, monocyte-derived dendritic cells (DCs) in our Multi-Organ-Chip (MOC). MOCs consist of a microchannel system connecting two culture compartments for cells or biopsies. A peristaltic on-chip micropump enables the circulation of the medium, allowing a cross-talk of different cell types. Utilizing this system, we applied different sensitizers directly to the medium and analyzed the activation of DCs based on the induction of CD86. Moreover, we performed topical application of the compounds using EpiDerm™ models instead of human epidermis.

Here, we report on the differences in DC activation for identical substances among our first-time developed MOC-based sensitization assay and a conventional static assay using only DCs, implying that our assay is a promising approach to improve the prediction of skin sensitizers.

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I-3-768

**How new technologies are promising to fulfil the Reduction and Replacement 3Rs objectives**

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In **vitro** research has changed dramatically over the past 10 years. Most researchers now recognise the inadequacies of traditional cell culture methods (on or in plastic or glass, under static conditions) using animal cells or immortalised cancer-derived cell lines. There is an increasing focus on faithfully replicating the **in vivo** environment so that **in vitro** results tell us accurately what is happening in the body. A variety of technologies and techniques are now employed to mimic **in vivo** conditions: 3D constructs to replicate tissue architecture; co-culture to allow different cell types to communicate the way they do in the body; flow to refresh nutrients and stimulate cells with physiologically-relevant shear stress. These new methodologies have facilitated the advancement of 3Rs objectives.

In this paper we describe a system that will allow the incorporation of more of these physiologically-relevant parameters. We consider the effects of a selection of factors such as oxygen concentration, glucose availability, pressure, motion and circadian rhythm on cells, discuss their relative importance and how they might be replicated **in vitro**. The paper concludes with a roadmap of how the “organ-on-a-chip” technology is likely to develop from the R&D phase through to a more routine testing capability.

I-3-784

**Kidney toxicity assessment and ADMET testing in a two-circuit microfluidic device**

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Kidney toxicity to date is badly predictable in cellular assays and animal experiments. Among all drug candidates failing because of organ toxicity the failure rate due to kidney toxicity is only 2% in preclinical studies but 19% in clinical phase III studies. Therefore, there is a translational gap and a need for improved preclinical models.

We developed a microfluidic device made up of two interconnected PDMS-embedded circuits mimicking the renal blood and urinary system. Each circuit can be operated at physiological flow rates by an on-chip-micropump. The interface between the circuits is a microporous PET-membrane that can be seeded with renal epithelial and endothelial cells. The device also includes culture spaces for intestinal cells and liver aggregates for ADMET profiling.

We could show viability and functionality of a human renal proximal tubule cell line (RPTEC/TERT1) for up to 10 days in the device. Furthermore we performed first substance exposure experiments with tobramycin and other known nephrotoxins.

Taken together, we developed a microfluidic device that is suitable for assessment of kidney toxicity. In the future this could be used to improve the translation from pre-clinical to clinical studies and determine the ADMET profile of substances to replace animal experiments.

I-3-792

**Microtissues meet microfluidics: a multi-parallel body on a chip concept**

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The next step towards more biomimetic **in vitro** models is the design of multi-organ devices which allows communication among different tissue types. Here, we present a novel “body on a chip” concept which not only allows interaction among different microtissue types, but is also robust and flexible thus enabling its routine use for substance testing. Spherical microtissues are interconnected by tilting the microfluidic chip, therefore avoiding tubing and external pumping technology. The microfluidic chip contains dedicated micro-chambers to immobilize pre-formed spherical microtissues, and continuous perfusion for cultivating is facilitated through interconnecting micro-channels. The chip design allows operating up to 48 devices in parallel, and replicates of various drug concentrations can be tested. A proof of concept study was performed by interconnecting rat liver and colorectal tumor microtissues on the chip. Interestingly, rat liver microtissues secreted elevated levels of albumin over the first 10 days on the chip prior to reaching equilibrium. For proof of feasibility, pro-drug activation of cyclophosphamide (CPA) on the chip was compared to static conditions. A significant impact of CPA on the tumor model was only observed with the chip; whereas, supernatant from static liver microtissues treated with CPA did not lead to significant anti-tumor effects.
Drug-induced hepatotoxicity is one of the most important reasons for the attrition of drug candidates. The availability of suitable human liver-based in vitro models allowing reliable metabolism screening and early detection of acute to chronic drug-induced liver injury has great interest for the pharmaceutical industry. Promethera Biosciences, a cell therapeutic biotech company, develops 2D/3D-cell-based assays based on human adult liver progenitor cells (Soka, 2011). Key hallmarks are their non-tumorigenic human origin, acquisition of metabolic capacity within the range of primary hepatocytes, long-term stability in culture, large-scale production yield, efficient cryopreservation and controlled differentiation & reproducibility (O’Brien et al., 2004). Both 2D/3D models form a spontaneous co-culture system of hepatocytes embedded in a cellular stroma. Successful hepatic differentiation-maturation is evidenced by expression of HNF4a, CK19, albumin, OTC and UGT1A at RNA and protein level (Buyl et al., 2014). Moreover, they display inducible and kinetic Phase I cytochrome P450-dependent enzymatic activity (CYP3A4/OH-Midazolam, CYP1A2/Acetaminophen, CYP2B6/OH-Bupropion, CYP2C9/OH-Diclofenac), Phase II paracetamol-sulfotransferase and bilirubine-glucuronidation conjugation capacity and active Phase III uptake (OATP) and efflux (MRP2) transport. Hepatic functionality was preserved for up to 1 month. Their substantial metabolic capacity, ease of use, & long-term stability make them suitable candidates as sustainable preclinical models for metabolism screening and chronic pharmaco-toxicological assessments.

References

In vitro generation of functional organoid structures resembling embryonic liver buds using differentiated, human cells
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Formation of 3D structures resembling liver buds in vitro has recently been described using stemcell-derived cell types (Takebe et al., 2013). Such structures can be transplanted into animals but may also prove useful for other applications, like ex vivo drug toxicity tests. Here we used human upcyte® cells to form liver buds in vitro. upcyte® cells are derived from primary human cells via genetic modification in order to transiently induce cell proliferation. When contact-inhibited, upcyte® cells differentiate into functional cells maintaining their cell-type specific characteristics.

We used defined mixtures of differentiated human upcyte® cells (hepatocytes, liver-sinusoidal endothelial cells and mesenchymal stemcells) that spontaneously formed liver buds in vitro which could be cultured for up to 30 days using a kirkstall bioreactor. These self-organized, liver-like organoid structures harbour healthy, living cells showing typical functional characteristics of liver parenchyme, including basal and drug-induced activity of several Cytochrome P450 enzymes. Within the bud the cells formed a typical “liver-like” architecture built up by polarized hepatocytes.

In summary we describe that 3-dimensional, functional liver structures can be generated in vitro using upcyte® cells and that these “mini-livers” are useful models to study long-term human liver function ex vivo.

Reference
HepaRG spheroid cultures in order to assess their suitability for the objective of the study was to characterize long-term 3D human hepatic models that can better predict DILI in humans. Therefore, taken all together, the results from our study suggest that the organotypic human HepaRG spheroid cultures may be a promising in vitro tool for DILI studies.

**Organotypic human HepaRG spheroid cultures for in vitro toxicity studies**

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Drug-induced liver injury (DILI) remains the main cause of acute liver failure (ALF) and post-market drug withdrawals. The well-documented limitations of pre-clinical in vivo animal studies and in vitro two-dimensional (2D) human hepatic models hinder the accurate prediction of DILI in humans. Therefore, there is a dire need for alternative in vitro human hepatic models that can better predict DILI in humans. The objective of the study was to characterize long-term 3D human HepaRG spheroid cultures in order to assess their suitability for in vitro toxicity studies. 3D HepaRG spheroids were obtained using the hanging drop technology. HepaRG microtissues were maintained for 4 weeks and assessed for liver specific morphology and function. The cultures were viable and maintained a stable size (diameter ≈ 250 μm) over the culture period in the absence of a necrotic core. The data illustrates that the cultures secreted albumin and urea throughout the culture period. Furthermore, cultures possessed both basal and inducible CYP3A4 enzyme activity, which is one the most imperative enzymes in drug metabolism and toxicology. Therefore, taken all together, the results from our study suggest that the organotypic human HepaRG spheroid cultures may be a promising in vitro tool for DILI studies.

**Characterization of heterotypic 3D human liver microtissues for drug-induced hepatotoxicity testing**

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Stable organotypic in vitro hepatic models might lead to a significant reduction of animal studies to assess long-term (4 weeks) toxicity of substances. 3D liver microtissues (MTs) cultures, composed of hepatocytes and Kupffer macrophages were systematically assessed for viability, liver-specific morphology, functionality and mRNA expression over 28 days. MTs demonstrated a 5x longer life span than the conventional 2D cultures maintaining consistent ATP content and morphology (CYP3A4, CD68, CK7 and BSEP). Albumin secretion was significantly higher in the MTs and there was continuous inducibility of an inflammatory response towards LPS exposure. Transcriptome analyses revealed differential regulation of 145 liver-specific genes in the MTs versus the 2D cultures over the culture period, including genes involved in innate liver-specific functions and ADME. CYP (3A4, 1A2, 2B6, 2D6, 2A6) enzyme activity was at least 2 fold higher and was maintained only in the MTs. Repeated, long-term exposure to Troglitazone and Tolcapone resulted in increased sensitivity (reflected by decreased IC50) over time, in contrast to their non-hepatotoxic structural analogues, Pioglitazone and Entacapone, respectively. Taken together, the results illustrate that the MTs maintain a differentiated liver-specific phenotype for at least 4 weeks and are a valuable model to study chronic and inflammation-mediated DILI.
air-blood barrier. In sharp contrast, we present an advanced in vitro model of a lung alveolus that combines an air-blood barrier and cyclic stretch to mimic the respiration. For this purpose, a thin, porous and elastic poly(dimethylsiloxane) (PDMS) membrane is sandwiched between two PDMS layers. Endothelial cells are seeded on the basal side of the fibronectin-coated membrane and epithelial cells on the apical side. Upon confluency, the epithelial cells are exposed to air and cyclically stretched for 48 h using an integrated actuation mechanism. Preliminary results show reproducible and homogeneous cyclic stretch patterns and increased tight junctions expressions. This in vitro lung alveolus model mimics the complexity of the human air-blood barrier in an unprecedented way, is easy to handle and may thus be an ideal tool for toxicology assessments.

**I-4b-240**

Next generation tissue models as a replacement for animal experiments

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Despite advances in the development of in-vitro-tissue-models, the number of endpoints in toxicity-testing which can be addressed with these models is limited. This is due to a lack of key cellular components and a restricted live time of the models (Groeber et al., 2011). In addition, the manual production process of current tissue-models results in significant inter- and intra-lot deviations. One reason for these limitation is that suitable technologies for the culture and production of tissue-models are missing. To overcome these pitfalls, the Fraunhofer IGB is achieving advanced culture systems and biomaterials which allow long term culture of complex tissue-equivalents. Using these technologies, we have developed the first full thickness skin-model with a perfused vascular network (Groeber et al., 2012). Furthermore, within an interdisciplinary consortium of biologist and engineers, we have established a fully automated production facility to generate epidermal models based on an open source reconstructed epidermis (OS-REp) (Lemper et al., 2013; Poumay et al., 2004)). During the product manufacture, all media changes and manipulations are automatically preformed which allows a maximum output of 8000 OS-REp per month (http://www.tissue-factory.com).

In our work we could create new technologies for the generation and production of tissue-models which is a vital requirement to increase the success of in-vitro-test-methods.

**References**


We have developed a medium throughput, high content in vitro model of male reproductive development using neonatal rat testes. This model includes a co-culture of testes cell types cultured with a three dimensional matrix, creating in vivo-like niches (3D-TCS). To determine the breadth of toxicity signals that the 3D-TCS can assess, we screened over 60 compounds for cytotoxicity. Using relevant exposures and experimental toxicity data to set initial screening concentrations, cytotoxic doses were identified for 31 of the 63 compounds. Cytotoxicity was observed for compounds which act through general toxicity mechanisms (such as oxidative stress) as well as through mechanisms more specific to male reproductive endpoints (such as endocrine disruption). For some compounds, cytotoxicity was a sensitive indicator of male reproductive toxicity at concentrations near relevant therapeutic levels. For other compounds such as vinclozolin, cytotoxicity was not a good predictor of reproductive toxicity, indicating the need for further analysis. Five compounds (arsenic, crizotinib, nicotine, valproic acid and vinclozolin) were then selected for future testing using additional endpoints, including effects on testosterone excretion and transcriptomic profiles. This model has allowed us to expand our mechanistic output while addressing the need to reduce animal use.

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**I-4b-508**

Human cell based 3D-functional vasculogenesis/angiogenesis test for identification of angiogenic and embryonic vascular disruptors and to be used as the vascular platform in tissue models

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Formation of vascular network is a crucial process during embryonic development, and it also contributes to the pathogenesis of numerous disorders such as cancer. Vasculogenesis and angiogenesis are two central mechanisms by which the vascular network is formed. During embryonic development vasculogenesis refers to in situ differentiation and growth of blood vessels, and angiogenesis comprises the growth of new blood vessels from the preexisting ones.

The developed test utilizes human adipose stem cells or fibroblasts and human endothelial cells to form 3D-coculture. The vascular construct was characterized using structural, gene expression and functional markers. The presence of 3D tube structure, younger and more mature vessels, basement membrane, lumen, extracellular matrix, adherence junctions between endothelia and pericytes has been proven. In the test both anti-angiogenic and angiogenic properties of a chemical and biological substances can be studied. The relevance of the assay in man was investigated by comparing the effects of a broad number of different types of reference chemicals with the published...
human data. The comparison showed a good concordance indicating that this human cell based test predicts the effects in man and has potential to replace animal tests, and to be used as vascular platform in tissue models.

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 from human, mouse and rat 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.

By employing different compounds in the neurosphere assay, we so far identified intracellular signalling pathways like Nrf2, the arylhydrocarbon receptor and epigenetic modification by histone deacetylases as well as pathways guided by cell surface receptors like integrins and the fibroblast growth factor receptor as modulators of human NPC development. Contributions of some of these pathways to processes of neurodevelopment underlie species-specificities. For enabling high content image analyses, we wrote algorithms for sphere evaluation.

In summary, 3D neurosphere cultures are useful for identifying toxicity pathways related to disturbances of processes relevant for human brain development. 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.

Organotypic tissue culture as a new in vitro model of human myocardium

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Human myocardial tissue is rarely available for in vitro studies, and stays viable only for short periods. These limitations should be overcome by a technique to prepare many vital slices from one tissue specimen, permitting cultivation and functional assessment over several weeks.

Specimen of hypertrophic (resected) or failing (explanted) myocardium were cut into 300 µm thick slices of 1x1 cm², which were analyzed immediately or cultured at a liquid-air interface for up to 28 d. Membrane potential, contractility, and viability were determined. To enable collection of specimen from 3 surgical centers, cold preservation was tested for transport.

Slices prepared <12 h after tissue retrieval developed paced contractions of 2.7 mN/mm² at up to 180 bpm, depending on diastolic strain and adrenoceptor stimulation. Action potentials displayed normal diastolic potential (-80 mV), amplitude, duration (330-380 ms), and responded to hERG and KATP channel manipulation. Cultured slices maintained viable >28 d, with only minor electrophysiological changes. Their contractility declined during the first week of culture and was detectable throughout 28 d. This time-course of contractility was reproduced when slices were prepared from tissues transported for 18-36 h.

Our findings emphasize the potential and feasibility of human heart slices as an in vitro model for myocardial contractility and electrophysiology.

Collagen vitrigel membrane chamber useful for fabricating 3d-culture models composed of epithelial, mesenchymal and/or endothelial cells and its advantages for ADME/Tox studies

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Pathways of chemicals in each organ in vivo are classified into two patterns; one is the pathway from epithelium to endothelium via mesenchyme in case that the chemical is exposed to the epithelial surface of cornea and skin, etc., and another is the pathway from endothelium to epithelium via mesenchyme in case that the chemical is administered directly into a blood vessel. Meanwhile, A collagen vitrigel membrane (CVM) we previously developed is composed of high density collagen fibrils equivalent to connective tissues in vivo and is easily handled with tweezers (Takezawa et al., 2004). Also, it possesses excellent transparency and permeability of protein with high molecular weight and consequently it functioned well as a scaffold.
that can facilitate the fabrication of a 3D-culture model excellent for cross-talking between the different types of cells by seeding them on its both surfaces (Takezawa et al., 2007). Recently, we developed a CVM chamber useful for fabricating tissue sheet-type and organoid plate-type culture models composed of one kind and more than two kinds of cells, respectively (Takezawa et al., 2012). From the viewpoint of extrapolating ADME/Tox in vivo of chemicals, we have developed vitrigel-EIT (Eye Irritancy Test) (Takezawa et al., 2011; Yamaguchi et al., 2013), CPT (Corneal Permeability Test), and LMTT (Liver Metabolism Toxicity Test) methods.

References

I-4c-236
Functionalized electrospun nanofibers for the development of a 3D skin model
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An appropriate simulation of the 3D environment in which tissues develop is essential to build up in vitro models. Electrospinning has been recognized as an efficient technique to produce scaffolds, which mimic the topography of the native extracellular matrix. Modification of electrospun fibers with a functional, amphiphilic macromolecule based on a star-shaped poly(ethylene oxide) derivate transforms hydrophobic fibers into hydrophilic fibers. Moreover, the attachment of cell-adhesion mediating peptides as the fibronectin binding side RGD is possible (Grafahrend, 2011). But also other ECM components as collagen and laminin sequences or a combination of different motifs can be bound to the macromolecule. Co-culture experiments with HaCaT cells and fibroblasts have validated that it is possible to create skin equivalents with these functionalized scaffolds. The keratinocyte cell line grows in several layers and expresses cytokeratin 10 in the stratified epithelium and cytokeratin 14 in the basal layers. Vimentin staining of the fibroblasts showed that the cells infiltrated into the electrospun membrane. It could also be shown that cells produce laminin, collagen and fibronectin, which illustrates that the matrix is remodeled. The establishment of a method to make specific cell adhesion on electrospun fibers possible offers great opportunities for the construction of biomimetic co-culture systems.

Reference

I-4c-473
Solubilized matrix from decellularized liver as a functional ECM to reproduce in vivo micro-environment in in vitro culture
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A novel cell culture technology to enable the effective screening for enormous varieties of candidate compounds for pharmaceutical drugs is desired earnestly. For this realization, development of a functional ECM to reproduce in vivo micro-environment in in vitro culture is important (Sellaro et al., 2010; Zhang et al., 2009). In this study, we developed a functional material as a scaffold for hepatocyte culture to reproduce in vivo micro-environment. In addition, 3D-culture system with this material was developed as the screening tool of pharmaceutical drugs.

Solubilized extracellular matrix derived from decellularized liver (L-ECM) was obtained by treatments with Triton X-100 (decellularization) and pepsin-HCl (solubilization). L-ECM solution was air-dried in multi-well plate for the cultivation of primary rat hepatocyte and liver-specific function of cell and then evaluated. In decellularized liver, 92.4% of DNA was removed from the native liver. Moreover, L-ECM stimulated the expression of liver-specific functions – including albumin secretion, urea synthesis and ethoxyresorufin-O-deethylase activity – of primary rat hepatocytes. Therefore, L-ECM has the potential to become an effective material to reproduce in vivo micro-environment. In future, we will investigate the effectiveness of L-ECM as a screening tool of pharmaceutical drugs.

References

I-4c-544
Design and fabrication of human skin by three-dimensional bioprinting
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3D bioprinting, a flexible automated on-demand platform for the free-form fabrication of complex living architectures, is a novel approach for the design and engineering of 3D human tissues. Here, we describe the use of 3D bioprinting for engineering human skin. In this model, keratinocytes and fibroblasts were used as constituent cells to represent the epidermis and dermis, respectively, and collagen to represent the dermal matrix of skin. Preliminary studies were conducted to optimize cell viability and cell density to mimic physiologically relevant attributes of human skin. Histology and immunofluorescence characterization revealed that printed 3D skin tissues were morphologically and biologically representative of in vivo...
human skin tissue. In comparison with traditional methods for skin engineering, 3D bioprinting offers several advantages in terms of shape- and form-retention, flexibility, reproducibility, and high culture throughput. It has a broad range of applications in transdermal and topical formulation discovery, dermal toxicity studies as well as in designing autologous grafts for wound healing. We are currently focused on enhancing the complexity of this model via the incorporation of secondary and adnexal structures such as the blood vasculature, and the inclusion of diseased cells to serve as a model for studying disease pathophysiology.

I-4c-667 *  

**Nanofibrillar cellulose hydrogel enables flexible 3D cell culturing**

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Various synthetic and natural hydrogels have been used as 3D cell culture scaffolds for different biomedical applications. From the immunological point of view, xeno-free scaffolds are highly preferred. We have recently shown that plant-derived nanofibrillar cellulose (NFC) hydrogel provides a flexible 3D culture system for several cell types. NFC consists of fibrillar glucan chains with a diameter in the nanometer range. Stiffness of the hydrogel can be easily modified to provide a desired matrix support for each cell type. Human hepatic cells, human embryonic stem cells, and human induced pluripotent stem cells formed 3D multicellular spheroids in NFC hydrogel. Hepatic cells showed liver-specific properties in 3D hydrogel culture: they secreted human albumin, formed bile canalicular-like constructions, showed efflux protein mediated transport into formed canalicular structures, and expressed higher level of CYP3A4 activity compared to 2D culture. 3D stem cell spheroids retained pluripotency and can be released from the hydrogel with a cellulase enzyme. The recovered spheroids can be used for further 2D or 3D culturing, or for analysis. In conclusion, NFC hydrogel offers xeno-free 3D culture scaffold, which may be applied in organotypic culture systems for drug testing and tissue engineering in the future.

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Session I-4: Novel 3D models – Poster presentations

I-4-097  

**The assessment of a Caco-2/CCD-18co co-culture model of the small intestine cultured on poly (ethylene terephthalate) nanofibre scaffolds**

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The caco-2 model of the intestine has enabled the prediction of drug permeability in vitro (Lennernäs et al., 1996), providing an indication of the expected oral absorption of new drugs in man and identifying candidates with poor pharmacokinetic profiles. Recent Literature suggest subtle environmental cues can alter the functional output of the Caco-2 model (Kim et al., 2012; Kim and Ingber, 2013; Yu et al., 2012) exposing scope to improve in vitro predictions. Nanofibre scaffolds which mimic the basement membrane structure can be produced by electrospinning; these were investigated as a scaffold platform for an in vitro model. Intestinal sub epithelial myofibroblast line CCD-18co cells were cultured on the basolateral surface of the nanofibre scaffold for 4 days prior to Caco-2 cell seeding on the apical scaffold surface to simulate the multicellular milieu of the intestinal mucosa.

The resulting platform was assessed for barrier functionality through TEER (Trans-epithelial electrical resistance), paracellular transport and drug permeability assays. Results show that Caco-2 cells cultured on nanofibre scaffolds in monoculture and in a co-culture with CCD-18co demonstrate reduced barrier formation which may be more comparable to the human intestinal barrier. Additionally Caco-2 monolayers cultured on nanofibre matrices exhibit increased paracellular transport to Lucifer Yellow compared to the traditional Transwell™ platform model.

**References**


I-4-166  

**New BEST – biomaterials enhanced simulation test**

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This work presents the development of new in vitro testing method for biomaterials in the closest to hostile conditions. The objective of this BEST – biomaterials enhanced simulation test – is to provide maximal possible yet realistic control and monitoring of chemical, biological, cytological etc. reactions, and leading to decrease of animal testing. Recently no combined in vitro solution exists capable of answering the
demands and needs of all stakeholders (patients, hospitals, biomat-
erials producers, pharmaceutical industry) at reasonable costs, speed and
safety (von Recum, 1998; Black, 1999). New challenges require more
consistent and holistic approaches to ensure reliability and safety of
the implants including those with ATMP.

Here the design of the BEST methodology and test equipment is
shown for the case of load-bearing implants such as orthopaedic and
dental ones (van Mow and Huiskes, 2005). The demonstration includes
based porous coated biomaterials at different conditions showing the
preferential potential for bone, cartilage or fibrous tissue formation.
The testing is supported by time- and frequency-domains simulation
with models in silico (Gasil et al., 2012).

The results show the importance of proper application of relevant
testing parameters vs. traditionally used protocols, leading also to re-
duction of specimens and faster screening of new materials formula-
tions with new method.

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14-172

An in vitro ocular test system for a detailed quantification of
the cellular damage in the corneal epithelium and stroma

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The study aims to develop a test system for the complete replacement
of the Draize Eye Irritation Test to allow the discrimination of all 3
GHS categories in one experiment. The test system is based on bio-
technologically produced 3-dimensional semi-cornea models, which
comprise both an epithelium and a stroma with collagen embedded
keratinocytes (Engelke et al., 2013). A collagen membrane was in-
serted between these two tissues for the separation and independent
assessment of damages in the epithelium and stroma after exposure
to potential eye irritants. Cell viability, assessed with the MTT assay,
was used as a toxicological endpoint. Acceptance criteria were defined
based on negative and positive controls. The prediction model which
was defined on the results of 30 test materials uses a single exposure
period and the combination of cut-off values in tissue viability from
both epithelium and stroma. As a result, 100% of the GHS 1 category,
88% of the GHS 2 category and 57% of the GHS unclassified test ma-
terials were predicted correctly. In conclusion, the test system predicts
and discriminates GHS 1 and GHS 2, but is over predictive for GHS
no category materials. The project is funded by the German Federal
Ministry of Education and Research (FKZ0316010).

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14-173

The initial depth of injury in 3-dimensional tissue models for the prediction of the eye-irritation
potential of chemicals

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Based on 3-dimensional reconstructed tissue models of the human eye
we aimed to establish a test method to reliably predict the eye-irritation
potential of chemicals for all GHS categories. We developed a tech-
nique to determine the initial depth of injury (DoI) in tissue models of
human cornea and conjunctiva by combining the MTT viability assay
with cryosectioning procedures. The formazan-free, metabolically in-
active area in the tissue after topical substance application, the visible
correlate of the DoI, is quantitatively analyzed on cryosectional images
with ImageJ software analysis tools. Our experiments revealed that for
most of the chemicals tested so far the DoI values increased in paral-
el with increasing eye-irritating potential of the chemicals. Therefore,
the test method allows us to distinguish between the cytotoxic effects
of different chemicals for all 3 GHS categories. However, in order to
establish a robust prediction model, a larger set of chemicals of differ-
ent chemical classes and drivers of irritation must be tested in future.
In conclusion, analyzing DoI in MTT-stained cryosections repre-
sents a promising tool to assess toxicological reactions in reconstruct-
ed corneal and other epithelial tissues.

The project is funded by the German Federal Ministry of Education and
Research (FKZ0316010).

14-204

A new 3D human airway tissue model for in vitro lung cancer
research (OncoCilAir™)

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Lung cancer is the most common cancer with about 1.4 million deaths
worldwide every year (Cancer Facts, 2014). Here we developed a fully
humanized in vitro lung cancer model, OncoCilAir™, which com-
bines primary human bronchial epithelial cells with lung adenocarci-
noma cell lines in order to replicate as closely as possible the in vivo
formation and progression of lung cancer. Cultured at the air-liquid
interface, this system displays proper differentiation features with a
pseudostratified columnar epithelium containing ciliated, goblet and
basal cells. High trans-epithelial electrical resistance, cilia beating and
production of mucus demonstrate the full functionality of the epithe-
lum. Remarkably, and in contrast to monolayer cultures, tumour cells
extended forming nodules into the adjacent tissue, a hallmark of hu-
mans lung cancer (Henschke et al., 1999). Dose response experiments
including the investigational drug Selumetinib showed that the system
could be effectively used to assess both drug efficacy and toxicity. In

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USA: Taylor and Francis.
A novel 3d-culture system activating hepatic function of HepG2 cells utilizing a collagen vitrigel membrane chamber and its application to liver metabolism and toxicity test

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A collagen vitrigel membrane (CVM) chamber we developed is a culture tool useful for oxygen supply and 3d-culture. Such a CVM chamber can provide a unique culture system enabling cell growth in culture medium (liquid phase) but also plastics (solid phase) or 5% CO2 in air (gas phase). Here, we designed above 3 different culture models of brain tumors in comparison to other culture conditions. A collagen vitrigel membrane (CVM) chamber we developed is a culture system in comparison to other culture conditions. A therapeutic concentration of 10 µM etoposide was identified which demonstrated toxicity to tumours while maintaining four-fold higher viability in normal brain tissue. This proof-of-concept study offers the opportunity to perform biorelevant safety and efficacy screening and to reduce animal experiments.

Offbeat to mainstream: high-throughput, user-friendly three-dimensional cell culture models of brain tumors

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Current cell culture models using two-dimensional monolayers are of limited value in predicting in-vivo response and cannot compete successfully with animal models. Multicellular spheroids utilising human tissue offer improved predictive potential through better representation of tumour biology. We present a suite of assays in 96-well format that allow for rapid, affordable and biorelevant spheroid screening of drug delivery platforms.

Human brain tumour medulloblastoma cell line UW228-3 and foetal brain tissue stem cell neurospheres were cultured in xeno-free conditions in 96-well ultra-low attachment plates. They reproducibly formed a single spheroid per well (100-900 µm, CV 5-10%). A set of three mechanistically different methods for spheroid health assessment (volume, metabolic activity and acid phosphatase enzyme activity) were validated against cell numbers in healthy and drug-treated spheroids. In addition, fluorescently marked tumour and normal tissue were cultured together forming co-culture spheroids, exposed to biorelevant etoposide concentrations and the viability of both populations was assessed separately for each population using flow cytometry and multiphoton microscopy.

A therapeutic concentration of 10 µM etoposide was identified which demonstrated toxicity to tumours while maintaining four-fold higher viability in normal brain tissue. This proof-of-concept study offers the opportunity to perform biorelevant safety and efficacy screening and to reduce animal experiments.
I-4-352

Human organotypic nasal epithelial tissue culture as an in vitro model to evaluate effects of cigarette smoke


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In line with the reduction, refinement, and replacement framework of animal use and to overcome the limitations of species translation, the development and use of human in vitro models, that closely mimic in vivo biology, is of great importance.

To assess the effect of cigarette smoke (CS), human organotypic nasal epithelial tissue cultures were exposed for 28 min at the air-liquid interface to air or to different dilutions of mainstream CS (under Health Canada smoking regime and nicotine doses of 0.15 mg/L or 0.25 mg/L). Time- and dose-dependent CS effects were evaluated by measuring multiple endpoints (cytotoxicity, CYP1A1/IB1 enzyme activity, inflammatory marker secretion, histological and transcriptional changes).

Exposure to CS resulted in increased CYP1A1/IB1 enzyme activity and release of various inflammatory markers. In addition, using gene expression data and a network-based systems biology approach, significant perturbations of biological processes such as apoptosis, inflammation, cell proliferation, cellular stress and senescence were shown and quantified over different post-exposure time points.

Our results demonstrate that human nasal organotypic tissue culture could be a suitable model for mechanistic and toxicological assessment of CS effects on the respiratory tract and for comparative studies of reduced-risk products.

I-4-368

Pre-validation of the Hen’s Egg Test for Micronucleus-Induction (HET-MN): final evaluation of data

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The HET-MN assay is distinguished from other in vitro genotoxicity assays by toxicologically important properties such as absorption, distribution, metabolic activation, and excretion of test compounds. As a promising follow-up approach to supplement existing in vitro test batteries for genotoxicity, the HET-MN recently underwent a formal pre-validation.

After the method was developed at the University of Osnabrück it was transferred first to the laboratories to Henkel following an inter-laboratory study analyzing 14 test substances in both laboratories. In 2010 the method was transferred to the third (Federal Institute for Risk Assessment, BfR) and fourth lab (Harlan Cytotest Cell Research). In the first phase of the transfer cyclophosphamide and 7,12-dimethylbenzanthracene were examined. In the second phase, three compounds (ampicillin, carbendazim, acrylamide) were tested in a blind study in the laboratories of the BfR, Harlan, and Henkel.

In the final pre-validation a balanced data set of 20 chemicals were tested blinded. They comprised different chemical classes and mode of actions covering the groups of the true positives, true negatives and misleading positives.

The data with a promising outcome will be presented with regard to reproducibility and predictivity.

The work was funded twice by the German Ministry for Research and Education.

I-4-369

3D Skin Comet assay: status quo of the ongoing validation

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In 2012, Cosmetics Europe joined forces with the German Federal Ministry of Education and Research (“BMBF”) with the goal to validate the 3D Skin Comet assay. Whereas the BMBF project worked on a number of full thickness (FT) skin models the CE project had originally focused on an epidermal tissue. When the team compared three models, Phenion® and EpiDerm™ FT were selected to enter the validation phase, based on statistical evaluation of results from the method transferability phase, and taking into account inter- and intra-laboratory variability and the number of valid experiments. The validation will include 30 chemicals, of which 8 are already tested. The initial testing phase, which is presented here, focused on inter- and intra-laboratory reproducibility and is demonstrating high predictive capacity for the 8 chemicals tested. Additional efforts have been made to establish and validate the most suitable measurement of cytotoxicity in the FT models.

As the Comet assay detects a broad spectrum of DNA damage that may give evidence for mutations the final aim of the validation study is to provide a new approach to be used as a follow-up for positive results from the current in vitro genotoxicity test battery.

I-4-381

Development of an immune-competent model of human upper airway epithelium

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Respiratory diseases remain a major cause of morbidity highlighting the need for better understanding of their pathophysiology to enable the development of novel therapies. To address such need scientists often rely on animal models however the poor physiological relevance of these models to human is widely acknowledged. This highlights the requirement for biomimetic models of human airway that can be used as platforms for disease modelling and testing drugs. Lung epithelial cells are the first line of defence against airborne pathogens and allergens. Here we hypothesised that co-culturing lung epithelial cells, fibroblasts and dendritic cells in 3D and under physiological conditions could allow simulating in vivo conditions and facilitate better understanding of the airway epithelium homeostasis.

Using this 3D co-culture we studied immune-modulatory properties of epithelial cells in response to bacterial extract. Our data shows differential regulation of a key immune regulatory enzyme namely indoleamine 2,3-dioxygenase in epithelial cells cultured at air-liquid interface (ALI) compared to submerged cultures. These differences are likely due to changes in TLR-4 expression and formation of a functional barrier in ALI. We suggest such 3D co-culture can provide a physiologically relevant tool for investigating the pathophysiology of different inflammatory conditions in human airway.

I-4-397

Global proteomic analysis of acetaminophen toxicity in 3D human liver microtissues

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I-4-398

Biological assay device of neurologic cells with in situ observable gel culture system composed of ECM-modelized matrix

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In recent years, neurologic cell culture is performed as alternative of animal experiments which evaluate influences on central nervous system (CNS) of various chemical agents. Culture system which mimics CNS is desirable for the evaluation, but neurologic cells are generally cultured in non-physiological monolayer condition (Lai et al., 2012). Therefore, we developed heparin-crosslinked collagen gel which was inspired by ECM composition of CNS (Watanabe et al., 1995; Inatani et al., 2003). Furthermore, we aimed to construct a novel 3D culture system which enables in situ observation. Heparin was chemically cross-linked to collagen, and ECM-modelized matrix was developed. Neural stem cells (NSCs) were embedded into ECM-modelized matrix gel and cultured with bFGF. Furthermore, NSCs were embedded into the thin-layered gel of 0.1 mm in thickness. Immunohistochemical analysis was performed. Embedded NSCs in ECM-modelized matrix showed greater proliferation and neurite outgrowth than those in collagen gel. In addition, NSCs in ECM-modelized matrix gel are more sensitive to anesthetic than those in monolayer. These results indicated that ECM-modelized matrix made in vivo-like culture environment. Thin-layered gel makes much easier to observe embedded cells than conventional gel culture of 2 mm in thickness. These results suggest constructing bios assay device with in situ observable 3D culture system composed of ECM-modelized matrix.

References


The evaluation of spheroid culture of human hepatocytes for drug induced hepatotoxicity


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Drug induced hepatotoxicity is the most frequent reason for discontinuing development of drug candidates and post-market drug withdrawals. It is difficult to predict human hepatotoxic potentials in preclinical safety studies because of the species difference. Primary human hepatocytes are considered the main choice to evaluate hepatotoxicity; however hepatic functions, especially cytochrome P450 activities, decrease under conventional monolayer culture conditions. Recently, three-dimensional cultured hepatocytes (hepatocyte spheroids) have been a focus of attention to the ability that enabled a long-term evaluation.

In this study, we investigated human hepatocyte spheroids to assess the feasibility in a drug induced hepatotoxicity assay. The spheroidal formation and hepatic functions (e.g., activities of drug-metabolizing enzymes and albumin secretion) were maintained for several weeks. Under this culture condition, the spheroid was exposed with well-known hepatotoxic drugs, resulted in a concentration-dependent elevation of AST activity and depression of albumin secretion by the long-term exposure of several drugs. Considering activities of drug-metabolizing enzymes were sustained during culture period, certain metabolites would involve in the toxicity.

In conclusion, we established an in vitro system to evaluate human hepatotoxicity by using spheroid culture method. This approach will be useful for preclinical safety assessment in the early stage.

In vitro 3D scaffold-free osteoarthritis model – multiple application options

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Osteoarthritis (OA), a leading cause for disabilities and financial burden on society, is characterized by a complex interplay of inflammatory processes and cartilage degradation. To study underlying mechanisms and new therapeutic approaches, small animal models are widely used whereas the applicability to the human is questionable. However, to our knowledge the existing 3D cell model is only restricted able to unify the complex pathogenesis and inconvenient to handle (e.g., bioreactors). Based on the scaffold-free 3D cartilage transplant (SFCt) technology (Fzmb GmbH), we generated an in vitro OA model that consists exclusively of chondrocytes and their metabolic products. The SFCt’s achieves diameters up to 1.5 cm and thickness between 1-3 mm and are treated with arthritic conditions. Moreover, the advantage of this model is the extended durability and the possibility to produce constructs in parallel from one human donor facilitating reproducibility. In addition, it can be used in pharmacological screenings, in situ modeling, biomaterial development and transplant testing. Here, we will present first results of our study showing significant differences in histological, biochemical, molecular biological and biomechanical analysis between the arthritic SFCt models, indicating our model as advantageous and promising alternative to existing small animal models.

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Human cell-based functional 3D-angiogenesis test for identification of inhibitors of angiogenesis

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Angiogenic response of 36 selected test chemicals were studied by using human in vitro angiogenesis test. The chemicals included 14 drugs, 8 additives used in food or personal care products and 14 environmental chemicals.

The test method consisted of a co-culture of human fibroblasts and endothelial cells in the culture medium with specific exogenous growth factors to induce formation of tubular structures and tubular networks. During the test, the co-culture was exposed to the test chemicals at a concentration range of 10 µM to 10 mM (or limited by solubility). After 24 h, neutral red uptake assay was performed to obtain IC50 cytototoxicity value. The concentrations producing 80% or more viability were selected for angiogenesis assay. The extent of tubule formation was quantified microscopically after 6 days in culture.

The inhibitory concentration on tubule formation (EC50) varied broadly among chemicals studied ranging from 0.6 µM (Cladribine) to 1788 µM (Diethanolamine) showing the potency of the angiogenesis test for chemical rankings. 10 chemicals did not show any an-
Evaluation of human hepatocytes cultured by three-dimensional spheroid systems for drug metabolism


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Drug-induced hepatotoxicity is the major reason for discontinuing clinical trials. To evaluate hepatotoxicity, in vitro studies using a primary culture of human hepatocytes and human hepatic microsomes, and in vivo studies using animals have been performed. However, these assays have several problems such as the involvement of reaction intermediates in the development of toxicity due to species differences in drug metabolic enzymes. In this assay we evaluated whether the three-dimensional spheroid culture of human hepatocytes reflects human metabolic profiles (Ohkura et al., 2014). Sequential metabolic reactions by phase I and then phase II enzymes were found in diclofenac (CYP2C9 and UDP-glucurononyltransferases (UGTs)), midazolam (CYP3A4 and UGTs) and propranolol (CYP1A2/2D6 and UGTs). Moreover, lamotrigine and salbutamol metabolism were metabolized to lamotrigine-N-glucuronide and salbutamol 4-O-sulfate, respectively. These metabolites, which are human specific, could be observed in clinical studies, but not in conventional hepatic culture systems as seen in previous reports. In addition, mRNA of drug-metabolism enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT2B7, sulforhodase 1A1 and glutathione S-transferase pi 1) which were measured by qRT-PCR, were expressed in the human hepatocyte spheroids. In conclusion, these results suggest that human hepatocyte spheroids are useful in hepatotoxic assays to estimate drug metabolism pathways.

Reference

In vitro human alveolar tissue model for pulmonary drug delivery and toxicology applications

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Reliable in vitro human models are needed to assess airborne environmental agents or inhaled therapeutics. To address this need we developed an in vitro air-blood barrier model from primary human alveolar epithelial cells, pulmonary endothelial cells and macrophages. Endothelial cells and alveolar epithelial (ATII) cells were isolated from human lungs. The alveolar model was constructed by seeding endothelial cells on the underside of a microporous membrane and ATII cells onto the top membrane surface. Co-culture continued at the air-liquid interface (ALI) until barrier development occurred. Finally, human monocyte-derived macrophages were seeded onto the apical surface. Confocal imaging of differentiated cultures demonstrated staining for cytokeratins 7 and 19, and carboxypeptidase M (ATII cell markers), tight junction proteins ZO-1 and occludin. The endothelial cells stained positive for von Willebrand factor and e-cadherin. Macrophages were visualized with Celltracker dye. The model produced a robust barrier demonstrated by maintenance of TEER>400 Ω*cm² for up to 30 days. Drug transporter gene expression including ABC family efflux transporters BCRP, MRP1 and MRP2, and organic cation uptake transporters OCTN1, OCTN2 and OCT3 was demonstrated by RT-PCR. This new in vitro human alveolar model shows promise for in vitro pulmonary toxicology and inhaled drug delivery investigations.

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 many 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 tissue 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 chemical (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, hist-
Development of normal human cell based small intestinal (SMI) tissue models that allow integrated approaches to study drug safety, inflammation, and restitution are needed. The validity of Caco-2 cell based studies and animal testing is in question due to lack of physiological relevance. Therefore, our principal goal is to develop a human relevant, quality-assured, medium- to high-throughput testing strategy to prioritize chemicals for (developmental) neurotoxicity testing. This poster evaluates the functionality of SMI tissue models reconstructed from normal human primary SMI epithelial cells and fibroblasts. Reconstructed SMI tissues were characterized morphologically, molecularly, and functionally. Inflammatory responses were examined by TNF-α plus IFN-γ exposure. Wound closure and re-epithelialization of the epithelium was monitored following injury of the tissue. Analysis of the SMI tissue revealed: 1) columnar epithelial cells, 2) a physiological TEER value of 100-180 Ω•cm², 3) expression of epithelial markers, efflux transporters, and brush borders. Studies using 2 P-gp substrates, ranitidine and talinolol, demonstrated active transport while warfarin did not. Treatment of the SMI tissue with TNF-α plus IFN-γ induced an increase in proinflammatory cytokines/chemokines (IL-6, IL-8 and GRO-α). Confocal and H&E staining of injured SMI tissues showed cooperation of epithelial cells and fibroblasts in wound healing process. This SMI tissue will have pre-clinical applications and will reduce the use of animals for experimentation.

References

Using human organotypic tissue slices in cancer research
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Cancer is one of the major causes of death worldwide. Research to improve therapy is mostly done with xenograft models by injecting (human) tumor cell lines, e.g., into the flanks of mice or rats. This means a great burden for the animals, especially when the endpoints of the experiments are represented by Kaplan-Meier-curves which depict the time point where half of the animals in the study died. It also involves problems like the lack of cellular heterogeneity or inter-species differences. Therefore, only few animal studies can be successfully translated into a clinical setting for humans.

We have previously established a human test system consisting of 3D-tissue slice cultures of tumor tissue from surgeries which can be kept in culture for several weeks and used for radio- and chemotherapeutic experiments. So far, this system is used for glioblastoma, squamous cell carcinoma and gastric cancer tissue from resections as well as fat from plastic surgery. In these settings, we can monitor response to known therapies, test new compounds, analyze cell proliferation or death, or track behavior of special cell types or live imaging over time. With our model system, animal testing could be reduced and species differences are eliminated.

For further reading see Merz et al., 2013 and Gerlach et al., 2014.

References
**The importance of normalization and standardization of cytotoxicity assays for 3D cell models**

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**Introduction:** Cytotoxicity is often the first endpoint measured in vitro to estimate non-cytotoxic or sub-cytotoxic concentrations that can be further studied for more specific and complex endpoints. Lately it has been recognized that 3D cell models with increased cell-cell interactions are essential to mimic the in vivo situation. However, cytotoxicity assays for 3D models require standardized protocols, often optimized from traditional monolayer cultures.

**Aim:** Evaluate cytotoxicity assays for a 3D neuronal cell model.

**Method:** The dopaminergic cell line LUHMEs, were cultured in neuronal differentiation media under constant gyratory shaking, for 12 days to form 3D aggregates. Size measurements of each aggregate and cytotoxicity assays (DNA quantification, Lactate dehydrogenase, Resazurin, Neutral Red Uptake (NRU) and cell number quantification) were performed in 384-well plates.

**Results:** Resazurin and NRU showed the best correlations with aggregate diameter (0.8634 and 0.7334, respectively). In addition, the estimation of number of cells showed a good correlation (0.7869) in the exponential growth curve by aggregate’s volume. DNA quantification assays were not considered optimal for 3D models.

**Conclusion:** Cytotoxicity assays for 3D models need to be carefully selected and standardized. The establishment of these models can contribute to reduce costs, time and animal use for toxicity testing of chemicals.

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**BIOMEMS for accurate in vivo – in vitro correlation**

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In the last five years we successfully validated the use of ultrathin ceramic membranes issued of state of the art in micro fabrication as support for cell growth (Halamoda et al., 2013). The 500 nm thin membranes have by design excellent transport properties. Consequently we focused our effort in their integration into systems conceived for translocation assays, novel drug carrier absorption and particulate exposure models. We present here a family of devices ranging from an insert like system, compatible with commercial multiwell plate (SIMPLI-well, Patent EP, 2011), to dynamic automated bioreactors, all suitable for 3D models of biological barriers. Their relevance is improved by the reduced interference of the mechanical support. System health can be continuously and non-invasively monitored by reliable integrated micro electrodes for TEER measurement. Metabolic reactions can be spectrometrically observed by inducing plasmonics to the same micro porous membrane and making it highly selective in the MIR range. User-friendliness inspired our engineering: if SIMPLI-wells are compatible with routine practises, the most complex version of these bioreactors goes towards automated standalone machine with bio-systems compatible with a “cartridge”-like approach requiring no specialised operators for standard toxicology test and environmental monitoring.

**References**


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**Development of a new reconstituted human oral mucosal model to assess the oral irritation testing**

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In this study we developed a novel three-dimensional human oral mucosal model (HOM model) based on two different cell sources. One is immortalized human oral keratinocyte cell line (HOM-IHOK model) and the other is human normal buccal keratinocytes (HOM-NBK model).

Immunohistochemistry and barrier function testing were employed to characterize these newly developed model system. For further reading see Chai et al., 2010; Klausner et al., 2007; Liu et al., 2010; Moharamzadeh et al., 2008; Moharamzadeh et al., 2012.

**References**


Session I-5: Bioreactors

Co-chairs
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Session I-5: Oral presentations

I-5-210

Optical cell separation by photodegradable hydrogels for three dimensional cultures

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Cell separation techniques contribute to analyze cell profiling such as genome information. Several studies reported that cells express their native phenotypes in 3D culture compared to two-dimensional culture. Thus, we require the tool for 3D culture which can separate target cells, especially animal testing alternative. Herein, we propose the method of optical cell separation by photodegradable hydrogels in 3D environment. In previous study, we synthesized photocleavable cross-linker NHS-PC-4armPEG, and it applied to form photodegradable hydrogels which composed of NHS-PC-4armPEG and gelatin. The hydrogels allowed cells to attach on the surface and to growth. For developing further biomimetic condition, we encapsulated cells into the photodegradable hydrogels through optimization of compositions. Cells could survive in the hydrogels for 96 h. In photodegradation, we irradiated the target area using a computer-controlled light irradiation system, which can irradiate according to the designed micropatterned images or arbitrary area under the microscope. Both of the irradiation system and photodegradable gels successfully separated cells in the hydrogels. Furthermore, the separated cells could growth on a culture dish. The minimum resolution was estimated at 20 mm, which should separate each cells. This method expected to become a novel strategy in animal testing alternative.

I-5-238

A three dimensional (3D) perfusion bioreactor-based model of colorectal cancer for chemotherapeutic assessment

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In this study we addressed the suitability of a perfusion bioreactor to sustain 3D colorectal cancer cell growth and to test established treatment regimens as compared to 2D cell culture or xenografts. At variance to 2D, 3D-perfused constructs were characterized by heterogeneous tissue-like structures of proliferating and apoptotic cells and expression of typical tumour marker, similarly to xenografts. Treatment with clinically relevant concentrations of 5-FU had no effect on cells number cultured in 3D-perfusion or in xenografts constructs, in contrast to 2D cultures. In perfused cultures only a marginal effect on the expression of BCL-2 apoptosis-resistance gene was observed, while significant down-regulated in 2D. The combination of ABT-199 (Souers, 2013) and 5-FU induced additional cytostatic and cytotoxic effects in 3D-perfusion but not in 2D cell cultures. Interestingly, 3D-perfusion partially showed similar responsiveness to 5FU and BCL-2 expression in colorectal tumors in patients undergoing neo-adjuvant treatment. Our data consistently indicate that 3D perfused cultures efficiently mimic phenotypic and functional features observed in animal models and clinical specimens. These in vitro models may have the potential to reduce animal use for testing drugs with critical translational relevance for diagnostic purposes as well as to address fundamental issues in human tumor cell biology.

Reference

I-5-333

Microfluidic perfusion culture system for culturing human induced pluripotent stem cells under fully defined culture conditions

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Human induced pluripotent stem cells (hiPSCs) are promising cell sources for drug discovery and are expected to reduce the number of animal experiments. For drug screening applications using hiPSC derived cells, it is important to use a culture system that accurately controls the proliferation and differentiation of hiPSCs. However, two problems can lead to inaccuracies in the conventional drug screening system: 1) instability of culture conditions caused by using conventional multi-well culture plates, in which the medium is replaced daily, and 2) masking and distorting of screening results caused by using undefined culture medium and extracellular matrix.

Here, we developed a pressure-driven microfluidic perfusion culture system for controlling hiPSC states under defined extracellular matrix and culture medium conditions. The growth rate of hiPSCs under perfusion culture conditions was higher than that under static culture conditions in our microfluidic system. Immunocytochemical analysis showed that the self-renewal and differentiation of the hiPSC-
SCs was successfully controlled. The effects of three anti-tumor drugs on hiPSCs using our microfluidic system were the same as that using a 96-well plate.

Our system is suitable for high-throughput drug screening systems using hiPSCs with the added benefit of eliminating the inaccuracies caused by unstable and undefined culture conditions.

1-5-407

Autonomous bioreactor modules for disease models and detection of systemic toxicity

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Advanced systems based on bioreactors and scaffolds are an essential step towards the development of more predictive and ethical alternatives to animal experiments. Size, modularity, automation, monitoring and essential design are crucial because these elements will ease the transition from old technology and accelerate their acceptance into mainstream research. Based on these requirements, the interconnected transparent sensorised “lego” bioreactors designed in our labs have been used to generate physiologically relevant disease and toxicity models which recapitulate systemic responses impossible to observe in standard cell cultures.

The disease model is an interconnected bioreactor circuit with i) adipose tissue in 3D in 3 different concentrations representing normo-weight, over weight and obese body mass indices, ii) human hepatocytes on porous collagen scaffolds and iii) monolayers of human endothelial cells. High adiposity and elevated glucose levels induce systemic and endothelial inflammation in the circuit, as observed in overweight and diabetic humans (Iori et al., 2012). Using similar technology a three-tissue circuit for monitoring the absorption, distribution and metabolism and toxicity of nanoparticles was developed in the context of the EU project InLiveTox (Ucciferri et al., 2014). Based on these requirements, the interconnected transparent sensorised “lego” bioreactors designed in our labs have been used to generate physiologically relevant disease and toxicity models which recapitulate systemic responses impossible to observe in standard cell cultures.

References


1-5-419

Cell function induction using perfusion culture

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One of major elements causing inadequate organ/tissue-specific functions of cultivated cell is that the physicochemical environment for the cell culture is totally different from that in vivo. We have looked at the perfusion of culture medium, which is intimately related to nutrition supply and waste removal, and hydrodynamic stimuli. We are developing novel microchips where cell culture environments are precisely controlled.

In this talk, I will present the advantages of the perfusion culture taking our works for instance. It has been well-known that the spheroids formed with HepG2 express higher hepatic function. The spheroids can be uniformly formed under perfusion culture. The growth rate of hiPSC under perfusion culture is higher than that under static culture. The vascular endothelial function can be induced using HUVEC under the perfusion culture at a shear stress larger than 10 dyne per square centimeter. The former two results can be considered in relation to the nutrition supply and waste removal, and the last one is due to the effect of shear stress.

References


3D multi-compartment bioreactor technology for in vitro pharmacological studies as an alternative to animal testing

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The relevance of animal models in the prediction of human drug effects is limited by species-dependant differences in hepatic metabolism and susceptibility to toxic effects of xenobiotics. Human in vitro liver cell models could provide a suitable alternative to in vivo experiments by exhibiting human-relevant functions.

Our approach is based on a dynamic four-compartment culture technology that approximates the natural environment of the cells in the organ. Cells are cultivated within a three-dimensional network of hollow-fiber capillaries that serve for decentralized medium supply and oxygenation. Down-scaled laboratory versions of the technology allow reducing the needed amount of cells and reagents for in vitro research (Zeilinger et al., 2011; Hoffmann et al., 2012; Lübberstedt et al., 2012).

Primary human liver cells were cultivated in bioreactors under serum-free conditions. The toxicity of diclofenac used as a model drug was investigated in the bioreactor system using two concentrations of the substance (0.3 mM, 1 mM). The results show a dose-dependent toxic effect of diclofenac application, as determined by analysis of enzyme release, glucose metabolism and urea production.

In conclusion, the bioreactor technology allows stable cell cultivation for in vitro studies on drug metabolism and toxicity. Thus, the device provides a promising in vitro model for pharmacological studies on human hepatocytes.

References


Session I-5: Poster presentations

I-5-271

Rapid fabrication of engineered liver tissue using novel fibroblast system

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Reconstructed liver in vitro and in vivo from isolated hepatocytes have a great deal of potential in drug screening. However, fabrication of vascularized liver tissue was difficult because it has to be controlled configurations and types of cells. In this study, we challenged establish of novel fibroblast system for controlling of extracellular microenvironment using cell sheet technology and fabrication of subcutaneous human liver tissue in mouse.

Human hepatocytes were inoculated onto human dermal fibroblast monolayer on a temperature-responsive culture dish (UpCell; Cell-Seed Inc.). Hepatocytes adhered onto fibroblast monolayer within at least 2 hours of culture and harvested engineered hepatocyte/fibroblast sheet (eHFS). Vascularized-human liver was fabricated under the skin of mouse by transplantation of EHSF and showing significantly higher synthesis activities of liver-specific proteins in vivo than transplanted hepatocyte-only sheets. These results will be caused by high-syntheses of angiogenic factors from EHSF.

In this study, we established rapid fabrications of EHSF and vascularized human liver tissue under the mouse skin without prior induction of angiogenesis. This human liver model could be used for drug screening with easy in vivo observation and collecting of subcutaneous liver tissue.

I-5-298

Human fibroblast remodelling when cultured in 3D and under flow

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3D cultures of different tissues have been shown to be physiologically relevant; however under static conditions distribution of nutrients and removal of waste in 3D is not efficient. Furthermore, most cells in vivo are exposed to forces due to flow which influence their function. There is therefore considerable interest in the development of perfusion systems that enable the maintainance of 3D cultures and provide more physiologically relevant culture conditions. In this study we examined wound healing in 3D cultures of human lung fibroblasts that were cultured in a Quasi-Vivo® perfusion bioreactor and made comparison with static cultures of the same cells. For wound healing studies cells were exposed to NaOH and the healing process was monitored via cell proliferation and extra cellular matrix (ECM) production for 7 days. Our data clearly show 3D cultures under flow conditions proliferate at a significantly higher rate, produce and deposit higher levels of ECM proteins and show faster recovery after injury. Our data also suggest these events are likely to be driven by higher production of fibroblast growth factor and IL-6 under flow conditions. This perfusion culture system could provide a useful platform for studying fibroblast remodelling, a key event in many lung pathologies.

I-5-336

Engineering hepatic tissues with perfusable vascular structures

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The ability to reconstruct biomimetic tissues in vitro may be beneficial for the alternative to animal testing and regenerative medicine. One of the most important issues in the fabrication of tissues is the lack of approach for engineering vasculatures for delivering oxygen and nutrients throughout engineered constructs. This study proposes an approach to fabricate endothelial cell-lined microchannel using electrochemical cell transfer and induce subsequent self-organization of cells.

Oligopeptide was designed to spontaneously adsorb and form a dense-molecular layer on a gold surface and to be desorbed from the surface electrochemically. Cells adhering on a gold surface via the oligopeptide layer were detached within 5 minutes electrochemically. We applied this approach to cylindrical gold rods; Human umbilical vein endothelial cells (HUVEC) were transferred from rods to the internal surface of microchannels (9500 mm) in a hydrogel, resulting in the formation of HUVEC-lined perfusable vasculature. These vasculatures were maintained vascular functions such as vascular endothelial barrier. In the following perfusion culture, HUVECs spontaneously sprouted into a hydrogel. Furthermore, when we encapsulated hepatic cells in a hydrogel, liver-specific functions, such as albumin secretion and ammonia removal, increased overtime. This vascularized hepatic tissue fabrication approach may provide more biomimetic in vitro testing for drug development.

I-5-420

Protection from cell death in multicellular spheroids

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Forming multicellular spheroids is attractive for cell-based assay because various functions are enhanced. However, the center part of the
Development of a perfusion chamber for the cultivation of 3D skin constructs

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Reconstructed skin models, as an alternative to animal testing, show higher permeability and reduced mechanical resistance due to a less efficient skin barrier in comparison to human skin in vivo (Schäfer-Korting et al., 2008; Küchler et al., 2013). To overcome these obstacles, we constructed a perfusion chamber aiming to ensure a constant nutrient supply and to provide shear forces which normally occur in vivo. In the perfusion chamber the reconstructed skin is grown at an air-liquid interface with a constant, laminar flow below the dermis equivalent. Histological analysis of first skin models grown in the perfusion chamber revealed a significantly thicker stratum corneum in comparison to the skin models grown in the classic transwell setup. In order to determine the influence of the perfusion chamber on the skin lipid order of the skin models, Fourier transform infrared spectroscopy was performed. The constructs grown in the perfusion chamber exhibits more ordered lipids chains in comparison to the control. Furthermore, skin models grown in the perfusion chamber showed reduced skin permeability for the OECD reference substance testosterone compared to control models. In conclusion, cultivation of reconstructed skin in perfusion chambers is a promising method in order to improve the quality and skin barrier function of reconstructed skin.

References


2D and 3D cultures of the human hepatocarcinoma cell line HepaRG were established in an EU-funded project NOTOX to allow system-wide study of long-term repeated-dose toxicity effects. Using well-designed media long-term cultivation over 4 weeks is possible, both in 2D microtiter plate and 3D perfusion cultures with minimal loss of viability and hepatolytic enzyme activities. Solid spheroids are formed within a few days developing bile canalicular structures, clearly visible in electron microscopy. In vivo MRP2 activity assays show active secretion into bile canalicular structures. Long-term toxicity tests revealed clear differences between 2D and 3D cultures for test compounds as bosentan, valproic acid and chlorpromazine. Cultures are characterized using various omics methods, e.g., metabolomics, fluxomics, transcriptomics, proteomics, as well as by different imaging methods. Data are used for modelling pathways and microtissue, i.e., spheroid, geometry. The goal is, to eventually support safety assessment by directed in vitro experiments combined with systems oriented models suited for toxicity prediction.

The need of innovative technologies for new 3D relevant in-vitro models and the answer of Ivtech

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New relevant in-vitro models are priorities in pharmaco-toxicology, cosmetic and food research to reduce the animal tests. Therefore, in-vivo models show ethical issues, are not time and cost effective and are progressively showing scientific limitations: for instance they fail in detection of pathogens that are species specific (Mazzoleni et al., 2009). The search of more relevant pre-clinical models forced the researcher to move from 2D to 3D in-vitro models in order to maintain the phenotype of cells (Lovit et al., 2013; Mattei et al., 2014). Even if the significant progress in material science, the metabolic requirement of 3D tissues is higher than a 2D culture and the scaffold is a limitation in nutrients transport. Dynamic cell culture chambers are then required to assure the gas/nutrient supply, waste elimination, mechanical stimulation of cells, study of cross talk between different tissues and real time monitoring of cells. Nowadays the only systems that meet all these specifications are the Ivtech technologies. Ivtech is an innovative Italian start-up that grows up to solve the needs of in-vitro...
experts, offering and customizing several type of transparent, dynamic and modular cell culture systems, organizing workshops and training. The goal is to expand the 3D approach and permits a significant evolution towards highly relevant in-vitro models.

References

1-5-747
Improving in vitro tests of orthopedic implant materials
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New orthopedic implants must be extensively tested – for safety and for mechanical stability after implantation – before market entry. Current in vitro analyses of implant materials are used essentially to screen for acute toxicity and biocompatibility. Improved in vitro tests with good predictive value for osseseintegration are needed if the number of in vivo tests in this field is to be reduced.

We are developing 3D in vitro testing methods for tissue scaffolds for bone replacement. A small bioreactor has been developed that allows the scaffolds to be tested for long periods of time (weeks) under physiologically-relevant dynamic mechanical loads. The bioreactor is placed inside a standard incubator, which ensures suitable conditions for cell culture. Cell culture medium is passed through the bioreactor for oxygenation of the sample over long incubation times.

First results show good growth of cells – SaOs-2 and mesenchymal stem cells – on scaffolds in the bioreactor. They also highlighted difficulties in monitoring cell growth and imaging cells within the scaffold. The next steps will focus on developing techniques for cell seeding and monitoring cell growth in the scaffold in order to optimize culture conditions.

1-5-780
Bioengineering approaches for the development of novel 3D in vitro models for pre-clinical applications
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The need for physiologically relevant cellular systems, which provide insight into the mechanisms and pathways by which chemicals exert their effects on cells and organisms is particularly perceived for preclinical phases of drug development, namely for drug screening, target validation and toxicology.

Our work has focused on the development of human 3D cell models, making use of stirred-tank bioreactor culture systems for expansion, differentiation and maturation of clinically relevant cells. Herein, we will present results concerning the establishment and refinement of cultures systems for (1) the differentiation of human neural stem cells in functional and mature neurospheres enriched in neural cell subtypes; (2) differentiation of pluripotent stem cells towards highly pure and functional cardiomyocytes; (3) Donor-derived hepatocytes and hepatic cell lines as source of functional hepatic models. The presented systems were phenotypically and functionally characterized/validated.

Engineering of 3D microenvironments yields in vitro models with improved physiological relevance and culture in computer-controlled bioreactors enables reproducibility and robustness, making these bioprocesses suitable to be applied in the pharmaceutical industry. Thus, the newly developed 3D in vitro models increase the collection of tools available for preclinical drug development, contributing to increase its efficiency and to accelerate drug development pipelines.

1-5-805
Use of dynamic 3D primary hepatocyte culture to understand the effects of constitutive androstan receptor activators in primary rat and human hepatocytes
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A variety of substances including pharmaceuticals, plasticizers and crop protection agents contain Constitutive Androstan Receptor (CAR) activators. Activation of CAR is known to cause hepatocellular proliferation in rodents, but their effect upon human hepatic cells is unclear. Using both traditional two dimensional static culture and a 3D dynamic bioreactor (LiverChip™) the effects of CAR activators upon gene expression and cell proliferation were examined in primary rat and human hepatocytes. Gene expression data indicate that dynamic 3D culture is more effective than traditional 2D static culture at maintaining rat hepatic phenotype in vitro up to 7 days, as evidenced by maintenance of expression of hepatocyte specific genes including HNF1a, HNF4a CAR and CYPs. Rat hepatocytes were shown to exhibit increased proliferation by MTT assay following treatment with phenobarbital for 24 hours whereas treatment of primary human hepatocytes with the selective human CAR activator CITCO had no effect on proliferation. Given the lack of proliferative effects of CAR activators in human cells, we have subsequently focussed upon whole transcriptomic and miRNA screens comparing the effects of CAR activators in rat and human hepatocytes.

1-5-814
Dose metric considerations of cationic and anionic surfactants in in vitro cytotoxicity assays
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In vitro concentration-effect relationships are used for ranking toxicants and extrapolating to in vivo toxic doses. In vitro concentration-effect relationships are traditionally based on nominal concentrations. However, the nominal concentration may not represent the concentration of the chemical causing toxicity in cells in in vitro cell-based assays. The extent to which a chemical partitions into cells will depend on its neutral fraction, volatility, stability and binding affinity to medium constituents, well plate plastic and cells. The aim of this study was to determine how the cytotoxicity ranking of primary, secondary, tertiary and quaternary amines as well as (perfluor)carboxylates and alkyl sulfates varying in carbon chain length depends on the dose metric used to construct concentration-effect relationships in a basal cytotoxicity assay using the rainbow trout gill cell line, RTgill-W1. Results indicate that charge shielding and lipophilicity determined the in vitro bioavailability as well as cytotoxicity of the test chemicals. Moreover, toxic potency rankings of the chemicals were dependent on the dose metric used and effect concentrations based on cell-associated concentrations were least dependent on in vitro setup.

Session I-6: High-content imaging

Co-chairs
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Session I-6: Oral presentations

1-6-597

Use of High Content Imaging (HCI) to screen for developmental neurotoxicity

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Because the molecular events leading to developmental neurotoxicity are not well defined, methods are being developed to assess key neurodevelopmental processes such as cell proliferation, differentiation, neurite growth, and synaptogenesis. Many of these processes can be assessed at the cellular level using fluorescent markers (e.g., immunostaining or expression of fluorescent proteins) combined with microscopic imaging. With the advent of HCI these phenotypic changes can be measured in an unbiased, high throughput format. Neural cells show dramatic changes as they differentiate, and the formation and extent of the specialized process such as axons and dendrites can be quantified using image analysis algorithms which measure cell size and shape. Assessment of the multiple cell types that arise from neuroprogenitor cell differentiation including glia (astrocytes and oligodendrocytes) and numerous types of neurons (e.g., glutamatergic, GABAergic) can be delineated and quantified after immunostaining godendrocytes) and numerous types of neurons (e.g., glutamatergic, proprogenitor cell differentiation including glia (astrocytes and oli-

1This abstract does not necessarily reflect U.S. EPA policy.

1-6-757

Intravital multiphoton tomography for non-invasive in vivo analysis of human skin

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Replacement is the first worthwhile strategy in basic and clinical science by circumvention of the detour via animal models if avoidable. Multiphoton tomography (MPT) of endogenous fluorophores is such a high-content imaging tool for non-invasive “optical biopsies”, i.e. high resolution in vivo examination of human skin directly at patients’ bedside (Koenig and Riemann, 2003). Several biomolecules like NA-DH, melanin, collagen or elastin, showing autofluorescence or second harmonic generation, can be visualised with a penetration depth of 150 μm. These molecules provide information about subcellular morphology, epidermal architecture and physiological conditions of the skin and can indicate changes in cell metabolism (Su et al., 2011). Additional parameters like fluorescence decay times (MPT-FLIM) or spectral shift of the emitted fluorescence could be used for objective diagnosis and a therapy follow-up in skin diseases during repetitive clinical visits.

Therefore, MPT-FLIM application offers the possibility to directly examine the individual etiopathology of skin diseases, primary in vivo tracking of applied therapeutic agents and an ad-hoc molecular analysis of intraepidermal and -dermal therapeutic response in a non-invasive manner. As a unique feature, this technique envisions new in vivo and ex vivo parameters that were already successfully used to detect and monitor pathophysiological skin alterations in patients and histological sections prior to clinical manifestation (Huck et al., 2011; Seidenari et al., 2012).4

References
Towards a high throughput microscopy pathway in toxicity reporter platform for chemical safety assessment

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Adaptive cellular stress responses are paramount in the healthy control of cell and tissue homeostasis after cell injury during hypoxia, oxidative stress or unanticipated side-effect of medications and other chemical exposures. To increase our understanding of chemically-induced adaptive stress response pathway activation and its contribution to safety assessment a time-resolved, sensitive and multiplex readout of chemical-induced toxicological relevant cellular stress responses is essential. For this we develop a platform containing a panel of distinct adaptive stress response fluorescent protein reporter cell lines. These are used for automated high content live cell imaging and quantitative multi-parameter image analysis to elucidate critical adaptive stress response pathway activation that can contribute to human chemical safety assessment. To conserve the endogenous gene regulatory programs, we tag selected reporter target genes with GFP using BAC-transgenomics approaches. Here we demonstrate the functionality of individual BAC-GFP pathway in toxicity reporter cell lines to their respective specific model compounds. The application of these reporters in chemical safety assessment in relation to drug-induced liver injury will be discussed. We anticipate that ultimately a phenotypic adaptive stress response profiling platform will allow a high throughput and time-resolved classification of chemical-induced stress responses assisting in the safety assessment of chemicals.

This work is part of the MIP-DILI project supported by the Innovative Medicines Initiative (grant agreement n° 115336), and the FP7 SEURAT-1 DETECTIVE project (grant agreement 266838).

Using high-content imaging to analyze cell-state trajectories and biological tipping points for chemical exposures

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Translating results obtained from high-throughput screening to risk assessment is vital for reducing dependence on animal testing. We studied the effects of 976 chemicals (ToxCast Phase I and II) in HepG2 cells using high-content imaging (HCI) to measure dose- and time-dependent perturbations in p53, JNK, oxidative stress, cytoskeleton, mitochondria, and cell cycle. A novel computational model was developed to describe the dynamic response of the system as cell-state trajectories based on multidimensional HCI datastreams. Cell-state trajectories produced by 10 concentrations (0.4 to 200 µM) of 976 chemicals showed resilience of the HepG2 system in many cases, however, we also found “tipping points” in system recovery. Further analysis of trajectories identified dose-dependent transitions, or critical points, in system recovery for 340/976 chemicals. The critical concentration was generally 5-times lower than the concentration that produced 50% cell loss. We believe that HCI can be used to reconstruct cell state trajectories, and provide insight into adaptation and resilience for in vitro systems. With additional research, cellular tipping points could be used to define an in vitro point of departure (PoD) for risk-based prioritization of environmental chemicals.

This work does not reflect U.S. EPA policy.

High-Content Imaging as a tool for nonclinical drug safety investigations

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In pharmaceutical research and development drug safety is not only addressed in the regulatory testing battery in the preclinical and clinical phases by doing in vivo studies. More and more in vitro toxicity tests are used to support the selection of drug candidates with a better toxicity profile in early screening approaches or are used to investigate toxicity mechanisms of a drug candidate in development in parallel to the regulatory testing battery. For this, high content imaging (HCI) is a valuable basic tool in pharmaceutical toxicology. We use HCI in several projects for different purposes and with various cellular systems. To show the broad field of HCI application in our industry, the talk will present examples where and how we used HCI with respect to, e.g., phospholipidosis, respiratory epithelium toxicity and cardiotoxicity. The examples will show different level of complexity ranging from simple cell line monocultures and single endpoints to multicellular 3D models and multiple read outs.

This work is part of the MIP-DILI project supported by the Innovative Medicines Initiative (grant agreement n° 115336), and the FP7 SEURAT-1 DETECTIVE project (grant agreement 266838).
Telemetric monitoring in preclinical drug development

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During preclinical development of new chemical entities (NCE), different animal models are necessary to determine efficacy and safety prior to first administration to humans. In the past anesthetized or restrained animals were used for the measurement of parameters of vital organ functions like blood pressure, ECG, EEG or body temperature. The use of radio-telemetry techniques such as invasive implanted or non-invasive external telemetry allows the continuous monitoring of such parameters in awake, freely moving animals which are chronically instrumented with specific telemetry devices. Those techniques allow the assessment of pharmacodynamic effects of a NCE without interaction with anesthetics over a long observation period. Furthermore animal welfare is enhanced by reduction of stress or the possibility for social housing. In addition the telemetry technique contributes to the concept of 3Rs by the refinement of methods and the reduction of animals due to the re-use of animals.

The presentation gives an overview of various telemetry methods in different animal species with special focus on cardiovascular safety assessment in rodents. Since regulatory guidelines like ICH S7 (Anon, 2000) recommends the use of unanesthetized animals, the collection of data using telemetry techniques is preferred in Safety Pharmacology investigation (Leishman et al., 2012).

References
Anon (2000). ICHS7A. ICH Guidance

Continuous blood glucose levels and food intake after RYGB surgery in rats

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Glucose levels in the blood change rapidly in response to various stimuli, including eating, physical activity and stress. Current practices to assess glucose responses in laboratory rodents typically involve frequent blood sampling and analysis with glucometers. Not only can such sampling practices, which often involve anesthesia or stress, obscure glucose measurements, but important information can also be lost or overlooked in between sampling intervals. Developing technology facilitates remote collection of continuous blood glucose levels in freely moving rats. Using an implantable telemetric glucose sensor (Data Sciences International), we tested whether glucose excursions following a meal are altered in a rat model of Roux-en-Y gastric bypass (RYGB). Rats were adapted to BioDAQ food intake monitors and glucose levels were analyzed in relation to spontaneous meals and during refeeding after a 6-hour fast. Analysis of glucose excursions during refeeding showed that RYGB rats had reduced preprandial baseline glucose, reached lower minimum periprandial glucose, and had a larger range of glucose values during this periprandial period. We conclude that RYGB alters the glucose response to a meal, and demonstrate the utility of an implantable glucose sensor to collect clinically relevant data that are otherwise difficult to obtain with current glucose measurement practices.

Simultaneous use of implanted and jacketed external telemetry – a contribution towards the 3R’s

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The introduction of telemetry in freely moving animals (Chui et al., 2009; McMahon et al., 2010), reducing the need for instrumented anesthetized models, has made a considerable contribution towards the 3R’s. Socialization can be maintained during data recording, thus further improving animal welfare.

For ethical, scientific and economic reasons, the objective of a rational study design is to collect the maximum amount of relevant scientific data with a minimum number of animals, while maximizing data quality and robustness. Developments in telemetry technology over recent years have permitted substantial progress in meeting these criteria.

A combination of implanted and jacketed external telemetry enables investigation of all safety pharmacology core battery investigations within a single study. Cardiovascular and respiratory parameters are measured continuously and central nervous system function is evaluated in a functional observational battery (FOB) at relevant time-points. Single or repeated blood sampling allows exposure level measurement or full pharmacokinetic profiling, respectively. According to the characteristics and pharmacological properties of test items, complementary analyses can also be performed. This presentation will focus on the use of implanted and jacketed external telemetry in cynomolgus monkeys and how these approaches may be combined to improve safety pharmacology assessment.

References
EEG telemetry as a gold standard for rodent studies in neurodegeneration

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Telemetric recording of biosignals is a state-of-the-art method for monitoring physiological functions in awake and freely moving laboratory animals, while minimizing stress artifacts. EEG activity, as well as blood pressure, heart rate, blood flow, electrocardiogram, respiratory rate, sympathetic nerve activity, body temperature, and many other biological signals can be studied in a wide range of animal species, including rodents (rats and mice), dogs, rabbits, gerbils, hamsters, monkeys, guinea pigs, and pigs (see for example Greene et al., 2008). Stress and anxiety are reduced in animals that are freely moving and unhindered by external hardware. For this reason, telemetry allows to reduce the number of required subjects in chronic and longitudinal studies (http://cdn.intechopen.com/pdfs-wm/21096.pdf). Anxiety levels may act as hidden variables in experimental studies, and we here report different examples of experimental models in which anxiety can be decreased using telemetric recording. In particular we hereby present data obtained in different experiments that point out:

a) anxiety-modulated susceptibility to convulsive drugs in mice;
b) anxiety-modulated cognitive performance in mice and rats;
c) sleep/wake cycle alterations as a tool for eliciting early mild cognitive impairment.

Potential applications of telemetric recordings will be discussed in relation to the above-mentioned data.

Reference
Session I-7: Poster presentations

I-7-033

Dual pressure telemetry in rats; measuring interventricular asynchrony in experimental pulmonary hypertension

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Radio-telemetry provides an alternative means of obtaining physiological measurements from freely moving animals, without introducing stress artifacts. This surgical procedure describes the implantation of a new radio-telemetry dual pressure and biopotential transmitter, right ventricle (RV) and left ventricle (LV) and RV and aorta in rats. The success rate of the surgical procedure for the RV&LV technique was 8 out of 12 but none of the animals survived at the end of the experiment at 9 weeks. For the RV&Aorta technique the success rate was 6 out of 13 and the overall success rate was 3 out of 13. In experimental Pulmonary Hypertension (monocrotaline model), the adaptation to an elevated RV systolic pressure leads to ventricular asynchrony and cardiac inefficiency. The aim was to assess the possibilities to monitor disease progression and interventricular asynchrony in monocrotaline rats using dual telemetry. In RV&Aorta rats, the QA-interval of the RV showed a sudden increase after monocrotaline treatment, whereas the LV range is maintained. Implantation of telemetry for measuring both pulmonary as systemic pressures is feasible, however the RV&LV technique is not advised because of a high rate of complications. In combination with electrocardiography, dual pressure telemetry can be used to evaluate ventricular contractility.

I-7-325

Correlation of infrared thermography vs. rectal body temperature measurements in baboons (Papio spp.)

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One of the most clinically relevant and objective physiologic parameters is body temperature. Traditional methods for measuring body temperature in non-human primates may require sedation, physical, or mechanical restraint which can alter results. The objective of this study was to compare body surface temperature using infrared thermography (IRT) with rectal temperatures using standard medical thermometers. In conjunction with another study requiring general anesthesia of time-mated baboons, eight adults and six of their newborn offspring were examined on multiple occasions by simultaneously recording rectal body temperatures and body surface temperature using a hand held IRT camera. All paired measurements were compared using linear regression analysis. The results showed a statistically significant correlation between IRT and rectal thermometer temperatures. The most consistent body temperature recordings using IRT were from non-haired anatomical regions (face, sex skin). IRT is a noninvasive and accurate method for measuring body temperature in baboons and should be considered as an alternative to traditional methods.

Enhanced monitoring of disease studies involving laboratory mice using radiofrequency identification technology

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Appropriate monitoring of laboratory animals is critical to refining experimental studies and should be carried out by experienced and compassionate staff. This monitoring can be enhanced by the use of automated technologies if applied appropriately (Hawkins, 2014). There are now a number of automated technologies capable of monitoring the most widely used laboratory species- the mouse (Richardson, 2012). Automated monitoring can be used to identify specific behaviours indicative of pain (Miller et al., 2011) and in progressive disease studies to identify objective biomarkers of disease progression- critical for the implementation of appropriate humane endpoints (Franco et al., 2012). Advantages of radiofrequency identification (RFID)-based systems when studying mice include the small size of the transponders and the capacity to use these systems to monitor socially housed animals. Additionally, RFID based systems that measure body temperature can be combined with those that monitor other behaviours such as drinking.

This presentation will describe how RFID technology was used to enhance monitoring of ongoing studies involving mice with liver disease or cancer. Examples will include using body temperature as a biomarker of disease progression (Hunter et al., 2014) and studying non-nutritive visits to drinking areas as an early indicator of disease.

References


Development of the mCM – mobile circulatory module – for ex-vivo physiological lung tissue for breathing simulation

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The REACH regulation (Regulation (EC) No 1907/2006) and Cosmetics regulation (Regulation (EC) No 1223/2009) of the European Union pressurise the manufacturers of chemicals and cosmetics to use alternatives to animal models. The developed lung simulator “i-Lung” provides a mean for alternative lung simulation. Active spontaneous breathing cycles of different lung equivalents like porcine lungs, instead of latex bags, David et al. (2013) allow research on breathing mechanisms. The used pig lungs are taken from the conventional slaughtering process and are therefore not part of an animal based study. In order to provide a physiologically and anatomically relevant model, the explanted lung tissue has to be maintained in an appropriate state and is used as lung equivalent for respiratory simulation including aerosol in- and exhalation (Forjan et al., 2012). Therefore, the presented mCM is being developed. The module allows the nourishing of the lung tissue during transport and simulation using a roller pump in an insulated housing and giving sensor data of fluid pressure, temperature and flow. The data is transmitted wirelessly using international medical IT standards to an Android based tablet and thereby enables standardised telemonitoring (Frohner et al., 2013) of the lung tissue. Further development will include cell-tissue based evaluation of the tissue status and use of the model for working-place safety measurements.

References
Theme II – Predictive Toxicology

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Session II-1a: Pathways approaches in toxicology

Co-chairs
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Session II-1a: Oral presentations

II-1a-123

Pathway activation from tissue samples as a predictor of in vivo toxicity profile

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Pharmaceutical safety assessment has historically had good success in avoiding serious injury to first in human volunteers and patients. Present guidance from regulatory authorities specify reporting animal studies dosed high enough to induce toxicity up to predefined limit dose levels. Forward looking toxicologists anticipate that a finite number of toxicity pathways may be defined in in vitro biological culture systems that would encompass all risk of human toxicity, and that these in vitro tests could replace the need for animal studies. As technologies advance, it may prove useful to demonstrate the translatability of in vitro and in vivo toxicity pathways by showing predictivity of in vitro systems for same-species in vivo equivalents. As a first step, we retrospectively analyzed in vivo gene expression patterns from animals dosed for 5-7 days with a compound for activation of pathways of toxicity. This pathway activation in vivo was evaluated for its ability to predict toxicity in the same tissue and same species in repeat dose toxicity studies. All authors are employees of AbbVie. The design, study conduct, and financial support for this research was provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

II-1a-613

Cell-based risk assessment relies on a quantitative understanding of toxicity pathways

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At its core, the paradigm shift in toxicity testing now taking place relies on the power of modern molecular biology to identify perturbations in cellular function, and to integrate these perturbations into a quantitative systematic framework (Bhattacharya et al., 2011). In this explanatory framework, cells and computational systems biological models are used to understand how biological pathways act and interact to maintain cellular homeostasis. Toxicant-induced alterations in biological pathways are the signals used to develop models of the dynamics of disruption of cellular functions, and are key to distinguishing adaptive versus adverse responses, and points of departure for risk assessment (Boekelheide and Andersen, 2010). The critical biological pathways that are most responsive to toxicant exposure have been called toxicity pathways, and the ongoing description of their dynamics is a key step forward (Andersen et al., 2011). Requirements for success of this cellular framework for risk assessment include a focus on human biology.

Adeleye, Y., Andersen, M., Clewell, R. et al. (2014). Toxicology, Epub ahead of print. doi: 10.1016/j.tox.2014.02.007

Can cancer safety assessment be conducted solely on the basis of in vitro studies?

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"Toxicity Testing in the 21st Century (TT21C): A Vision and A Strategy" called for a change in toxicology; focusing on evaluating changes in signaling pathways using human relevant cells rather than relying on high dose animal studies. To support this transition, we have been working to provide practical examples of how the TT21C vision can be implemented to facilitate chemical safety assessments for several toxicity pathways, including the DNA damage response pathway. The project studies p53-mediated DNA damage stress response in human cells to determine the response circuitry and the dose-response behaviour for pathway activation following chemical induced DNA damage. This research has three overarching goals: (1) map the key determinants of cellular fate following DNA damage, (2) identify dose-dependent thresholds associated with adaptation and toxicity (clastogenicity) and (3) perform a safety assessment based on predicted regions of safety. Initial work involved validation of the in vitro model and collection of dose-response data at the gene (transcriptomics), protein (p53, p-p53, p-H2AX, MDM2, etc.), and cellular (cell cycle arrest, apoptosis, micronucleus) level using prototype chemicals. Biokinetic models have been used to facilitate in vitro to in vivo extrapolation and the determination of safe levels of human exposure.

Reference
Adeleye, Y., Andersen, M., Clewell, R. et al. (2014). Toxicology, Epub ahead of print. doi: 10.1016/j.tox.2014.02.007
broad coverage of cell types, and an understanding of how endocrine, extracellular matrix, and intercellular interactions modify cellular responses. Taking this systematic cell-based approach to perturbed human biology, an approach that relies on mechanisms and pathways, has inherent value, contributing both to improved toxicity testing and to the fundamental molecular elucidation of human disease.

References

II-1a-696
Using adverse outcome pathway analysis to identify gaps in high-throughput screening for thyroid disruption
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Common findings in safety studies include rodent thyroid histological abnormalities, increased gland weight, and/or tumors. The presence of these rodent thyroid abnormalities may suggest a chemical-induced perturbation of thyroid hormones (THs) that is relevant across species. A primary toxicological concern is that maternal THs modulate neurodevelopment. Adverse outcome pathway (AOP) analysis demonstrates that TH disruption occurs via several molecular-initiating events (MIEs), including: inhibition of glandular iodine uptake, TH synthesis, and/or peripheral deiodination of THs; increased hepatic catabolism of THs; and, interaction with TH receptors. However, high-throughput screening (HTS) assay data are unavailable for many MIEs. The need for HTS assay development is highlighted by comparison of thyroid-related HTS data from the US EPA ToxCast program and the in vivo thyroid endpoints reported in ToxRefDB. Analysis of the added predictivity of a new high-throughput assay for thyroperoxidase inhibition, as well as the inclusion of other available HTS assays for markers of hepatic catabolism, will also be discussed. Assay development for thyroid-disruptor screening based on AOPs would provide predictive tools for new chemical entity development, drastically reduce animal tissue use, and inform prioritization testing schemes.

II-1a-712
Advancing integrated approaches to animal-free chemical safety assessment using an Adverse Outcome Pathway framework
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Reducing and ultimately replacing animal testing for determining complex human health and environmental effects of chemicals depends on our ability to effectively combine information derived from multiple complementary alternative methods. Although considerable scientific challenges have yet to be overcome, the rapid emergence of increasingly sophisticated experimental and computational tools is paving the way towards credible solutions to predictive toxicity. However, relying on conventional trial-and-error approaches to identify the optimum method-combinations for different protection goals seems a futile pursuit since we simply have too many tools in our toolbox. Progress towards reduction and replacement of animal testing is far more likely if the design of integrated approaches is based on understanding of the underlying biological system and the toxicological pathways that lead to its failure. The Adverse Outcome Pathway (AOP) framework provides a systematic, practical, and scientifically sound approach to describe the sequential chain of causally linked key events, occurring at different levels of biological organisation, that lead to an adverse health or ecotoxicological effect. An AOP represents a conveniently packaged distillation of curated mechanistic knowledge that can be readily used to design integrated testing strategies and to guide method development and validation.

Session II-1b: Pathways approaches in toxicology
Co-chairs
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Mathieu Vinken, Vrije Universiteit Brussel, Belgium

Session II-1b: Oral presentations

II-1b-010
Development of an adverse outcome pathway from drug-mediated bile salt export pump inhibition to choledastic liver injury
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Adverse outcome pathways (AOPs) have been recently introduced in human risk assessment as pragmatic tools with multiple applications. AOPs intend to provide a mechanistic representation of pertinent toxicological effects. AOPs are typically composed of a molecular initiating event, a series of intermediate steps and key events, and an adverse outcome (Vinken, 2013; Vinken et al., 2014). In this study, an AOP framework is proposed for cholestasis triggered by drug-mediated inhibition of the bile salt export pump transporter protein. For this purpose, an in-depth survey of relevant scientific literature was carried out in order to identify intermediate steps and key events. The latter include bile accumulation, the induction of oxidative stress and inflammation, and the activation of specific nuclear receptors. Collectively, these mechanisms drive both a deteriorative cellular response, which underlies directly caused cholestasis injury, and an adaptive cellular response, which is aimed at counteracting cholestatic insults. AOP development was performed according to OECD guidance, including consideration of the Bradford Hill criteria for weight of evidence assessment and the OECD key questions for evaluating AOP confidence (Vinken et al., 2013). The postulated AOP is expected to serve as the basis for the development of new in vitro tests and the characterization of novel biomarkers of drug-induced cholestasis (Vinken, 2013).

References

Il-lb-376
Constructing, quantifying, and validating an adverse outcome pathway for vascular development toxicity

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Embryonic vascular disruption (Knudsen and Kleinstreuer, 2011) leads to a range of adverse prenatal outcomes. The adverse outcome pathway (AOP) for embryonic vascular disruption was recently entered into the AOP wiki and accepted as part of the OECD workplan. The AOP was built based on molecular initiating events (MIEs) affecting genes from critical pathways (hypoxia/growth factor signaling, chemokine networks, extracellular matrix interactions and vessel remodeling/stabilization) with evidence of abnormal embryonic vascular development in the mammalian phenotype browser of the Mouse Genome Informatics database (http://www.informatics.jax.org). EPA ToxCast high throughput screening data (Kavlock et al., 2012) for assays mapping to targets in the AOP were used to prioritize >1000 chemicals for their potential to disrupt vascular development. A subset of these chemicals are being tested for developmental effects across a wide range of vascular-specific model systems. Preliminary results from functional validation of AOP targets, quantification of MIEs and key cellular events (Kleinstreuer et al., 2013), and compound hazard predictions will be discussed.

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References

Il-lb-468
Building on shared experience to advance practical application of pathway-based toxicology: repeat-dose liver toxicity

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In January 2013, the Human Toxicology Project Consortium convened a workshop to exchange experience regarding the usefulness of pathways as an organizing principle for applying different types of information at all levels of biological organization, using two liver-specific pathways (fibrosis and steatosis) as examples. Conclusions included that applicability of the pathway depends on the confidence in, and completeness of, the pathway, where qualitative pathways are useful for prioritization and read across but more developed pathways may be used quantitatively. Additionally, the quality assurance methodologies described in OECD guidance to assess data quality causal relationships, and completeness are appropriate and essential. Also, pathway development should be an iterative, interactive, multidisciplinary and public process. Confidence in the prototype liver pathways can be increased by including information from traditional toxicological sources such as histology and knowledge from pathologists and clinicians familiar with the biological outcomes at the tissue and individual levels. Bioinformatics and modeling approaches can be used to both inform the understanding of the pathway and to identify potential predictive markers or signatures for liver toxicity. This presentation will discuss workshop findings and recommendations as well as recent developments since the workshop.

Il-lb-499
Promises and challenges in constructing an adverse outcome pathway for chemical sensitization of the respiratory tract

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An adverse outcome pathway (AOP) framework for screening for potential endocrine disruption

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The US EPA is applying an adverse outcome pathway (AOP) framework for screening potential endocrine disrupting chemicals. Rather than considering a single molecular initiating event (MIE) and subsequent events leading to a specific adverse outcome, a more holistic approach is being used to evaluate chemical interactions along the estrogen (E), androgen (A), and thyroid (T) hormone pathways. Endpoints of EPA’s Endocrine Disruptor Screening Program (EDSP) assessment tools (e.g., in silico models, in vitro and in vivo assays), including MIEs, as well as intermediate and terminal events, are mapped to key events along the AOP. Linking assessment tools to steps along an AOP will assist in evaluating interactions and adverse consequences of exposure to endocrine disrupting chemicals. Examples of the pathway concept and maps of endpoints to steps along the AOP will be provided, with recognition that chemicals may activate multiple MIEs and/or hormone pathways with varied outcomes across different life stages and taxa. This effort is intended to illustrate the utility of this conceptual framework for linking assay endpoints with outcomes of regulatory interest. This, coupled with estimates of exposure can be used for risk-based prioritization of chemicals for EDSP screening.

This abstract does not necessarily reflect US EPA policy.

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**Session II-1c: Oral presentations**

**II-1c-212**

**PPARγ-related hepatotoxic mode-of-action: quantitative characterization and in silico study of the molecular initiating event involving receptor activation**

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**Aim:** In line with the new predictive toxicology concepts a MoA from ligand-dependent dysregulation of PPARγ to nonalcoholic fatty liver disease has been developed (Al Sharif et al., 2014). In this study we aim at refining and quantifying the proposed toxicity pathways by evaluating the causal relationships within the MoA (OECD, 2013) and at in silico modeling of the molecular initiating event (MIE) associated with the PPARγ activation.

**Methods:** The MoA is assessed according to the AOP/OECD principles. Identification and quantification of structure-activity relationships are performed to model interactions of PPARγ with its agonists. Results: In this study (i) key events were evaluated by weights-of-evidence and their contribution to the MoA was assessed, (ii) PPARγ ligand database was created with structural and biological data that could be used to characterize the chemical space of PPARγ agonists, and (iii) MIE-based in silico models were developed.

**Conclusion:** This is the first attempt to quantify the developed MoA. Further efforts are necessary to refine and update it considering the wide range of downstream events, the complex interconnections of PPARγ with other transcriptional regulators and the tissue specificity of the effects (liver vs. adipose tissue). Structural descriptors of importance for the PPARγ agonistic activity were identified.

**Acknowledgments to:** (i) the European Community’s 7th Framework Programme, (ii) the National Institute for Public Health and the Environment, Bilthoven, The Netherlands, and (iii) MIe-based models were developed.
Our data demonstrate a highly stable and reproducible method for the cellular systems in normal and pathophysiological conditions.

Metabolomics in vitro: a new approach for systemic toxicity – first applications for chemical grouping

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The use of alternatives to animal testing has considerably increased in the last years but is still mostly limited to topical applications. Metabolomics in vitro is a novel approach to identify systemic toxicity by determining modes of action and (dis)similarities in the profiles of chemicals. In vitro metabolomics allows for the acquisition of quantitative information about the multi-parametric metabolic response of the cellular systems in normal and pathophysiological conditions. Our data demonstrate a highly stable and reproducible method. The purpose of this study was to investigate if toxicological equivalence between pure enantiomers and their corresponding racemates can be demonstrated using this technology. We therefore exposed HepG2 cells at two concentrations for 48 h to different, hepatotoxic, active metabolites. The results obtained indicate that there was no bioequivalence of both the pure and racemic compounds. The results obtained indicate that there was no biologically relevant difference between the two forms of the respective compounds. As such equivalence had been demonstrated in the past using in vivo metabolomics, the present results can be seen as a proof of concept for the use of this in vitro technology. We thus demonstrate straightforward applicability and conclude that in vitro metabolomics is (more than) a promising tool to address systemic toxicity.

Pathway level interpretation of toxicogenomics data

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A major goal of toxicogenomics is the prediction of hazard (and ultimately risk) of chemicals based on genome-wide transcriptomics either derived from microarray or alternatively from high-throughput sequencing experiments (van Delft et al., 2012). Expression signatures derived from single gene markers are highly variable and thus lack stability across large numbers of chemicals. To address this point we have developed an approach that evaluates transcriptomics data at the level of entire pathways rather than isolated gene markers (Yildirim et al., 2011; Doktorova et al., 2013). In our presentation we demonstrate, based on data from the field of carcinogenicity and liver toxicity, that this approach improves hazard prediction to a large extent. Furthermore, we describe a novel resource, ToxDB, that holds functionality for network based interpretation of toxicogenomics data. ToxDB combines statistical network methodology and utilizes the ConsensusPathDB, a large resource of human molecular interactions and pathways (Kamburov et al., 2013). ToxDB can be used as a tool for deriving more stable network module signatures for hazard prediction and adverse outcome pathway identification.

References


Pathways of toxicity as a predictive tool for safety assessment


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The National Research Council report, “Tox-21c” has created an atmosphere of departure in the US. It suggests moving away from traditional (animal) testing to modern technologies based on toxicity pathways. The current developments on OECD level are to organize our knowledge on hazard manifestations as Adverse Outcome Pathways (AOP). The concept of Pathways of Toxicity (PoT) is part of the AOP and describes the molecular definition of mechanism and the perturbed networks.

The NIH is funding a transformative research grant, led by CAAT that involves several members of the Tox-21c panel. The goal is to develop a public database of pathways, the Human Toxome, to enable scientific collaboration and exchange.

An area of toxicology where Tox-21c and the Human Toxome could have a significant impact is developmental neurotoxicity (DNT). Current animal tests for DNT have several limitations: high costs, time consuming and uses large numbers of animals. Consequently, only very few substances have been tested for DNT. This is a concern as evidence shows that exposures to environmental chemicals contribute to the increasing incidence of neurodevelopmental disorders in children. DNTTox-21c is a CAAT project funded by the FDA that aims to find pathways of developmental neurotoxicity using a metabolomics approach.
Allergic contact dermatitis (ACD) is a skin disease triggered by repeated contact to an allergen. The prerequisite for the development of ACD is sensitization. Several in vitro methods including dendritic cell line activation (e.g., mMUSSt, h-CLAT), keratinocyte activation (e.g., LuSens, KeratinoSens™) and in chemico (e.g., DPRA) assays have been developed to assess the skin sensitizing potential of compounds. Although these are not stand-alone methods, they can be used in an integrated testing strategy (ITS) preferably representing different steps of the adverse outcome pathway for skin sensitization to replace animal tests. One promising strategy that was shown to have a good overall accuracy is to use the results in a weight of evidence (WoE) approach based on a 2-out-of-3-assessment (Bauch et al., 2012). To expand the existing data set, 40 additional compounds representing different chemical classes and formulations (including acrylates, surfactants, isocyanates, polyethylene imines, plant extracts and agro-chemical formulations) was tested in mMUSSt, h-CLAT, LuSens and DPRA followed by a WoE assessment. The results were compared to in vivo data. When excluding polyethylene imines, the accuracy of the WoE approach remains high. The results of this project help define the applicability domain of this ITS in which reliable predictions can be expected.

Reference

**II-1-285**

**Development of an alternative testing strategy for the Fish Early Life-Stage (FELS) test: results of the AOP ‘Acetylcholinesterase inhibition leading to motor activity impairment’**

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The Fish Early Life-Stage (FELS) test (OECD 210) is the primary guideline used to estimate chronic toxicity of chemicals. Industry and regulatory institutions express the need to develop alternative testing strategies for environmental impact assessment focusing on non-animal alternatives and mechanistic information (Ankley et al., 2010; Scholz et al., 2013). The goal of the CEFIC LRI-ECO20 project is to develop an alternative testing strategy to reduce the need for 30 days FELS tests. The project puts forward 4 adverse outcome pathways (AOPs) linking molecular initiating events to apical endpoints. Preliminary results of the AOP “Acetylcholinesterase (AChE) inhibition leading to motor activity impairment” will be presented for chlorpyrifos, chlorpyrifos-oxon, and carbaryl using a 5 days Zebrafish Embryo...
Toxicity (ZFET) test. AChE was inhibited and movement frequency was reduced in zebrafish larvae after 5 days exposure to each of these 3 compounds. These experiments with zebrafish larvae analysing locomotor activity and AChE inhibition are further extended to cover key events at different organisational levels. Therefore, neurite outgrowth in a human neuronal cell model and motoneuron development in zebrafish are under investigation to elaborate on this AOP and demonstrate whether any of these approaches, or combinations will contribute to predict chronic FelS toxicity of selected organophosphates and carbamates.

This research is funded by CEFIC Long-Range Research Initiative.

References

II-1-289
An adverse outcome pathway based approach to evaluate novel tobacco products and electronic nicotine delivery devices in vitro
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Novel “Heat-not-Burn” tobacco products and electronic nicotine delivery devices are expected to be associated with reduced risk of smoking-related disease compared to traditional cigarettes. In vitro models have excellent potential to minimise reliance on animal data to predict disease risk. Our challenge is to develop an appropriate and meaningful suite of in vitro models to complement animal and human studies, which are able to, collectively, evaluate the potential for reductions in risk.

Adverse outcome pathways are a useful 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 map out a pathway of events which could ultimately result in tobacco-related disease risk. Furthermore, we propose a suite of in vitro models based upon these pathways, which collectively, could be used to compare the relative biological effects of “Heat-not-Burn” tobacco products and electronic nicotine delivery devices against traditional cigarettes.

This approach could be useful as part of a weight of evidence package for the comparative risk assessment of future tobacco and nicotine based products.

II-1-501
Application of alternative toxicity test methods for safety assessment of active pharmaceutical ingredient intermediates
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Chemical synthesis of active pharmaceutical ingredients (APIs) involves handling with many intermediates. In order to establish an appropriate strategy for safety evaluation it is important to identify their risk and to settle precaution to make the process safer for workers. The aim of this research was to explore the potential of the alternative test methods for toxicity testing of four (5, 8, 9 and 17) intermediates of synthesis of highly potent APIs with respect to their applicability for hazard assessment.

The cytotoxicity was evaluated on three fibroblast cell lines: mouse 3T3 by Neutral Red assay and on mouse L929 and Chinese hamster V79 using the standard MTT assay. On the basis of midpoint toxicity values, the sequence was intermediate 5 > intermediate 9 > intermediate 8 > intermediate 17. Differences in sensitivity between the NR and MTT assays were not marked.

In vitro skin corrosion potential of intermediates was examined us-
ing the in vitro reconstructed human skin model EpiDerm™. It was concluded that all 4 intermediates are considered to be non-corrosive to the skin.

The predictive potential of in vitro tests was evaluated in comparison with the results of a set of alternative in vivo tests.

This work was supported by Ministry of Education SR Agency for Structural Funds of EU in frame of project code ITMS: 26240220061.

C24:1-ceramide may be a novel lipid biomarker for eye irritation in 3D human corneal epithelial model, MCTT HCE™

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Eye irritation test is mandatory for cosmetics and pharmaceuticals. Draize rabbit eye irritation test has been widely used. However, due to an invasive experimental procedure, alternative in vitro tests replacing Draize test are being actively needed. Recently, 3D reconstituted human corneal models receive enormous attention since they are morphologically and physiologically similar to human cornea. These 3D models are employing cell viability as a common endpoint for eye irritant but extra biomarkers are necessary to improve test performance. Here, we explored new lipid biomarkers for eye irritation using MCTT HCE model. Surfactants were selected as eye irritants since they are used in cosmetics and pharmaceutical products. Three irritants; sodium lauryl sulfate, benzalkonium and tritonX-100 were selected as representative surfactants. After treating 3 irritants at different concentrations on the model, we extracted lipid in supernatant using methyl-tert-butyl ether. And we quantified the amount of ceramides and fatty acids, representative lipid components in outer surface of body, using sensitive LCM/MSMS method. It was found that among diverse fatty acids and ceramides, C24:1Cer was significantly increased by three eye irritants. Moreover, C24:1Cer was increased by 3 irritants in a dose-dependent manner suggesting that it can be a novel lipid biomarker for eye irritants.

Using adverse outcome pathways for regulatory applications

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Adverse Outcome Pathways (AOPs) offer a pathway-based toxicological framework to support hazard assessment and reduce uncertainty in regulatory decision-making. Here we present four case studies demonstrating different levels of knowledge and confidence to examine how the AOP concept can be used as a qualitative and quantitative tool in hazard and risk assessment for either human health or ecological concerns. We also present a conceptual model that enables quantitative integration of data into a pathway context, based on the biological relevance of an event to the outcome, the strength of evidence for a causal relationship between key events, and the ability of a key event to infer that a chemical will cause the adverse outcome. The model involves weighting values assigned to each event and subsequent analysis of the relative contribution of each event to the overall weight-of-evidence assessment. The utility of the model is demonstrated by examining the pathway for mitochondrial fatty acid beta-oxidation inhibition leading to steatosis. For AOPs to be useful they should result in a risk assessment with higher confidence compared to current approaches. This necessitates the development of quantitative solutions to AOP assessment and this study offers one such approach.

The experience of using the alternative in vitro methods for testing and safety assessment of goods for children, detergents, cosmetics, including raw nanomaterials

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The alternative in vitro methods are widely used at the safety assessment of the products In the Hygiene and Epidemiology Center in the city of Moscow (further the Center).

About 40% of all toxicological studies conducted in the Center fall to the share of alternative in vitro methods.

Generally these work are conducted for control in the market of Russia, Belarus, Kazakhstan domestic and foreign products such as cosmetics, household chemicals and detergents, goods for children, toys, sanitary pads and tampons. Among alternative methods of 58% it is the share of cytotoxicity tests with use of cattle sperm; 30% – the share of cytotoxicity with photobacteria model and 12% – ex vivo irritation modified HET-CAM test method with Doppler ultrasonography on CAM vessels.

Now in collaboration with Research Institute of Medical Equipment, Lomonosov Moscow State University, The Republic Hygiene and Epidemiology Center of Belarus with support of manufacturers, in particular Unilever Company, SPLAT and Colgate-Palmolive, we conducted the studies in order to compare the results of acute toxicity and mucosa irritation effects received on various test – models from oral care products and raw materials, including those which compounds containing nanoparticles (Ag, Zn, Ti, hydroxyapatite).
Session II-2: Systems biology

Co-chairs
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Session II-2: Oral presentations

II-2-362

In vitro data combined with human disease data to improve toxicological hazard assessment: the ASAT Knowledge Base

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In line with the Assuring Safety Without Animal Testing (ASAT) principle, risk assessment may ultimately become feasible without the use of animals (Fentem et al., 2004). ASAT assumes that activation of human disease mechanisms in in vitro models can be used for toxicological assessment. Therefore, the goal of the present research was to demonstrate the integration of public data from the human disease domain with in vitro toxicology. Two diseases, associated with chemical exposure, were considered: hepatocellular carcinoma and allergic contact dermatitis (ACD). Data were retrieved from online sources (e.g., GEO, CTD) and expert knowledge. A Knowledge Base for curation, storage and modelling of the data was developed. Using the Knowledge Base for ACD, it was possible to discern sensitizing from non-sensitizing chemicals, as defined by enrichment of disease gene sets in in vitro toxicogenomics datasets. Interestingly, the strongest sensitizers most profoundly activated these gene sets. The ongoing incorporation of (reverse) PBPK models in the Knowledge Base to judge the relevance of in vitro concentrations, in relation to realistic in vivo exposure scenarios, will be presented. Finally, the expansion with models towards other disease areas (cholestasis) will be discussed.

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Reference

II-2-395

Development of a mechanistic based model for Nrf2 and oxidative stress in the context of the Adverse Outcome Pathway framework

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Cellular responses caused by prolonged increases in ROS production and oxidative stress are detrimental and have been associated with numerous adverse toxicological outcomes in relation to chemical induced toxicity. Cells have evolved complex adaptive responses designed to regulate and maintain cellular homeostasis and limit the consequential increased risk of toxicity. Determining the point when this homeostatic control becomes dysregulated and shifts from an adaptive to an adverse state is critical to facilitate decision making within an AOP framework. To better understand the mechanism of action in relation to adaptive and adverse effects at low doses for oxidative stress, we report on studies to develop an integrated Nrf2 and cellular outcome systems model. Time- and dose-dependent changes in biomarkers for ROS production, alterations in GSH, and Nrf2 activation were measured in a human keratinocyte (HaCat) cell line following chemical exposure. The downstream effects on cytotoxicity, lipid and protein damage were measured to understand the cellular consequences at varying exposure concentrations and duration. These data are being integrated into a computational pathway model of Nrf2 response to predict the low dose response behaviour of the system and assess where physiologically adaptive/adverse responses are observed, for non-animal based safety assessment of pro-oxidant chemicals.

II-2-602

Using alternative approaches to prioritize testing for the universe of chemicals with potential for human exposure

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One use of alternative methods is to target animal use at only those chemicals and tests that are absolutely necessary. We discuss prioritization of testing based on high-throughput screening assays (HTS), QSAR modeling, high-throughput toxicokinetics (HTTK), and expo-

Reference
Percellome toxicogenomics project as the 3R-toxicology and the foundation of in vitro- and in silico-toxicology

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Percellome Toxicogenomics Project, using fewer animals exposing to lower doses for one time or short period of time, was initiated in 2001, aiming at mechanistically reinforcing the “safety (uncertainty) factor” used for the extrapolation of animal data to humans, and eventually replacing and making the process in silico. The project was designed not to miss any unpredicted toxicity. For this need, a normalization method designated as “Percellome” (Kanno et al., 2006; Aisaki and Kanno, 2011), was developed for microarrays and Q-PCR to generate absolute copy numbers of mRNAs per one cell (in average). Quantified mRNA data of mouse liver (4 time points x 4 dose levels, n=3, 48 per chemical) are obtained on more than 100 chemicals and data-based with the data from repeated dosage, various organs, inhalation, etc. Data was visualized in 3D surface graphs (time x dose x mRNA copy number per cell) corresponding to the probesets of Affymetrix MOE430 2.0 GeneChip and subjected to comprehensive analysis by a series of in-house software. We report case studies on estragole (Kanno et al., 2012) and pentachlorophenol (Kanno et al., 2013), discovering unreported networks of PPAR-alpha and interferon signaling networks, respectively. Further strategy, including systems biology (Garuda Project, http://www.garuda-alliance.org/), for the foundation of the alternative methods will be discussed.

References

In vitro to in vivo translational biomarkers

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There is need to develop better biomarkers with improved target organ specificity, improved sensitivity and which provide deeper mechanistic insight, for both preclinical and clinical testing. The use of human in vitro cell cultures is being realised as a rich resource for the discovery of novel mechanistically based biomarkers with potential transferability to in vivo. Here I will provide examples of liver, renal and cardiac biomarkers which have been either discovered in vitro or where such systems have been used to elucidate key mechanistic information. Many of these biomarkers are more than innocent by-
Session II-2: Poster presentations

II-2-036

Urinary glycosaminoglycan excretion in rats with cyclophosphamide-induced haemorrhagic cystitis

V. Sobolev and V. Shmurak

It is shown that concentration of glycosaminoglycans (GAGs) in human and animal urine changes when suffering from mucopolysaccharidosis (Fischer et al., 1998), rheumatoid arthritis (Kery et al., 1992), glomerulonephritis (Mitsushashi et al., 1993), interstitial cystitis (Akçay and Konukoğlu, 1999). In this work we evaluate the level of excretion of glycosaminoglycans in rat urine suffering from hemorrhagic cystitis induced by cyclophosphamide (CYP).

Experiment was conducted on 20 adult female Sprague Dawley rats that were divided into 4 groups of 5 animals each. First group was used as an intact control. All other animals (groups 2-4) were given cyclophosphamide (100 mg/kg) by intraperitoneal injection. One hour prior the injection and later once a day for 3 days, the rats from groups 2 and 3 were given a solution of glycosamine hydrochloride (GHCl) (10 mg/kg and 100 mg/kg, respectively) intragastrically. The rats from group 4 did not receive glycosamine hydrochloride. 24 hour urine was collected daily before the experiment and for 24 hour after injecting CYP to measure glycosaminoglycans. Measurements were done with using 1,9 dimethylmethylene blue (DMMB) (Grant et al., 2006).

Spectrophotometrically measured GAG excretion in urine showed no statistically significant differences between healthy rats and rats with hemorrhagic cystitis induced by cyclophosphamide. GAG excretion in rats that received glycosamine hydrochloride for 24 hour also showed no difference.

References


II-2-095

Enlightening the intracellular metabolic phenotype of neuronal cells: prospects of transient isotope tracer experiments

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Motivation: Neurons respond highly sensitive to toxin exposure by modulating their cellular phenotype (Henry et al., 2002). To identify the primary targets of the toxins it is, thus, pivotal to quantify alterations of the intracellular in vivo reaction rates, the fluxome, being the integrated functional output of all “ome” levels (Hua et al., 2007). Up to now, the majority of toxicity studies with animal model cells determine only a limited number of fluxes in combination with selected labeling data (Henry et al., 2002). Recent advances in fluxomics technology enable the systematic model-based evaluation of comprehensive transient 13C-profiles derived from cellular intermediates (Wichert and Nöh, 2013).

Results: A large-scale metabolic network model with atomic resolution for neuronal cells is built. In a case study, central metabolic fluxes are calibrated with the transient labeling data from state-of-the-art GC-MS devices. By means of global sensitivity values we investigate the impact of the quality and the quantity of the data with respect to the flux determination problem. This provides a powerful and universal tool to understand the functional relations between measured data and flux values. A guideline is derived to design tracer experiments that are capable to resolve carbon flow in neuronal cells.

References


II-2-518

Chemical grouping and read across: a biology based approach using metabolomics

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MetaMapTox is a metabolomics database, containing >500 data rich chemicals, agrochemicals and drugs. It has been built using 28 day studies in rats (OECD 407) with blood metabolomics after 7, 14 and 28 days of treatment. 120 metabolome patterns have been established, associated with target organ toxicity and modes of action. With these patterns early detection of toxicity can be obtained from routine or early phase studies. This enables development of new compounds with a low toxicological profile and helps to reduce the number of animal studies necessary to do so. To reduce the high number of animal studies be performed under the REACH, grouping of chemicals and read-across to data rich ones is the best option. Currently, QSAR models are driving the selection process of chemical grouping. ‘Omics technologies such as metabolomics can help to optimize the chemical grouping process by providing biologically based criteria for toxicological equivalence. This is based on the MoA identification and by whole metabolome comparison to the 500 reference compounds. The combined evaluation of this information is a powerful tool for biology based grouping of chemicals. Going from QSAR to QBAR (quantitative biological activity relationship).

The SEURAT-1 (Safety Evaluation Ultimately Replacing Animal Testing-1) research cluster is comprised of seven EU FP7 Health projects and is co-financed by Cosmetics Europe. The SEURAT-1 strategy is to adopt a mode-of-action framework to describe repeated dose toxicity to derive predictions of in vivo toxicity responses. ToxBank is the cross-cluster infrastructure project which provides a web-accessible shared repository of research data and protocols. Experiments generate dose response data over multiple timepoints using different omics platforms including transcriptomics, proteomics, metabolomics, and epigenetics over different cell lines and a common set of reference compounds (details available at wiki.toxbank.net). Data is also generated from functional assays and bioreactors and supplemented with in silico approaches. This complex and heterogeneous data is consolidated and harmonized through the ToxBank data warehouse in order to perform an integrated data analysis. We describe for 14 reference compounds the meta-analysis of currently public data including Open TC-GATEs human in vitro liver data of the reference compounds including reactive compounds (e.g., acetaminophen, CCH), mitochondrial disruptors (e.g., Rotenone), promiscuous binders (e.g., valproic acid, amiodarone), nuclear hormone receptor ligands (e.g., tamoxifen, WY14643), selective binders (e.g.,fluoxetine) and cardiotoxins (e.g., Doxorubicin, Nifedipine). Adverse events of interest that are represented include cytotoxicity, fibrosis, steatosis, cholestasis and phospholipidosis.

System II-3: Computational modeling and chem-informatics

Co-chairs
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Session II-3: Oral presentations

II-3-356
Utilization status and future development of HESS repeated dose toxicity prediction system
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Hazard Evaluation Support System Integrated Platform (HESS) (Sakuratani et al., 2013; Hayashi and Sakuratani, 2012) is a system for predicting repeated dose toxicity of chemicals based on category approach, which was developed by national project in Japan, and was released free of charge from our website (http://www.safe.nite.go.jp/english/kasinn/qsar/ess-c.html) in June 2012. HESS is compatible with OECD (QSAR) Toolbox and the workflow of HESS is similar as that of OECD (QSAR) Toolbox.

We have been showing that highly reliable prediction can be obtained by using HESS for several groups of chemicals. However, because the prediction by category approach is case by case, it is necessary to accumulate case studies and continuously upgrade the system in expanding the applicability of the system.

Over four hundred users were registered as HESS so far. We have associated actively with HESS users through regular training course of HESS, and so on. Considering feedbacks from users we have explored methods of effective utilization of HESS and the direction of further improvement of HESS. In this presentation, we will introduce current utilization status of HESS and recent activities for future development of HESS.

References

References
Quantitative high throughput profiling of the Tox21 10K compound library for environmental hazards

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The goal of the U.S. Tox21 program is to shift the assessment of chemical hazards from traditional animal toxicology studies to target-specific, mechanism-oriented approaches based largely on in vitro cell-based assays. In Tox21 Phase II, a collection of approximately 10K chemicals (http://www.epa.gov/ncct/dsstox/sdf_tox21s.html) is being tested in triplicate against a battery of nuclear receptor and stress response pathway assays in a quantitative high throughput screening (qHTS) format. To date more than 48 million data points have been generated from approximately thirty assays. The robustness of the data was evaluated by the reproducibility of the triplicate assay runs and the 88 compounds that are intentionally duplicated in the 10K library. Several approaches were used to minimize assay artifacts and cytotoxicity interference often associated with in vitro assays. The structure features of the 10K compounds and their activity measures from the assays were evaluated as descriptors to build predictive models for in vivo toxicity endpoints. The in vitro compound activity profiles generated from qHTS were assessed further for their applicability as signatures of toxicity mechanism and utility in prioritizing compounds for more in depth toxicological testing.

Use of QSAR tools for hazard identification of genotoxic impurities in pharmaceuticals

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The focus of the latest legislative and governmental efforts is to establish simple screening tools for identifying chemicals most likely to cause adverse effects without experimental testing. The use of quantitative structure-activity relationship (QSAR) is a powerful in silico technique that should be considered for prioritizing chemicals for subsequent experimental verification. Genotoxicity is the highest concern for human health among all toxicological endpoints, because genotoxic chemicals have potential to cause cancers at very small level. In ICH, we are developing the new guideline, “Assessment and Control of DNA-Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (MT)”, which addresses a way to characterize the genotoxic hazard of impurities in pharmaceuticals and to assess their carcinogenic risk. The guideline focuses on DNA-reactive impurities which can be detected by Ames assay. In silico QSAR methodologies could be employed to predict the Ames mutagenicity. Negative results from two complementary QSARs (expert rule-based and statistical) are sufficient to demonstrate the none-mutagenicity and does not require further testing. The ICH-M7 guideline recommendations provide a state-of-the-art approach for assessing genotoxic impurities to avoid unnecessary experimental testing.

A knowledge-informed chemotype approach to mining the ToxCast/Tox21 chemical-data landscape

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Toxcast and Tox21 chemical libraries currently exceed 2000 and 8000 unique chemicals, respectively, and span a broad diversity of chemical use-types, functionality, and toxicity mechanism and endpoint space. These libraries function as mechanism probes across hundreds of high-throughput in vitro bioassays. Structure-activity relationship (SAR) models and structure alerts that carry historical chemical-toxicity inferences can be projected onto this chemical landscape as a way of incorporating prior knowledge, thereby aiding in the detection of significantly enriched patterns and associations within and across the in vitro and in vivo data landscapes. A set of public ToxPrint chemotypes are being used to create a common platform for storing and communicating such associations, and for profiling and comparing structure inventories. Examples will be presented using such knowledge-informed features to convey associations within structure subsets pertaining to metabolic activation of rat carcinogens, disruption of mitochondrial membrane potential, and induction of cleft palate. The large and growing in vitro, in vivo, and computed property data profiles associated with ToxCast/Tox21 chemicals are also providing a means to expand the concept of molecular similarity beyond that of chemical structure alone, to build on prior knowledge and inform read-across approaches.

Abstract does not reflect EPA, FDA, or NIH policy.

Read-across at the crossroad of chemoinformatics and regulatory science

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Improving the predictivity of methods for human safety and risk assessment remains a key interest of regulatory programs. A diverse array of in vivo, in vitro, and computational methods are being evaluated to profile compounds for potential impact on human health. Along with conventional toxicity experiments, the new paradigm of regulatory science brings high throughput screening assays and toxicogenomics to enrich the biological domain. Profiling compounds to fill data gaps is especially promising as a pragmatic and viable non-testing method. From a broader perspective, read-across can be considered as a data gap filling process, where diverse data sources, including animal toxicity, bioassays, analog- or category-based classifications along with quantitative structure activity relationship models are combined and assessed. The key is to find data relevant to the target endpoints based...
Abstract does not reflect EPA or FDA policy.

II-3-857

Being more certain about uncertainty in computational toxicology modeling

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II-3-074

Inhibition screen of OATP1B1, OATP1B1*15 and OATP1B3 combined with computational modeling to predict OATP-mediated drug-drug interactions

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Human organic anion-transporting polypeptide 1B1 (OATP1B1) and OATP1B3 are important hepatic uptake transporters. Early assessment of OATP1B1/1B3-mediated drug-drug interactions (DDIs) is therefore important for successful drug development. A promising approach for early screening and prediction of DDIs is computational modeling. In this study we aimed to generate rapid, Bayesian prediction models for OATP1B1, OATP1B1*15 and OATP1B3 inhibition.

The inhibitory potential of 640 FDA-approved drugs (10 μM) on the uptake of [3H]-estradiol-17β-D-glucuronide (1 μM) was measured in HEK-OATP1B1, HEK-OATP1B1*15 and HEK-OATP1B3 cells. Using a cut-off of ≥60% inhibition, 8% and 7% of the 640 drugs were potent OATP1B1 and OATP1B1*15 inhibitors, respectively. Only 1% of the tested drugs significantly inhibited OATP1B3, which was not sufficient for Bayesian modeling. Modeling of OATP1B1 and OATP1B1*15 inhibition revealed distinct (structural) features that enhance the probability of a compound binding these transporters. The overall performance of the model for OATP1B1 and OATP1B1*15 was ≥80%, including evaluation with a true external test set.

Our Bayesian classification model represents a fast, inexpensive and robust means of assessing potential binding of new chemical entities to OATP1B1 and OATP1B1*15. This model may be used to rank compounds in early drug discovery and avoid late-stage transporter-mediated adverse effects.

II-3-127

Development of a curated database of in vivo estrogenic activity

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Mandated testing to identify potential estrogen-active chemicals will involve thousands of chemicals, cost millions of dollars, and take decades to complete using current validated methods. High-throughput screening (HTS) assays may streamline this process by quickly and cost-effectively identifying estrogen-active chemicals. Access to a comprehensive database of high-quality in vivo data is critical to effectively validate in vitro and in silico models and HTS assays, as well as enable more targeted in vivo testing. We created a reference database by searching the scientific literature and identifying in vivo studies with endpoints indicating estrogen activity. Data from the studies were extracted and compiled using a standardized ontology. An R script was developed to evaluate the quality of the data in an efficient and standardized manner by modified Klimisch criteria. Data that were classified as reliable were added to the database, which will be publically available on the NTP website (http://ntp.niehs.nih.gov/go/40658).

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66

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Novel methods are presented for the estimation of six physicochemical properties (octanol/water partition coefficient, water solubility, boiling point, melting point, vapor pressure and bioconcentration factor) of environmental chemicals using simple binary molecular fingerprints. Quantitative structure-property relationship (QSPR) models were developed using four approaches with differing complexity: multiple linear regression (MLR), random forest (RF) regression, partial least squares regression (PLSR), and support vector regression (SVR). Genetic algorithms (GA) and RF methods were employed to select the most information-rich subset of descriptors for obtaining reliable and robust regression models. MLR, PLSR, and SVM exhibited satisfactory predictive results with low prediction errors and all substantially outperformed RF. The approach in which MLR was coupled with GA for descriptor selection was superior to all other approaches and achieved high correlation coefficients between the calculated and experimental data (R² > 0.90). This study demonstrates that (1) molecular fingerprints are useful descriptors, (2) GA is an efficient feature selection tool from which selected descriptors can effectively model these properties, and (3) simple methods such as MLR give better results than more complicated methods.

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Computational approaches for predicting protein targets for chemical toxins based on molecular similarity and inverse docking

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Chemical toxins often affect multiple protein targets. Although large amounts of toxin-target information are available from experimental approaches and databases, a great deal of specific information remains unknown. Computational methods provide a means to uncover novel interactions and provide new concepts for testable hypotheses in future experiments. We developed two tools for predicting protein targets for chemical toxins. The first method is a toxin-based approach. It is based on the idea that similar molecules affect similar targets. OpenEye molecular similarity search Tools and Toxin-Target database (t3db) (Lim et al., 2010) were used to predict possible protein targets for chemical toxins that have analogs in the t3db. This method can be applied to predict novel targets for toxins that are already in t3db. The second approach is a protein-based approach and uses inverse docking of a toxin against thousands of protein structures. X-ray crystal structures of human proteins were downloaded from Protein Data Bank and OpenEye docking tools are used to perform ligand-protein docking. This method is applied when a chemical toxin of interest has low molecular similarity to toxins in the t3db. Examples of application of these tools will be presented such as identification of hemoglobin as a potential target for toluene.

Reference
mation (R² = 96.2%) were obtained at the first step (398 samples) (Yuta, 2013). The KY methods used in these screenings could realize a complete classification, in spite that a large number of samples were used and nega/posi samples were highly overlapped. In general, prediction rates are relatively low as compared with classification rates. The same applies to our results. However, it should be noted that a perfect classification can always be achieved by the KY methods. Prediction rates may be improved in the near future by further modifying the KY methods.

References

II-3-505
Issues relating to the availability and standardisation of data for nanomaterials to assist in risk assessment
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As for “traditional” toxicology, computational approaches are being increasingly considered to support the risk assessment of nanomaterials. A basic requirement to develop in silico models, however, is the availability of high quality experimental toxicity and structural/physicochemical property data. These data should be easily accessible, ideally in an open, electronic format and, most importantly, in a unified form. As part of increasing global efforts, such as the various projects under the umbrella of the EU NanoSafety Cluster and those in the USA and Japan generating and collecting data, the EU NanoPUZZLES Project is systematically applying a standardised format, ISA-TAB-Nano. The aim is to organise the data, also to allow a comparison between data from different sources and thus give added value to a database. Especially as part of larger efforts such as the NanoSafety Cluster, this possible comparison of different data collected for the same nanomaterials will allow for the bringing together of the dispersed efforts to characterise nanomaterials to support safety assessment. Thus, particular attention has been given to metadata standardisation through the use of ontologies and assessment of the data quality.

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II-3-526
Applying cheminformatics approaches to support toxicity prediction and safety assessment of chemicals through the development of robust categories
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Cheminformatics approaches are increasingly important to support chemical risk assessment without relying on animal testing. In particular, the grouping of chemicals into categories allows for data gaps to be filled via read-across. The key aspect to this approach is the definition of biologically meaningful chemical similarity. Structural categories have been defined, based on organic reaction mechanisms, for two key Molecular Initiating Events: covalent bond formation with proteins and DNA. These structural fragments have been shown to be useful for grouping chemicals, some of which are alerts for endpoints such as skin and respiratory sensitisation, hepatotoxicity and genotoxicity. They have been refined to chemotypes and made available through ToxPrint.org for use with the freely available ChemoTyper software (http://chemotyper.org). Chemotypes have the advantage of being able to include physicochemical property information or even quantitative structure-activity relationship (QSAR) equations in addition to structural information, and thus allow an improved method for grouping chemicals into categories suitable for making read-across predictions. These chemotypes are applied to inventories of diverse chemicals of cosmetics, food additives, drugs, and pesticides for comparison of chemical and biological space to support safety assessment of chemicals.

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Using Adverse Outcome Pathways to support mechanistic in silico modelling: examples with organ level toxicity

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Adverse Outcome Pathways (AOPs) are increasingly being used to organise mechanistic information related to an adverse effect. AOPs provide a way to organise data that allows the link from the molecular initiating event (MIE) to the adverse effect to be evidenced. As such, they add a new dimension to in silico, or computational, predictive toxicology. The MIE is the basis by which the the chemistry underpinning the model can be interpreted and the complete AOP provides the mechanistic justification that links the MIE to the effect. The aim of this study was to use knowledge of the AOPs for adverse effects to the liver to identify structural features of molecules related to toxicity. To this end a large database of compounds associated with liver toxicity was analysed. This has allowed for the identification and definition of over 100 structural fragments, some which have been coded into relevant chemotypes, for effects such as phospholipidosis, cholestasis and reactive hepatotoxicity. These alerts and chemotypes provide a robust means of category formation allowing for read-across which may be justified in terms of the MIE.

Acknowledgement: EU COSMOS Project (grant agreement 266835).

Acute oral toxicity modeling for mechanistic and toxicological mode of action

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Based on a category approach, quantitative structure activity relationships (QSAR) were developed for acute oral toxicity (AOT) of chemicals (in rats). Chemicals were grouped in different toxicological categories based on their chemical interaction mechanism and toxicological mode of action.

In this work, analyzing the toxic potency of chemicals, two general types of toxicity were defined: basic and excess. Basic toxicity is considered as the minimum toxicity caused by non-reactive chemicals. The toxicity of chemicals destroying some critical processes in the cells or the whole organism deviates from the basic toxicity. These chemicals revealing an excess toxicity usually have highly reactive groups or act by specific interaction mechanisms. Solubility parameters were found to correlate adequately with the potency of these chemicals. However, they can also reveal just basic toxicity because of their limited bioavailability.

This AOT model is implemented in the TIMES platform. Currently it contains about 2500 training chemicals classified in 67 toxicological categories. We will discuss how this model overcomes the limitations of current models by integrating multiple but specifically derived QSAR models for each toxicological category. Moreover all predictions are to be supported by mechanistic justification for the mode of action, example chemicals and an applicability domain indication.

A mathematical model for a reproduction-related adverse outcome pathway in fathead minnows (Pimephales promelas)

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A fundamental knowledge gap exists between experimental toxicity tests and long-term human health or wildlife population effects. Recently, an Adverse Outcome Pathway (AOP) has emerged as a potential solution to this problem by providing a framework for causal linkages between a molecular initiating event (MIE, e.g., receptor-ligand binding) and population level outcomes (e.g., reduced fecundity) across many levels of biological organization (Ankley et al., 2010). The aim of this research is to quantitatively link an MIE to population level impacts through biologically based mathematical modeling. We developed a first-generation mathematical model of a reproduction-related AOP for a nonsteroidal aromatase inhibitor, fadrozole, that predicts changes in fecundity for the female teleost fathead minnow (Pimephales promelas). The model was developed in Matlab® and links two existing models: one representing dynamic interactions at the transcriptional and hormone level in the hypothalamic, pituitary, gonadal (HPG) axis (Mayo et al., 2012), and an oocyte growth dynamics model (Li et al., 2011; Watanabe et al., 2012). Given an aquatic fadrozole exposure profile, the quantitative AOP model predicts dynamic impacts to fecundity by mechanistically modeling: (i) transcriptional effects resulting from aromatase inhibition; (ii) their effects on steroidogenesis; (iii) vitellogenin production triggered by estradiol; and (iv) vitellogenin-dependent oocyte growth and maturation.

References
Session II-4a: Risk assessment – Nano

Co-chairs
Harvey Clewell, The Hamner Institutes for Health Sciences, USA
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Session II-4a: Oral presentations

II-4a-157
Search for and implementation of integrated testing strategies in nanotoxicology
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Integrated testing strategies (ITS) are heavily discussed as tools for reducing animal numbers in toxicology and for testing complex effects. Safety testing of nanomaterials raises several serious issues, of which one is the sheer impossibility of testing all existing and future nanoparticle preparations in vivo. On the other hand, nanomaterials are already in wide use and their ecologic and health risks must be assessed properly. Our project raises the question whether ITS can provide a solution for this dilemma. For this purpose, we seek suitable in vitro approaches within the Swiss National Research Programme 64 (Opportunities and risks of nanomaterials) for possible inclusion into an ITS (proof of concept). With this, we will attempt to convince authorities in Switzerland to rely on similar approaches that use animal testing only as a last resort. Simultaneously, we are raising the question whether EU-financed programmes concerning themselves with risk assessment of nanomaterials routinely and efficiently build into an ITS (proof of concept). With this, we will attempt to convince authorities in Switzerland to rely on similar approaches that use animal testing only as a last resort. Simultaneously, we are raising the question whether EU-financed programmes concerning themselves with risk assessment of nanomaterials routinely and efficiently build into an ITS (proof of concept). With this, we will attempt to convince authorities in Switzerland to rely on similar approaches that use animal testing only as a last resort. Simultaneously, we are raising the question whether EU-financed programmes concerning themselves with risk assessment of nanomaterials routinely and efficiently build into an ITS (proof of concept).

II-4a-447
Cytotoxicity of eleven metal oxide nanoparticles
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In the current study we analysed 11 metal oxide nanoparticles (NPs) for their potential toxic effect using human epithelial cell-lines A549 and Caco2 as in vitro nanotoxicity models for alveolar and intestinal physiological barriers. The particles were produced by flame pyrolysis method and were tested on cell lines in the concentration range of 3.1-100 µg/ml. Solutions of respective metal salts were analysed simultaneously, to evaluate the toxic impact of ions dissolved from NPs. As a toxicity endpoint, 24 h inhibition of viability measured by NRU (OECD validated non-animal alternative for acute systemic cytotoxicity) was used. The results were compared with microscopic analysis and transepithelial electrical resistance measurements (TEER) to monitor the integrity of cell monolayer.

II-4a-430
Comparison of cytotoxicity of engineered nanoparticles in mouse BALB/c 3T3 fibroblasts, rat NR8383 macrophages and human U937 monocytes
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The aim of this study was to compare the cytotoxicity of engineered nanomaterials (silver (Ag), gold (Au), titanium (TiO2), silica (SiO2) nanoparticles (NP), and carbon nanotubes (CNT)) in mouse BALB/c 3T3 fibroblasts, rat NR8383 macrophages and human U937 monocytes. Cells were grown in standard culture conditions (37°C, 5.0% CO2) in 96-well plates and exposed to different concentrations of dispersed nanoparticles for 24-48 hours, after which the cytotoxicity assays were performed. BALB/c 3T3 cell viability was assessed using NRU assay (OECD GD 129), and NR8383 and U937 cell viability was assessed using WST-1 assay, which is suitable for suspension cells. The movement, morphology and uptake of nanoparticles were followed during exposure with live cell imaging (Cell-IQ).

AgNP was toxic to all cell cultures studied, whereas the toxicity of CNT, SiO2 and TiO2 seemed to be more cell-specific: NR8383 macrophages were most sensitive to the nanoparticles followed by BALB/c 3T3 and U937 cells suggesting potential immune-related toxicity of nanoparticles. AuNP did not affect the viability of any of the cell cultures. Live cell imaging revealed differences between cell cultures in the uptake of nanoparticles, as well as in changes in cell morphology and motility.
Biological impact of modified inhalable carbon black nanoparticles assessed in cell and tissue culture models

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Carbon black nanoparticles (CBNP) have a large surface area to volume ratio and potentially cause adverse health effects after inhalative exposure. Biological effects of four CBNP modifications with different surface properties were assessed in human pulmonary cell lines (16HBE14o−, Calu-3, A549) and precision cut lung slices (PCLS) of mice, rats and humans. Viability was assessed by LIVE/DEAD® staining and WST-1/8 assay. Pro-inflammatory immune responses in PCLS were quantified using ELISA. CBNP-induced formation of reactive oxygen species (ROS) was assessed by flow cytometry using the DCFH-DA assay. The effect of CBNP exposure on transepithelial electrical resistance (TEER) was investigated in Calu-3 cells. CBNP Printex® 90, acetylene soot, and benzo[α]pyrene nanoparticles were nearly non toxic in cell lines and PCLS. However, the dose-response relationships varied considerably between cell lines. 9-Nitroanthracene showed a toxic effect at 50 μg/ml in PCLS. The inflammatory response was assessed in tissue lysate and decreased intracellular cytokine concentration. Increased ROS formation was observed in cell cultures. The cytokine concentration. The large numbers and varieties of nanomaterials. the toxCast program for prioritizing nanomaterial hazards and informing targeted testing due to the impracticality of using traditional toxicological testing on the large numbers and varieties of nanomaterials. The ToxCast program at the US EPA has used various high-throughput assays and developed computational tools to help assess potential toxicity and identify toxicity pathways of hundreds of traditional chemicals. We investigated the compatibility of selected ToxCast cellular, high-throughput screening assays on engineered nanomaterials, with the ultimate goals of identifying toxicity/biological pathways affected by nanomaterials and finding correlations among nanomaterial physicochemical characteristics, testing conditions, and nanomaterial toxicities/bioactivities. Au, Ag, CeO₂, CuO₂, TiO₂, SiO₂, and ZnO nanoparticles, their ion and micro counterparts, carbon nanotubes (CNTs), asbestos, and pesticides containing nano-Cu(O) were screened at 6-10 concentrations each. A total of 262 bioactivity/toxicity endpoints in cells and zebrafish embryos were measured. Cellular stress and immune response pathways were the most common pathways affected. NM’s core chemical composition was more important than size for bioactivity. Ag, Cu, and Zn (nano and ion samples) were the most active materials with nano and ion counterparts producing similar profiles, suggesting ion shedding was a key factor in mechanism of action. While 3 asbestos samples had similar immune response profiles, 6 CNTs had profiles distinctive from asbestos We demonstrated that HTS assays can identify affected cellular pathways, predict targets, and may be useful for ranking NMs for specific purposes. This abstract does not necessarily reflect EPA policy.

In vitro testing of engineered nanomaterials in the EPA's ToxCast program

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High-throughput and high-content screens are attractive approaches for prioritizing nanomaterial hazards and informing targeted testing due to the impracticality of using traditional toxicological testing on the large numbers and varieties of nanomaterials. The ToxCast program at the US EPA has used various high-throughput assays and developed computational tools to help assess potential toxicity and identify toxicity pathways of hundreds of traditional chemicals. We investigated the compatibility of selected ToxCast cellular, high-throughput screening assays on engineered nanomaterials, with the ultimate goals of identifying toxicity/biological pathways affected by nanomaterials and finding correlations among nanomaterial physicochemical characteristics, testing conditions, and nanomaterial toxicities/bioactivities. Au, Ag, CeO₂, CuO₂, TiO₂, SiO₂, and ZnO nanoparticles, their ion and micro counterparts, carbon nanotubes (CNTs), asbestos, and pesticides containing nano-Cu(O) were screened at 6-10 concentrations each. A total of 262 bioactivity/toxicity endpoints in cells and zebrafish embryos were measured. Cellular stress and immune response pathways were the most common pathways affected. NM’s core chemical composition was more important than size for bioactivity. Ag, Cu, and Zn (nano and ion samples) were the most active materials with nano and ion counterparts producing similar profiles, suggesting ion shedding was a key factor in mechanism of action. While 3 asbestos samples had similar immune response profiles, 6 CNTs had profiles distinctive from asbestos We demonstrated that HTS assays can identify affected cellular pathways, predict targets, and may be useful for ranking NMs for specific purposes. This abstract does not necessarily reflect EPA policy.
Consumer risk assessments for personal care products without the use of data generated in animals

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For more than 10 years, we have been working with others to develop novel ways of assuring consumer safety that are human-relevant and do not rely on animal data. There has been a great deal of progress in realising this goal and, for many areas of consumer safety, prototype case study risk assessments are being developed. These are based on the initial framework described by the US NRC (NRC, 2007) and the Adverse Outcome Pathway framework currently being explored by the OECD.

Consumer safety risk assessments for cosmetic ingredients are always exposure-driven. Pathways-based risk assessments require an understanding of the kinetics of ingredients following consumer exposure within the skin (for dermally applied ingredients), the lung (for respirable ingredients) and the systemic circulation. This kinetic exposure information can then be used together with in vitro “point of departure” data for pathways of concern to allow quantitative in vitro in vivo extrapolation. The pathways we have examined using this framework to date include P53-mediated DNA damage (Adeleye et al., 2014), skin sensitisation (MacKay et al., 2013), nr2 activation, mitochondrial toxicity and lung fibrosis. Each of these prototype consumer safety risk assessments demonstrates the practical applicability of the AOP framework and highlights the areas where future research is needed.

References
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Non-invasive monitoring of drugs and toxic agents based on Raman laser spectroscopy

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In pharmaceutical drug evaluation, cosmetic substance compatibility as well as toxicology testing there is an increasing demand for non-invasive yet highly sensitive cell recognition methods. Emerging technologies to identify cells or to monitor cell state and fate are vibrational spectroscopic methods such as Raman spectroscopy. Raman spectroscopy provides information of the bio-molecular status of cells non-invasively, nondestructively, and under physiological conditions. Cells remain undisturbed for ongoing cultivation or downstream analysis. This is especially important to monitor, e.g., cell/drug interactions or to perform cell based toxicology tests.

A challenge for cell based testing is the availability of stable cell cultures. Xellulin® a novel hydrogel based matrix was used as scaffold for cultivation of Smooth Muscle Cells (SMC’s). SMC’s can be kept vital for several months without splitting and still exhibiting in vivo cell morphology.

Raman spectroscopy was used to compare the vitality of SMC’s cultured under different conditions. Confluent SMC’s after 10 days cultivation on standard tissue culture plates were compared with SMC’s cultivated on Xellulin® having reached confluence three weeks ago.

This novel combination of stable cell growth and culture technology with non-invasive Raman spectroscopic monitoring has huge potential for effective and animal-free toxicology testing and drug qualification.

Can a retrospective analysis of SCCS toxicological data contribute to the design of a new approach for the safety assessment of cosmetic ingredients with low oral bioavailability?

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Risk assessment of cosmetic ingredients is usually based on existing in vivo oral toxicological data and newly generated in vitro results on dermal absorption. This strategy is straightforward for substances with a reasonably high oral bioavailability. This approach, however, is not applicable without knowing the extent of oral systemic availability for topically applied compounds with a low oral bioavailability. In addition, for these substances usually the full set of toxicological data is not available and further in vivo testing is not possible seen the strict animal testing and marketing bans present in the Cosmetic Regulation 1223/2009. Therefore, a retrospective analysis of the toxicological results, present in the opinions (Annex substances, 2000-2014) of the Scientific Committee on Consumer Safety (SCCS) and its predecessors was performed. This data shows that an initial set of physicochemical properties (e.g., molecular weight, log P, H-bond donors and acceptors, melting point), preferably enriched with (available) kinetic and toxicological parameters (e.g., dermal absorption) could allow a pragmatic conclusion to be reached, on a case-by-case basis,
for cosmetic substances with low oral bioavailability. Consequently, the required data for the safety assessment of these substances, identified using the above approach, could be limited to local toxicity and genotoxicity/mutagenicity testing.

II-4b-347

Human skin-derived stem cells as a novel cell source for in vitro hepatotoxic testing

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Hepatic toxicity induced by pharmaceutical compounds is one of the major causes of acute liver failure. The current human hepatic screening methods are based on scarcely available primary hepatic cells or cell lines derived from cancerogenously liver tissue. New developments in stem cell research create new possibilities as stem cells represent an inexhaustible cell source and have the ability to differentiate into multiple cell types. In this study, we evaluate the potential of human skin-derived precursors (hSKP) as an alternative cell source for hepatotoxicity testing. These cells can be easily isolated from human (fore) skin segments and have the capacity to differentiate into cells with hepatic characteristics (hSKP-HPC). Using a toxicogenomics approach we observed that when exposed to paracetamol, a known hepatotoxic compound, hSKP-HPC respond in a similar way as primary human hepatocytes in culture. Furthermore, we could show that exposure to sodium valproate, a well-known steatotic compound, induced production of microvesicular lipid droplets and enrichment of genes typical for hepatic steatosis. This methodology was more sensitive than human hepatocytes. In conclusion, our data shows that skin-derived stem cells could represent a suitable human-relevant preclinical model for in vitro testing of hepatotoxic compounds, mitigating the necessity of using primary human hepatocytes.

II-4b-361

Toxicogenomics as a WoE tool to investigate false positive responses obtained in the LLNA

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Skin sensitization represents the most common manifestation of immunotoxicity in humans, and hundreds of chemicals have been implicated as skin sensitizers. The murine local lymph node assay (LLNA), based on assessment of lymphocyte proliferation in draining lymph nodes, is a relatively recent method for hazard identification and potency assessment of skin sensitizers. Compared with previous guinea pig assays the LLNA offers animal welfare benefits (reduction and refinement). However, certain chemistries, such as long chain fatty acids, result in apparent false positive LLNA responses when compared with guinea pig and human data. A toxicogenomic approach has been applied to provide insight into the mechanisms that may explain these differential responses. Gene expression responses were evaluated in mice following exposure to equipotent doses of 9 chemical sensitizers and 7 false positives as per the standard LLNA dosing regimen. Differentially expressed genes between sensitizers and false positives were identified: genes involved in early T-cell development and DC/T-cell maturation or genes associated with pro-inflammatory processes, respectively. These gene expression profiles suggest differential cellular recruitment to lymph nodes following exposure to true sensitizers and false positives and provide a potential new endpoint that could identify false positives and enhance LLNA predictivity.

II-4b-777

Assessment of phototoxic potential of topically applied substances and formulations using 3-D reconstructed human skin models

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Reconstructed human skin models permit testing of various types of topically applied materials ranging from neat chemicals to final clinical formulations. The methods developed to date are based on measuring cell viability in the tissue with and without irradiation. Release of cytokines/chemokines into the medium can be assessed as well. Already in 1990’s, ECVAM pre-validation phototoxicity study on the EpiDerm™ model (Liebsch et al., 1997, 1999) showed that reconstructed human epidermis (RhE) models are capable of correct detection of the acute human phototoxines and non-phototoxines. Several studies with other commercially available reconstructed tissue models (EPI SKIN®, SkinEthic™) confirmed the findings. The sensitivity and specificity of assays developed with RhE models for prediction of acute skin phototoxicity are close to 100%. Use of RhE-based phototoxicity assays for prediction of the topical phototoxicity testing has been recently implemented also into the draft ICH Guidance Document on Photosafety Testing (S10). As shown in a feasibility study with EpiDerm, these models can also be used to assess the phototoxic strength of topical photo-toxins. The presentation will focus upon current 3-D skin models for the assessment of the phototoxicity potential of dermal formulations and will provide overview of RhE-based phototoxicity assays, their strengths, weaknesses and use for photosafety assessment in toxicology and pharmacology.

References

Session II-4: Risk Assessment – Poster presentations

II-4-041
Agglomeration influences cytotoxicity of nanovesicle formulations in vitro
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Cationic lipid-based nanovesicles (NVs) have been proposed as bio-compatible drug delivery devices. Although extensive studies have focused on drug release and penetration properties of nanovesicles, the potential toxic mechanisms of this kind of carriers have not been explained sufficiently and specially in relation to their size and the potential formation of aggregates. In this study we have developed cationic nanovesicular formulations containing different cationic lysine-based surfactants as surface modification agents and differing in the cationic charge position. Cytotoxicity was determined in four cell lines (3T3, HaCaT, HeLa and THP1) and different endpoints were assayed. The nanovesicles were characterized both in water and in cell culture at 0 h and 24 h incubation period. There are no correlation between the nanovesicle size and the cytotoxicity determined by the different endpoints. However, significant agglomeration was observed after 24 h incubation depending on the surfactant used for the formulation. In the worst case from 94 nm to 1780 nm and no agglomeration was observed in other cases. The results showed that agglomeration influences cytotoxicity, with less cytotoxic effect with higher agglomerates.

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II-4-113
Animal welfare observations and early toxicologic assessment tools used to improve selection of traditional drug discovery efficacy models
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Collaboration between disciplines early in the drug discovery process can reduce animal distress and numbers used, end non-productive research faster, and identify novel applications for alternatives. Here, the classic neuroscience reference compound, para-chlorophenylalanine (PCPA) was dosed to decrease central 5-Hydroxytryptamine (Serotonin) in rats to create a model of behavioral depression. During model development, patterns of illness and mortality were recognized by animal care staff. IACUC reassessment included veterinary, toxicology and pathology expertise. A comprehensive literature review of PCPA toxicity revealed renal and pancreas target organs, but no other organ specificity or mortality risk. Subsequent evaluations included characterizing different in vivo dosing routes and, in vitro, optimizing the formulation using the Hen’s Egg Test-Chorion Allantoic Membrane (HET-CAM) model. PCPA induced irritation was recognized during these evaluations. Injection site and visceral/parietal peritoneum inflammation was observed as were alternations in select clinical pathology parameters. The HET-CAM model revealed direct irritation by PCPA. These investigations demonstrated that PCPA, despite a long history of use in animal research models, induces local irritation, systemic inflammation, and related behavioral effects that confound accurate profiling of novel compounds intended as human anti-depression drugs. As a result, valid behavioral testing with this proposed model was deemed not feasible due to PCPA related irritation/toxicity.

II-4-041
Validation of the Slug Mucosal Irritation test: correlation with clinical data
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Local tolerance of pharmaceutical, industrial, household and cosmetic products needs to be evaluated prior to marketing them. In the past, animal studies were performed to assess this. Within the scope of alternative testing methods, the Slug Mucosal Irritation (SMI) test was developed. Mucus production (MP) was selected as endpoint to evaluate irritation, while released proteins and enzymes are an estimate for the tissue damaging potency of substances.

Several protocols of the SMI test have been developed in time. The recent Stinging, Itching & Burning test focuses on the prediction of clinical discomfort. Its applicability and clinical relevance for ocular and nasal applications were investigated by direct comparison with human results from 2 clinical trials. Both studies showed a significant positive association between immediate stinging sensations observed by the participants and the total MP of slugs (Spearman’s Rank correlation = 0.986, p<0.001 (ocular); = 0.963, p<0.001 (nasal)).

The SMI assay has proven to be a promising method for the evaluation of discomfort, irritation and tissue damage. It allows observation of reversibility and can predict subjective effects such as clinical discomfort. To our knowledge there is no other single method that can evaluate these parameters in one model.
Use of the zebrafish developmental screen and estimation of internal concentration to assess toxicity

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Environmental chemicals from the ToxCast™ Phase I chemical library were screened to assess developmental toxicity endpoints. Zebrafish embryos were immersed in media containing one of 309 chemicals tested, at concentrations ranging from 0.001-80 μM. The half-maximal activity concentration (AC50) for toxicity (lethality, non-hatching, tested, at concentrations ranging from 0.001-80 μM. The half-maximally were screened to assess developmental toxicity endpoints. Zebrafish environmental chemicals from the ToxCast™ Phase I chemical library with a mean AC50...others. The pyrethroids (n=12) were among the most toxic chemicals associated with developmental toxicity (eC50). toxicity potency rankings derived from AC50 and EC50 calculations were compared. Some chemicals were highly toxic regardless of how toxicity was expressed while use of EC50 values substantially affected the toxicity ranking of others. The pyrethroids (n=12) were among the most toxic chemicals with a mean AC50 of 4.01 μM. However, due to their high lipophilicity, the mean EC50 for the class was estimated at 843.25 μM. The ability of the zebrafish developmental screen to predict mammalian toxicity was assessed by examining the correlation between chemical potencies based on EC50 chemical class, and known in vivo effects.

This abstract does not necessarily reflect EPA policy.

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Leveraging small aquarium fishes to advance understanding of environmentally influenced human disorders and diseases

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Small aquarium fishes provide model organisms that recapitulate the development, physiology and specific disease processes present in humans without many of the limitations of rodent-based models currently in use. Fish models offer advantages in cost, rapid life-cycles, and external embryonic development. However, they remain relatively modest contributors to understanding the effects of environmental chemical exposures on human health. A workshop organized by NC State University, Duke University, NIH, NIEHS, EPA, and FDA explored how aquatic models could be used to (1) screen and prioritize compounds for further in vivo testing and (2) assess mechanisms of chemical toxicity and how this knowledge can impact environmental and human health. The workshop brought together experts from academia, industry, and government to develop a framework to assist in integrating toxicology data from aquatic models with testing initiatives currently underway to enhance risk and safety assessments of chemicals and pharmaceuticals. Workshop participants identified research initiatives that address current information gaps in risk and safety assessments for multi-organ toxicity, longitudinal studies to assess long-term consequences of chronic exposures, and the embryonic basis of adult disease.

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Evaluation of immunotoxicity of natural compounds by a combination of functional in vitro investigation and proteomics

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Natural products can affect the immune system leading to immune dysregulation. Immunotoxicity investigated in animals which have limited predictable time for humans should be replaced by combining functional cellular analysis, proteomics, metabolomics and bioinformatical analyses. Final aim is prediction of immunotoxicity of compounds using a database, in which cell biological, proteomics and metabolomics data are summarized to compound-target-pathway relations.

Here, the impact of three natural compounds known for affecting the immune system, Cannabidiol, Tulipaline and Primin, on Jurkat-cells is investigated. Cytotoxic potential was evaluated using NRU assay (control, Etoposide). Further characterization was done with IC50 and IC100, cell cycle, apoptosis, induction of intracellular reactive oxygen species (iROS) and proteome analysis.

IC50 values of compounds differed markedly. Of the three compounds Primin was most cytotoxic. Etoposide significantly induced iROS and apoptosis. Cannabidiol enhanced iROS, induced apoptosis and had little effect on cell cycle. Primin strongly induced iROS, apoptosis, decreased G1-phase. The impact of Tulipaline was less pronounced. 2D-gel-electrophoresis of intracellular proteins from Cannabidiol-treated cells revealed three different proteins (two induced, one repressed), which will be identified by mass spectrometry.

There is evidence to suggest that the compounds investigated have immunotoxic potential which has to be analyzed further.

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Integrated test strategy for cosmetics safety and efficacy assessment

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In vitro alternative test is the main approaches for toxicity prediction and development of active material for cosmetics. Under the pressure of regulations and science progress, non-animals test strategy should be established in inspection institution and enterprise. This series of research to try to establish the integration test strategy (ITS) for cosmetic toxicity and efficacy assessment and to provide technical services to industries. Since 2006, the Alternative Animal Test Group has established and using more than 10 test batteries for toxicology assessment, covered cytotoxic screening/acute toxicity prediction (3T3 NRU+DPC+KC NRU+ target tissue cytotoxicity), eye irritation (CAMVA+BCONHET-CAM+BCOMPEDMCK-FL-BCOP), skin irritation (keratinocyte +Episkin, TET+cytotoxicity), phototoxicity (3T3 NRU-PT+RBC-PT), developmental toxicity (EST+ER, EST+Leydig cell), skin sensitization and genotoxicity. Meanwhile, we are trying to set up in vitro efficacy ITS for screening ingredients of cosmetic materials, for example antioxidant (ring-CAM, fibroblast ROS), sunscreen (keratinocyte P53, inhibition ET-1) and whitening (anti-tyrosinase activity, inhibition keratinocyte uptake). AAT group also actively explore and innovate the toxicology tests of the 21st century. Through continuous training and promoting accept ITS concepts by enterprise and inspection agency, which accelerated and outspread the alternative methods in China. It is also helpful for the safety evaluation and efficacy claims of products.

* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

References
Use of in vitro assays to compare the effects of exposure to smoke and smoke extracts from a conventional cigarette and a reduced toxicant prototype

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British American Tobacco are developing a suite of in vitro assays to evaluate the effects of tobacco products on endpoints relevant to smoking-related diseases. Here we compare the responses of these assays to smoke derived from commercially available and reduced toxicant prototype (RTT) cigarettes.

Cells were exposed to mainstream smoke extracts in the form of particulate matter (PM), aqueous extracts (CSEaq), or to whole mainstream smoke (WS). The ability of the two test products to induce cytotoxicity, secretion of inflammatory mediators and oxidative stress in NCI-H292 bronchial epithelial cells or to inhibit wound closure in human umbilical endothelial cells (HUVECs), were compared.

In studies using PM there were no differences between the cigarette products in any of the assays (p>0.05). However, WS from the RTT cigarette induced significantly less cytotoxicity than WS from the conventional cigarette. Furthermore, preliminary data showed that CSEaq from the RTT induced less oxidative stress and lower inhibition of wound closure compared to the commercial cigarette.

These assays may be useful as part of a weight of evidence package for the comparative risk assessment of novel tobacco products. Further work will assess whether this approach can be utilised for the assessment of nicotine products.

Lentiviral transduction to drive a liver-derived rat epithelial cell line from biliary origin towards hepatocyte-like cells

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Before pharmaceuticals and other chemical compounds can be placed on the EU market, their safety needs to be ensured for man and his environment. Historically, toxicity testing is performed in vivo through tests with laboratory animals, mainly rodents. Yet, there is clear political incentive in the EU to minimize the number of animals involved in the chemical risk assessment process. However, to link newly obtained in vitro data to existing in vivo animal data, reliable rodent-based in vitro systems are needed. As such, we want to explore whether a rat epithelial cell line, called rLEC, can be reprogrammed into hepatocytes in order to assess in vivo-like liver toxicity. Our group recently found that rLEC exhibits properties of hepatic progenitor cells (HPC) and expresses HPC transcription factors including CEBPA, FOXA2 and OECUT1, but not the adult HNF4A and HNF1A. Yet, upon sequential exposure to hepatogenic growth factors and cytokines, rLEC is able to generate functional hepatic progeny. A major drawback of this technology, however, is the long culture time and high consumption of expensive growth factors. Therefore, in this study, we aim at generating functional hepatic cells by lentiviral transduction of rLEC with the hepatic nuclear factors HNF4A and HNF1A.

Optimization of an eye irritation assay for solid materials for hazard identification and labelling of chemicals

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The latest EU Cosmetics directives and REACH legislation necessitate reliable in vitro test methods. To address these requirements, the EpiOcular™ eye irritation test (EIT) was developed. The test utilizes a normal human organotypic tissue model and two separate protocols: one for liquid chemicals, and another for solids. The original EpiOcular-EIT protocol for solids discriminated between ocular irritants (“I”, GHS cat 1 and 2) and non-irritants (“NI”, no category) with 95.0% /78.9%/ and 87.2% sensitivity/ specificity/ and accuracy (SS&A). However, analysis of a larger dataset (Kolle et al., 2011) indicated that sensitivity of the solids protocol was lower than expected. Therefore, the test method for solids was further optimized. The optimized protocol which utilizes a longer exposure time, was tested 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% /68.4% /and 84.6% SS&A which meet all the acceptance criteria for validation. The assay has been involved in a formal, multi-laboratory validation study sponsored by the Cosmetics Europe under the auspices of ECVAM to assess the relevance and reliability of the assay with the goal of bringing it to formal validation.

References

Risk assessment of consumer products by use of alternative tox methods

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Risk assessment (RA) is a vital tool for establishing safety of consumer products for human use. Recent challenges have impacted the RA strategies for consumer products, ban on animal testing being the most significant development. These challenges have led to use of alternate Tox methods for qualifying various consumer products/ingredients by using in-silico prediction models including QSAR techniques,
read-across approaches, mathematical modelling to derive consumer exposure, TTC approach and validated non-animal methods data for local toxicity (Skin/eye irritation and corrosion, photo-toxicity), genotoxicity and dermal absorption. Few of the product categories viz. botanicals, aerosol products and laundry products involve complex ingredients/applications and hence need multiple alternate methods to derive exposure and assess risk to consumers. Novel botanicals qualification involves characterization of chemical composition, QSAR and TTC approach. For aerosol spray products, approaches such as mathematical modelling to derive the worst case scenario human exposure, read-across, in vitro methods, occupational toxicity data and TTC application is required. Laundry care products are qualified by estimating the direct and indirect exposure to chemicals which are mathematically derived with multiple parameters such as habits, dilution factors, assumptions on film layers on skin and transfer factors.

II-4-509
A method proposed to estimate the index of relevance using the statistical imputation approach
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Relevance is defined on the basis of the result of a validation study that can predict and indicate the toxicity of test substances. It is evaluated using the dataset from the inter-laboratory experiments in a validation study. All the test substances treated in a validation study should be used in all the participant laboratories. However, although all the planned test substances are used, different test substances are often used partially in each laboratory because of the limitations of cost etc. Thus, the dataset constructed for inter-laboratory analysis has a lot of missing data and might not be useful to evaluate the precise relevance.

This presentation aims to propose a new method to estimate the index of relevance in the case of a missing dataset. The proposed method involves including missing data to construct a pseudo complete dataset by using a statistical imputation method and calculate the index for relevance using the dataset. A simulation study was conducted to compare the sensitivity and specificity of the proposed method with those of the conventional one. The proposed method showed ideal performance. Therefore, the new proposed method would be more useful for determining the index of relevance.

II-4-561
Cytotoxic, cytoprotective and genotoxic evaluation of grape pomace extract against hydrogen peroxide insult
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Increasing interest in the beneficial effects of byproducts makes mandatory the performance of toxicological assays before any potential use (Ugartondo et al., 2007; Díaz et al., 2011). In this context in vitro methods appear as a very convenient strategy to evaluate both the possible adverse effects and beneficial activities. In the present study, an evaluation of the cytotoxic and genotoxic effects of a methanol grape pomace extract, besides its cytoprotective activity in front of peroxidative insult in 3T3 cell culture is described. MTT and NRU were used in order to evaluate cytotoxic and cytoprotective effects (Botta et al., 2013). The comet assay was performed to evaluate genotoxic and/or antigentoxicity effect of the sample or after oxidative insult with H2O2. The extract showed an IC50 of 441 and 465 µg/ml for MTT and NRU, respectively. Moreover, the extract prevented from H2O2-induced cytotoxicity in 3T3 cells (2 mM, 2 h), at concentration between 250-62.5 µg/ml. For genotoxicity test, the sample was proven at 100 µg/ml, and not produces any increase in the extent of DNA strand-breakage. We observed a slightly DNA-breakage preventing effect against H2O2 damage. The methanol extract of grape pomace is source of biological activity with several applications.

References

II-4-577
Isolated intervertebral disc cells as a platform for in vitro toxicology testing
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Introduction: For diagnostics of discogenic low back pain, local anaesthetics are often used, which may be cytotoxic to intervertebral disc cells. Studies so far have been mainly performed on animal cells. The study aim was to evaluate the effect of local anaesthetics on human intervertebral disc cells in vitro.

Materials and Methods: Annulus fibrosus and nucleus pulposus cells were isolated from human lumbar intervertebral disc fragments and exposed to various concentrations of lidocaine, bupivacaine and their mixture. Saline solution was used as a control. Three different dilutions (undiluted, 1:2 and 1:4) of anaesthetics were tested. The cells were treated for 6, 24 and 48 hours and examined with for viability.

Results: Nucleus pulposus cells were more susceptible than annulus fibrosus cells to the toxic effects of both anaesthetics. Lidocaine was more toxic with the final cell survival fraction of 0%, 10% and 20%. Bupivacaine presented less cytotoxicity with the final survival of 10%, 60% and 80%. Lidocaine-bupivacaine mixture showed an intermediate toxicological effect.

Conclusions: Lidocaine and its mixtures should be avoided due to its high toxicity to the intervertebral disc cells. Bupivacaine was less toxic, especially in 1:4 dilutions and may be recommended for the intradiscal diagnostics.
A review of the REACH legislation from the animal welfare perspective

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REACH is now seven years old. The first two registration deadlines have passed and over 12,000 chemicals have now been registered. Various stakeholders have been going through a period of review to see if the legislation has held up to their specific expectations. Here we assess how well REACH, and those involved in it, have upheld its central principles of “the promotion of alternative methods” and “animal testing as a last resort.”

In terms of animal numbers – so far – new animal tests have been fewer than expected. However, a proportion of these were done preemptively or where alternatives exist. There are issues with the appropriate use and acceptance of read across, in vitro tests, QSAR and weight of evidence approaches. The Agency has taken a narrow view of its role and will not reject testing proposals except in very limited circumstances. The Commission is funding alternative methods with a budget that appears to far exceed that of individual Member States but has failed to take swift action to ensure even those alternatives it has validated should legally be used. These issues need to be addressed urgently or where alternatives exist. There are issues with the appropriate use and acceptance of read across, in vitro tests, QSAR and weight of evidence approaches. The Agency has taken a narrow view of its role and will not reject testing proposals except in very limited circumstances. The Commission is funding alternative methods with a budget that appears to far exceed that of individual Member States but has failed to take swift action to ensure even those alternatives it has validated should legally be used.

Refined animal toxicity testing using unequally sized dose groups and Benchmark Dose analysis

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The Benchmark Dose (BMD) method has been recommended to replace the No Observed Adverse Effect Level (NOAEL) approach and is accepted in most regulatory settings. Standard protocols for toxicity testing are aligned with the NOAEL approach and suggest four dose groups of equal size.

We attempted to find the optimum study design for BMD analysis using designs with unequally sized dose groups. In total, 85 different designs were compared using group sizes of 30, 40, 50, 60 and 70 while keeping the total number of animals at 200. Dose-response data were generated by Monte Carlo simulations assuming a “true” dose-response curve with added variability. The quality of the dose-response analysis was assessed by comparing the Root Mean Squared Error (RMSE) of the estimated BMD for each design with that for the standard 50-50-50-50 design. The data was also evaluated from an animal welfare perspective.

The RMSEs tended to be lower with more animals in the lower dose groups, suggesting that the quality of dose-effect and dose-response analysis can be improved by distributing animals unequally among dose groups. Furthermore, moving animals from high to low dose may substantially reduce animal distress, since distress is more likely to occur at higher doses.

Metabolism simulation and toxicity prediction in the evaluation of food ingredient/contaminant safety

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During the safety assessment of food additives, metabolic knowledge becomes critical when in vivo data are unavailable for the specific compound. Inclusion of metabolism information into the in silico workflow is therefore a pre-requisite for the US FDA’s Chemical Evaluation and Risk Estimation System (CERES). The chemical space of food additives was profiled using public ToxPrint (https://toxprint.org) and a diverse set of metabolic chemotypes. Chemotypes are structural fragments encoded with physicochemical properties, and are considered as alerts when associated with a specific endpoint. As an example, the effect of metabolic chemotypes on Ames mutagenicity was analyzed against the food additives (http://1.usa.gov/1rwzlc5).

Although nearly 20% of the compounds were perceived as genotoxic carcinogens by chemotype alerts, only 4% of such compounds were actually predicted to be mutagenic under CERES mechanistic QSAR paradigm. Nearly 50% of the food additives predicted to be mutagenic were matched with chemotype alerts for genotoxic carcinogens. When repeating the analysis after removing the compounds that may be detoxified, the reliability of the genotoxic alerts is increased almost 3 times. This study demonstrates the value of the use of metabolic rules to reduce the false positive rate of structural rules.

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Comparison of in vitro and in vivo methodologies for eye irritation evaluation of cosmetic formulations

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In vitro protocols for evaluating eye irritation are widely used in the safety assessment of cosmetics. However, some techniques fail to identify weak irritants and numerous false negatives are observed. To overcome that restriction, application of the in vitro agar diffusion methodology – which uses SIRC cells for cell viability and growth inhibition analyses – was evaluated and compared to a usual in vivo approach (Guess et al., 1965). In vitro results showed that all selected products were harmful at the concentration of 100% (pure sample), but different classifications regarding the irritant potential were obtained with dilutions of 1% or IC50 (predetermined by cytotoxic-
ity). For in vivo studies, procedures were conducted under aesthetic and ophthalmic supervision, with previous application of make up to simulate real conditions of use. In each volunteer, one eye received a non-irritating solution while the other received an evaluated product (Cruz, 2003). Then volunteers answered a questionnaire to describe possible sensations, such as discomfort, weak irritation and/or burning. Comparative results evidenced a good correlation between in vitro and in vivo techniques, suggesting the agar diffusion as a good alternative, efficient and discriminative method for the safety screening of cosmetic formulations, especially those designed to be in contact with the eyes.

References


II-4-808

Cultured porcine cornea assay using confocal microscopy for high resolution detection and quantification of sub-mild ocular irritation

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A critical need exists for a non-animal ocular irritation assay sensitive to sub-mild ocular irritation. We have developed a novel assay, PorFocal, which can quantify individual dead corneal epithelial cells in porcine corneas using confocal microscopy. PorFocal uses phosphate buffered saline (PBS) as a negative control and 0.01% benzalkonium chloride (BAK) as a positive control. In 17 experiments, 0.01% BAK always caused more cell death than PBS, and statistically (p<0.05) more in 15 of 17 replicates. The PorFocal assay detected a significant dose-response with BAK dilution series treatment. Treatment with 0.01% BAK-preserved lubricant eye drops showed a significant 3-fold increase in cell death versus the preservative-free version. To examine potential of PorFocal to detect human eye sting of a known stinging chemical (avobenzone), we compared a low avobenzone (lA) to a high avobenzone (HA) sample. HA caused significantly more cell damage (7-fold increase) than lA. We compared PorFocal to an industry standard 3D reconstructed human tissue (RhT) ocular irritation assay. The RhT detected four of nine materials while the PorFocal assay detected eight of nine materials tested as statistically different (p<0.05) from PBS values. This indicates a great degree of sensitivity with PorFocal assay, not attainable by existing methods.

II-4-809

The replacement ocular battery (robatt): an integrated testing strategy for ocular irritation classification

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Alternatives to the Draize Rabbit Eye Test have been available since the 1980s but none have yet been fully successful due to no acceptable regulatory adoption to date. To address this need, we have developed an integrated testing strategy (ITS) for ocular toxicity testing: the Replacement Ocular Battery (ROBatt).

ROBatt incorporates four alternative ocular irritation assays – the Choriocapillaris Membrane Vascular Assay (CAMVA), the Bovine Cornea Opacity/Permeability test (BCOP), the Porcine Cornea Opacity/Reversibility Assay (PorCORA), and the Porcine Confoal Assay (PorFocal) into a logical testing approach for ocular irritancy potential ranging from non-irritant to ocular corrosion. A decision tree was devised that integrated these assays and allow for thorough evaluation and categorization of test materials. Fifty-two chemicals were selected from the ECETOC Technical Report No. 48 – Eye Irritation: Reference Chemicals Data Bank (Second Edition), supplemented by data provided by the FDA and EPA. These chemicals were tested to establish criteria that would lead to regulatory classification of chemicals with respect to ocular toxicity. The results are reported and compared with in vivo observations to evaluate ROBatt as an informative and efficient tiered testing strategy to categorize chemicals into regulatory classification without using the Draize test or employing live animals.

II-4-877

Cytotoxicity and cell adhesion evaluation of chitosan films with herbs actives for burns treatment

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Biopolymeric films have attracted much attention because of their potential biological properties, low toxicity, biocompatibility, antimicrobial activity and stimulation of healing, among others. Additionally chitosan promotes the activation of inflammatory cells in tissues and thereby accelerating granular cleansing of the wound and the re-epithelialization. These and other properties can be enhanced with the incorporation of herbal medicines. The objective of this study was to evaluate the cytotoxic and adhesion effect of chitosan films containing copaiba oils and Aloe vera in Balb/fibroblasts to verify the biocompatibility of these films. No cytotoxicity was observed by indirect or direct methods. In addition, cell adhesion assays showed that the films did not have any cell attached. All together these results and the mechanic and physical experiments showed that the biopolymeric film containing copaiba and Aloe vera have great potential to be used in wound healing process.
Session II-5: Discussion session: Application in decision making and testing strategies

Co-chairs
Silke Gabbert, Wageningen University, The Netherlands
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Session II-5: Oral presentations

II-5-133

Using in vitro HTS methods to identify endocrine disruptors

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Current testing for endocrine-active substances utilizes a battery of in vitro and in vivo screening assays. The Tox21 program includes multiple in vitro assays conducted in a high-throughput screening (HTS) format that are relevant to the estrogen receptor (ER) pathway and could be used to identify substances with potential ER activity in vivo. NICEATM compared results from 16 HTS ER pathway assays with published results from uterotrophic studies. We reviewed the literature for 1777 substances tested in ToxCast/Tox21 and identified 191 substances with uterotrophic data. Each study was scored based on adherence to a set of minimum criteria established by NICEATM for guideline-like protocols. To facilitate direct comparison for select ER-active substances, models were built using experimental data and structure-based predictions for metabolic clearance and plasma protein binding to estimate the oral equivalent doses that would result in steady-state blood concentrations equivalent to the HTS in vitro point of departure values. This strategy may be sufficient to screen for ER activity independent of in vivo tests.

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II-5-349

Evidence analysis in a Bayesian ITS

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When an ITS is used to integrate complex data for hypothesis building knowing strength of evidence is very useful to facilitate decisions. In the Bayesian Network ITS a hypothesis about a chemical’s potency is constructed by means of probabilistic weight of evidence. This makes it possible to run an evidence analysis to transparently and objectively assess strength of evidence and to detect possible conflicts. Specifically, the analysis tells us 1. How consistent is the full set of evidence with the hypothesis? 2. Which pieces of evidence support the hypothesis and which refute it? How strongly? 3. How consistent or inconsistent is each piece of evidence with all of the other evidence in the set? The analysis and the communication of it will be illustrated using BN ITS-2 for skin sensitization potency. In particular we will show 2 examples. In the first one there is a very consistent support of the hypothesis, thus an easy decision. In the second example we will show data in conflict and discuss how to resolve it. This makes practical application of BN ITS attractive as it is more than a prediction tool. Rather, it is a decision support tool.

II-5-353

A Bayesian approach to expert judgment of uncertainty in skin sensitisation risk assessment

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A preliminary question in skin sensitisation risk assessment is whether the chemical has the potential to be a sensitiser. Bayesian belief network approaches to this part of the assessment, which handles the disparate lines of evidence within a probabilistic framework, have been applied successfully (Jaworska et al., 2013; Rorije et al., 2013). When potential exists, risk assessors estimate the dose threshold (dose per unit area of skin exposed) at which the chemical induces a sensitisation response in humans (a potency measure). In the absence of in vivo data, the greater challenge comes in the quantification of uncertainty about this potency.

To make inferences about chemical potency, we used a Bayesian linear framework to model assessors’ expectations and uncertainties and to update those beliefs in the light of some competing data sources (Gosling et al., 2013). In producing a tool for synthesising lines of evidence and estimating hazard, we developed a transparent mechanism to help defend and communicate risk management decisions. In this talk, I will attempt to describe the principles of Bayesian modelling and formal processes for capturing expert knowledge. And, hopefully, I will be able to highlight their applicability where fast decisions are needed and data are sparse.

References
A vision for a concern-driven integrated approach for the (eco-)toxicological testing and assessment (IATA) of NMs, developed within the FP7-funded EU projects MARINA and NanoSafety Cluster, is presented (Oomen et al., 2014; Savolainen et al., 2013). The IATA begins by determining concerns for a given NM based on realistic exposure scenarios (Hristozov et al., 2014). In Tier 1 physico-chemical properties, “non-testing” information, and existing data are assessed. In Tier 2, a limited set of in vitro and in vivo tests, most predominantly short-term studies addressing acute effects and kinetic behavior, is performed applying standardized protocols for NM preparation and testing. The outcome of Tier 2 either indicates that the risk of the specific concern is sufficiently known or that further testing is required, providing specific indications for such testing. After each tier, it is evaluated whether the information gained permits safety assessment of the given NM at which point further testing is stopped. By effectively exploiting all available information, IATAs allow accelerating evaluation whether the information gained permits safety assessment of the given NM at which point further testing is stopped. By effectively exploiting all available information, IATAs allow accelerating assessment process and reducing testing costs and animal use.

In order to predict skin sensitizing potential and potency of many chemicals, we created a dataset of 139 test chemicals with sensitizing hazard and potency information more efficiently than traditional “check-list” approaches, where a pre-defined set of standardized in vivo or in vitro guideline tests is performed for every adverse response (Andersen et al., 2010). So far, research has predominantly focused on constructing testing strategies for selecting and combining most relevant pieces of toxicity information (Jaworska et al., 2013). However, efficient testing requires to balance information gains against costs at any stage of the assessment. Moreover, Gabbert and Weikard (Gabbert and Weikard, 2013) showed that the optimal order of testing and non-testing methods is exclusively driven by costs, including not only direct testing costs, but also expected private and societal costs of making errors, and animal welfare loss. Using a Bayesian Value-of-Information model we illustrate for the case of assessing skin sensitisation hazard (Bauch et al., 2012) of cosmetic ingredients how test information gains and costs can be integrated into an optimised testing scheme. Our results underline that efficient testing is not only determined by tests’ predictive performance and prior beliefs about a substances’ intrinsic properties, but also on the relationship of social benefits and costs of use.

References
Toward systems toxicology – modeling complexity from reductionist information sources

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The complexity of the human body and its response to toxicants can only partially be reflected in experimental systems, even in the ambitious human-on-chip approaches. However, they lead us to better cell cultures based on 3D perfused organo-typic cultures from human stem cells and the combination of organs can model fundamental interactions such as xenobiotic metabolism by the liver combined with organ toxicities.

At the same time, toxicity testing is increasingly embracing mechanism and assays reflecting them in a rather reductionist way. However, many hazard manifestations are based on several pathways of toxicity and the organism is a highly complex networked system. In order to predict hazard we need more than batteries of tests, but ways of integrating information. Integrated Testing Strategies are a first step to efficiently combine such information, but on a longer term modeling of responses in a Systems Toxicology approach will be necessary. Virtual experiments, as they are progressing as computational models for organs, embryos or entire patients, can then be compared to test results in order to refine our understanding of Adverse Outcome Pathways.

The state of the art in developing Human-on-Chip models, Integrated testing Strategies and the advent of more complex computational models relevant to toxicity prediction will be presented.

Session II-5: Poster presentations

Use of the in vitro Caco-2 assay to predict the oral absorption of aromatic amine permanent hair dyes

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Replacement of in vivo models for oral absorption is necessary for risk assessments of cosmetic ingredients due to the animal test ban. Oral absorption is an important pharmacokinetic parameter, and prediction is key to understand amounts of chemical delivered to systemic circulation. We compared data from in vivo oral absorption (rats) to the in vitro Caco-2 model, predicting the permeability (Papp) characteristics of 14 aromatic amine hair-dye molecules with varying chemical structures (phenylenediamine, aminophenol, and antrachinon types).

All but one of the hair dyes exhibited Papp values ≥ a high absorption reference (propranolol), suggesting that these would be well absorbed in vivo. The high oral absorption of these compounds (≥80%), was confirmed in vivo (rat). Moreover, the compound with a low Papp value (referenced to ranitidine for low absorption) was also poorly absorbed in vivo (5% of the dose), which would be expected due to the relatively high MW (460) and the presence of a bromide group.

In conclusion, the Caco-2 model is a useful alternative for determining intestinal absorption of hair-dye molecules with diverse chemical structures. In combination with other in vitro metabolism/skin penetration data, prediction and mechanistic interpretation will support the development of animal alternatives to predict in vivo absorption and disposition of chemicals.

A risk-warning method for reporting adverse events: an application of Bayesian statistics to the cosmetics industry

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Aims: Cosmetics companies frequently collect voluntary information on adverse events following the marketing of their products. The aim of this study is to propose a statistical method for the early detection of the risks of cosmetics by using such information.

Method: The proposed method is based on Bayesian statistics (Lee, 2004). The probability of risk associated with cosmetics is calculated and updated monthly from information about adverse events obtained from users of cosmetics products. When the probability of the risk of a cosmetic product exceeds a pre-specified probability, a warning is issued to reconsider the safety of the product. Thus, managers of cosmetics companies are always able to recognise the extent of risk of their cosmetics products as a probability value. The method is expected to guide the early detection of risks associated with marketing cosmetics.

Conclusions: In this presentation, we will report the results of a simulation study in order to reveal the performance of the proposed method.

Reference
**Comparison of 3 criteria incorporating variation of index for toxicity of the interleukin 8 luciferase Luc assay (IL-8 Luc assay)**

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The IL-8 Luc assay is one of the potential alternatives for the skin sensitization assay. In this assay, as an index for toxicity, the ratio of the means of measurements at a concentration with a chemical to that of measurements without the chemical is adopted. The cut-off value of the index is set at 1.4 based on results from studies with a high laboratory. Validation of this method showed low reproducibility. This may be due to the introduction of measurement variation in the criterion for judgment. Thus, we developed 3 criteria incorporating measurement variation and determined the results by using these criteria.

The first criterion is positive judgment in case that the ratio is over 1.4 and an index of evaluating appropriate for a kind of normalization of the measurement is over 0.05. The second criterion is positive judgment in case the 95% confidence interval of the lower limit of the ratio is over 1. The third criterion is positive judgment in case when not only the ratio is over 1.4 but also the lower limit of the ratio is over 1.

We will report the results of the comparison on the basis of the actual dataset.

**Relationship between IC50 and ID50 value in the Hand1-Luc Embryonic Stem cell Test (Hand1-Luc EST)**

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Purpose: The Hand1-Luc EST is one of the candidates to evaluate the developmental toxicity of chemicals with luciferase reporter assays using transgenic engineering mouse embryonic stem (ES) cells. In the test, the IC50 (the 50% inhibition concentration of the viability of cells) and the ID50 (the concentration of the test chemical that reduced the luminescence by 50%) are required for the judgment of the toxicity. For each experiment, it is necessary to verify that the IC50 value is higher than the ID50 value.

Methods: Statistical analyses were performed to determine the relation between IC50 and ID50 value using 21 chemical data obtained by 2 researchers for 3 times. We also estimated the difference in the mean of the logID50 from the one of the logIC50 by adjusting chemicals and experimenters with 95% confidence interval using the Generalized Linear Model (GLM).

Results: The adjusted difference of two means was 0.368. The 95% confidence intervals were [0.260, 0.475].

Conclusion: There was a high significance in not only verifying the IC50 and ID50 values but also in considering the relevance of the ID50 value in the evaluation of the differentiation inhibition.
Session II-6a: Updates on Research Activities from the USA

Co-chairs
Russell Thomas, EPA, USA
Ray Tice, NIEHS, USA

Session II-6a: Oral presentations

II-6a-614

**The future of Tox21: improving on coverage, relevance, and outreach**

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Tox21 is a multiagency collaborative effort by the NIEHS/NTP, NCATS, EPA and the FDA to shift the assessment of chemical hazards from traditional experimental animal toxicology studies to one based on target-specific, mechanism-based, biological observations largely obtained using *in vitro* assays, with the ultimate aim of improving risk assessment for humans and the environment. Limitations of the current evaluation of the 10K library in the high throughput screening program include the limited pathway coverage (i.e., focus on nuclear receptor and stress response pathways assays), the lack of biological complexity (i.e., the use of reporter gene assays using immortal cell lines), and the limited capability for xenobiotic metabolism. The goals of the next phase of Tox21 is to overcome these limitations by incorporating into the testing strategy more physiologically-relevant cell types (e.g., HepaRG cells, embryonic stem- and induced pluripotent stem cell-differentiated cell populations) and lower organisms (e.g., zebrafish, *C. elegans*) integrated with high content screening and high throughput transcriptomics platforms to assess chemical toxicity potential. Equally important are expanded efforts to make all data public and to increase stakeholder involvement by establishing formal and informal relationships with organizations and investigators interested in contributing data and/or data analysis tools to Tox21.

II-6a-879

**The U.S. EPA ToxCast program: moving from data generation to application**

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The U.S. EPA ToxCast program is entering its tenth year. Significant learning and progress have occurred towards collection, analysis, and interpretation of the data. The analysis of ~1,800 chemicals across ~700 high-throughput *in vitro* assays has shown that most environmental and industrial chemicals are very non-selective in the biological targets they perturb, while a small subset of chemicals are relatively selective for specific biological targets. The selectivity of a chemical informs interpretation of the screening results while also guiding future mode-of-action or adverse outcome pathway approaches. Coupling the high-throughput *in vitro* assays with additional *in vitro* pharmacokinetic assays and *in vitro-to-in vivo* extrapolation modeling allows conversion of the potency estimates to an administered dose (mg/kg/day). High throughput exposure models are also being developed that predict exposure potential based on key aspects of chemical fate and transport and personal use. Comparison of the administered dose to human exposure estimates provides a risk-based context. The lessons learned from this effort will be presented and discussed towards application to chemical safety decision making and the future of the computational toxicology program at the U.S. EPA.

This abstract does not necessarily reflect U.S. EPA policy.

II-6a-848

**Adverse outcome pathways can be critical to inform sound regulatory and policy decisions**

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Regulators must assure their toxicology toolbox keeps pace with advances in science and technology. Regulators must make safety decisions that potentially affect millions of consumers. This delicate balance between safety and avoiding restrictions on valuable products is a continuous, unique, and demanding challenge. Often regulators are forced to make decisions with less than an ideal amount of data. One such circumstance is environmental contaminants Regulators often don’t know enough about the mode(s) of action of these contaminants to eliminate uncertainty so policy decisions are needed to determine a safe level. ToxCast/Tox 21 data can be used as a starting point for the development of contaminant adverse outcome pathways (AOPs) that would provide valuable mode of action information. These AOPs taken together with other data would allow a regulator to answer critical regulatory questions in a transparent and scientifically defensible manner.

This abstract does not necessarily reflect U.S. EPA policy.
II-6a-930

The National Center for Advancing Translational Sciences (NCATS): overview of structure, processes, and phase II screening results for Tox21

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The Toxicology in the 21st Century (Tox21) program, a federal collaboration involving NCATS, The National Institute of Environmental Health Sciences (NIEHS), US Environmental Protection Agency (EPA), and US Food and Drug Administration (FDA), is aimed at developing better toxicity assessment methods. As part of the Tox21 production phase (Tox21 Phase II), the efforts at NCATS involve the use of an ultrahigh-throughput robotic screening system to test nearly 10,000 (10K) chemicals in cellular assays for their potential to disrupt biological pathways that may ultimately result in organism-level toxicity. The Tox21 robotic system combined with informatics efforts is capable of screening and profiling the collection of 10K chemicals in triplicate in a week. The Tox21 screening process will be described, along with details on the chemical library preparation, data processing, and screening results to date.

II-6a-942

ToxCast/Tox21: modern approaches for industry benefit

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Development of novel ingredients that can provide new functional benefits is the life blood of several industries, including the pharmaceutical and consumer product sectors. It is essential however that robust scientific approaches are used to assure the safety of these new ingredients for the patients and consumers who will use them, the workers who manufacture them, and the environment into which they may ultimately be disposed. Traditionally, toxicology data generated in mammals, fish and invertebrates, together with information on levels of human and environmental species exposure, have been used to enable informed safety decisions to be taken. The current reliance on animal data in these safety assessments is reflected in the majority of global regulations concerned with drug, consumer product and environmental safety. The ToxCast and Tox21 programs reflect the growing body of work aiming to modernize and improve this field; seeking to provide greater human health and/or environmental relevance and more efficient tools for safety assessments. The opportunities presented to Industry by these new approaches and their place in a framework for exposure- and pathways-based safety assessments will be discussed. Such in vitro tools combined with mechanistic chemistry information on ingredients allow the identification of potential biological targets, toxicological liabilities and mechanistic information for elucidation of adverse outcome pathways.

Session II-6b: Updates on Activities from Japan

Co-chairs
Yoshihiro Ohmiya, AIST, Japan
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Session II-6b: Oral presentations

II-6b-026

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’s 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 the possibility of 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 establishing methods for prediction or detection of carcinogenicity, immunotoxicity, 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.
Tox-omics project: development of methods to detect multi-toxic effects using gene expression analysis

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In Tox-omics project, we have been trying to develop a highly accurate and wide applicable prediction or detection systems of carcinogenicity, neurotoxicity, or other toxicological effects of chemicals in major organs with 28-day repeated dose study using rats. For carcinogenicity of liver, established prediction system named CARCINOscreen® showed a concordance of 94.4% with training data set. This system was validated by the test data with a concordance of 80% or more. Furthermore, gene expression data obtained from F344, Sprague-Dawley or Wistar-Hannover rats treated with known carcinogens were applied to the prediction formula, and all hepatic carcinogens were accurately predicted. In addition, key genes used in the prediction formula showed a common gene network contained tumor suppressor gene, p53 concerned with cell cycle, DNA repair, and apoptosis. For neurotoxicity, hepatotoxicity or nephrotoxicity, several biomarkers relevant to the phenotype are identified and developing detection systems. Furthermore, these key genes will be applied to Tox-In vitro project for developing in vitro test methods. These approaches will contribute to the 3Rs to evaluate of chemical safety based on toxicity mechanisms.

Towards the development of in vitro assay for hazard assessment using human/mouse artificial chromosomes

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Our human artificial chromosomes (HACs) have been generated mainly by a “top-down approach” (engineered creation). HACs with acceptor sites exhibit several characteristics required by an ideal gene delivery vector, including stable episomal maintenance and capacity to carry large genomic loci plus their regulatory elements, thus allowing the physiological regulation of the introduced gene in a manner similar to that of native chromosomes. Mouse artificial chromosomes (MACs) with acceptor sites were also created from a native mouse chromosome. Multiple integration sites were also loaded in HAC/MAC. The recent emergence of stem cell-based tissue engineering has opened up new avenues for various applications e.g., the development of a novel in vitro hazard screening system in combination with multicolor and secretion luciferase assay system. In this talk, our ongoing project, Tox-in vitro, will be introduced, i.e., liver, kidney and neuronal toxicities.

Development of novel in vitro neurotoxicity screening tests using mouse ES cells-derived neurons

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Several environmental chemicals affect nervous system, which causing damage to human health. Recently, social concerns are increasing for the effects on development of children’s brain because immature brain is considered to be sensitive to chemicals. We started to develop new, easily and sensitively detectable in vitro neurotoxicity tests using an engineered mouse embryonic stem (ES) cells. Our goal is to generate genetically engineered mES cells that express multi-luminescence marker genes as convenient reporters for neurotoxicity and establish efficient in vitro neurotoxicity tests.

We propose following three types of neurotoxicity tests based on the developmental stage; 1) the process of neuronal differentiation, 2) neurite outgrowth after mitosis, 3) specific neuronal and/or astrocytic toxicity in matured cell culture. To specify the neurotoxicity markers for reporter gene assays, we worked on DNA microarray analysis to search for candidate genes and tried to utilize the known neuronal and astrocytic marker genes.

In this session, we will talk about the progress of the project.

Generation of reporter cell lines by combined use of multicolor luciferases and artificial chromosome vector

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Reporter assay system using luciferases, which emit light by oxidizing its substrate luciferin, is widely used as a conventional tool to monitor cellular events, including gene expression. Recent advances in luciferase technology allow us to monitor the expression of multiple genes simultaneously using luciferases that emit different colors, namely, green-emitting and red-emitting beetle luciferases that act on a single luciferin (Nakajima and Ohmiya, 2010). Generation of stable cell lines carrying multiple luciferases, however, requires long time and complicated procedures. To improve the technical limitations, we utilize an artificial chromosome vector in which multiple transgenes can be introduced into the vector by site-specific recombination (Yamaguchi et al., 2011; Takiguchi et al., 2012). To verify capability of the vector, we generated fibroblast cell line expressing green- (as test reporter) and red-emitting (as internal control reporter) luciferases under the control of NF-κB response element and TK promoters, respectively. We successfully generated stable cells, in which luciferases were introduced into target sites of a mouse artificial chromosome vector, with high efficiency. The cells displayed high light intensity, and maintained basal promoter activities and response to TNFα during prolonged passages. The results indicate that the artificial chromosome vector serve as an effective tool for generating cell line and for monitoring multiple gene expressions.

References
Session II-7: Update from Europe – Alternative testing strategies program

Co-chairs
Catherine Mahony, Procter & Gamble, UK
Horst Spielmann, FU Berlin, Germany

Session II-7: Oral presentations

II-7-167
Can data sharing in the pharmaceutical industry contribute to 3Rs? – exploring the value of the IMI eTOX database
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The need to protect intellectual property leads to the fact that most preclinical safety data acquired during drug development are not publicly available. Enhanced data availability for compound comparison (“read-across”) or data mining to build predictive tools should enhance drug development, contribute to the reduction of animal use (3Rs principle) and also to regulatory risk assessment.

A consortium approach grouping a number of relevant partners was undertaken under the umbrella of the European Innovative Medicines Initiative (IMI). The eTOX (“electronic toxicity”: http://www.e-tox.net) consortium includes thirteen pharmaceutical companies, eleven academic institutions and six small-to-medium size enterprises (SMEs). The participating companies identified archived reports on systemic toxicity studies and manually extracted the data. In parallel a trusted organisation (“honest broker”) developed a toxicity database. Due to data heterogeneity a major effort was undertaken for data harmonization and ontology work. The steadily growing database currently contains >4000 reports with >1400 different chemical structures.

The database can be queried for all endpoints investigated in systemic toxicity studies including qualitative or quantitative data on dosing, clinical chemistry, histopathology, hematology and hemostasis. Although application of the database and the derived predictive tools within in the pharmaceutical industry started only recently use cases will be presented and the potential of the tools to contribute to the 3R principles will be discussed.

II-7-589
carcinoGENOMICS – main outcomes
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The EU FP6 carcinoGENOMICS project set out to develop a range of organotypical in vitro assays for predicting genotoxicity/carcinogenicity in vivo. Organs considered, were liver, lung and kidney. For creating classifying gene sets, whole genome gene expression analysis was the technology of choice. Per target organ, for the purpose of selecting optimal cell models, multiple rodent- and human-derived in vitro assays were considered, by challenging them with 15 prototypical compounds, data simultaneously generating training sets of predictive gene expression profiles. We were thus able to select the HepaRG model as best performing assay for predicting liver genotoxicity/carcinogenicity, and the RPTEC/TERT1 model for predicting kidney genotoxicity/carcinogenicity. These models were thereupon challenged with a further 15 prototypical compounds. Upon combining results from all 30 compounds, for both models, we found an accuracy of prediction of approximately 85%.

Although we were ultimately successful in developing a representative lung model by exploiting bronchoepithelial donor material, this unfortunately came too late in the course of the project for advancing this model.

Acknowledgement: EU COSMOS Project (grant agreement 266835).

II-7-504
Computational approaches for the safety assessment of cosmetics-related chemicals: results from the COSMOS Project
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Alternatives to in vivo toxicity testing for risk assessment are required to ensure consumer safety, as well as to respond to the full EU marketing ban of cosmetics tested on animals since 2013. This is also in line with other legislation such as the EU REACH and Biocides Regulations and the general 3Rs Principles. For complex chronic toxicity endpoints a 1:1 replacement of in vivo tests is not possible; instead, a combination of different approaches is sought. The EU COSMOS Project is developing computational approaches to support the safety assessment of cosmetics-related chemicals, focusing on repeated dose toxicity. These include the development of the COSMOS database with high quality data (http://cosmosdb.cosmostox.eu), based on a new Cosmetics Inventory and a number of toxicity endpoints; the extension of the threshold of toxicological concern (TTC) approach to cosmetics, considering issues such as dermal absorption and skin metabolism; toxicokinetics models; data mining to identify structural fragments for, e.g., hepatotoxicity, and their extension into chemotypes; and molecular modelling techniques to predict binding to nuclear receptors. Models developed are being implemented into KNIME workflows, a Web Portal also allows their execution through an internet browser.
Finally, liver and kidney assays were subjected to a coordinated inter-laboratory comparison which demonstrated that these in vitro tests are sufficiently robust. caninoGENOMICS data have been uploaded onto the diXa data warehouse (http://www.dixa-fp7.eu).

**II.7-630**

**ESNATS**

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ESNATS aims at developing a novel toxicity test platform based on embryonic stem cells (ESC) to propose a powerful alternative to animal tests. To this end, ESNATS has developed a unique battery of toxicity testing using human and mouse ESC-based systems subject to standardized protocols. The test battery focuses on selected reference compounds of pharmaceutical interest and unknown prenatal toxicity. Transcriptomics data were combined with phenotypic and functional readouts. The gene expression signatures were used to establish classifiers allowing the identification of compounds that act by a certain toxic mechanism or induce a specific phenotype. In a proof of principle study, the transcriptomics data revealed that the test compounds as valproic acid (VPA) and methylmercury chloride induce a “common response” which can be distinguished from “compound-specific” responses. Additionally VPA was used to analyze transcriptome changes upon transition from tolerated to cytotoxic drug levels in developmental toxicity assay that recapitulates the development of human embryonic stem cells to neuroectoderm. The findings suggest the use of the highest noncytotoxic drug concentration for gene array toxicogenomics studies.

All ESNATS data have been successfully uploaded onto the diXa data infrastructure, and ESNATS data are thus sustained.

**II.7-701**

**Identification of opportunities and limiting steps for reduction, replacement and/or refinement of animal experiments used in environmental risk assessment in European regulation**

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Animal experiments play an integral role in environmental risk assessment for the authorisation of chemicals, pesticides, biocides and pharmaceuticals in Europe. Development of alternatives has been so far relatively more advanced for human risk assessment. However, in 2013 the first international OECD guideline for environmental hazard based on an alternative method (TG 236 Fish Embryo Acute Toxicity Test) has been approved and international validation activities to establish a fish cell line-based guideline have been initiated.

EUROECOTOX (European Network for Alternative Testing Strategies in Ecotoxicology) is a European Union coordinated action, which has aimed at identifying the gaps and limiting steps for reduction, replacement and/or refinement of animal experiments in environmental risk assessment. EUROECOTOX has performed an analysis of regulatory requirements for ecotoxicity testing, novel strategies and approaches to reduce animal testing, potential bottlenecks for validating new methods and measures to accelerate development and validation of alternatives (Scholz et al., 2013). A detailed analysis and statistics of currently available approaches and limitations such as availability and accessibility of animal test data will be presented, supplemented by experimental findings. Moreover, specific reference has been made to the Adverse Outcome Pathway concept and its relevance to guide development of alternatives in ecotoxicology.

**Reference**


**II.7-835**

**Safety evaluation ultimately replacing animal testing – evolution of the SEURAT strategy**

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SEURAT-1 is a major European private-public research consortium that is working towards animal free testing with the long-term strategic target “Safety Evaluation Ultimately Replacing Animal Testing” (SEURAT). A research strategy based on generating and applying knowledge of mode-of-action was formulated and applied within SEURAT-1. The aim is to provide a blueprint for future implementation of mechanism-based integrated toxicity testing strategies into modern safety assessment approaches that draw from a variety of sources including high-throughput data streams, chemoinformatics, tissue constructs, and computational models. SEURAT-1 will end in December 2015 and the research strategy will be brought to the proof-of-concept level based on case studies. A possible follow-up programme should address the long-term SEURAT target while taking into consideration the achievements of SEURAT-1. To help guide the transition from SEURAT-1 to an independent follow-up programme, a planning group was established to i) elaborate the scope and aims of a follow-up research initiative based on the SEURAT vision and strategy and ii) to define an outline research programme and the required components to reach the aims. The deliberations and recommendations of the planning group will be communicated...
with the intention of informing prospective consortia pursuing future research initiatives in the safety assessment field.

II-7-902

The ChemScreen project to design a pragmatic alternative approach to predict reproductive toxicity of chemicals

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II-7-291

SEURAT-1: towards mechanism-based repeated dose systemic toxicity testing

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SEURAT-1 is a major European private-public research consortium that is working towards animal free testing and the highest level of consumer protection, co-funded by Cosmetics Europe (EUR 25 million) and the European Commission under the 7th Framework Programme (EUR 25 million). A research strategy was formulated around harnessing knowledge about toxicological modes-of-action and an organisational model was developed that marries crowd-sourcing with individual excellence.

The SEURAT-1 consortium combines expertise in cell culture for the preparation of stable human cell lines with the establishment of sophisticated experimental systems such as organ simulating devices for in vitro toxicity testing. The experimental work is linked with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration. The focal point of these joint activities is given by proof-of-concept studies (case studies) on three levels, demonstrating that (i) mode-of-action theory provides a solid foundation for mechanistic understanding of adverse effects at the subcellular scale (theoretical level), which (ii) can be converted into the development of animal-free Innovative Toxicity Testing methods (product level) that will (iii) ultimately support regulatory safety assessment (application level). The presentation will focus on recent achievements that have been made by the SEURAT-1 consortium.
Session II-8: Meeting new regulatory challenges following the cosmetic ingredients ban – Cosmetics Europe’s research programme on alternatives

Co-chairs
Patric Amcoff, Cosmetics Europe, Belgium
Horst Wenck, SCT AAT & Beiersdorf, Germany

Session II-8: Oral presentations

II-8-084
Meeting new regulatory challenges following the cosmetic ingredients ban – Cosmetics Europe’s research programme on alternatives

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As part of a dedicated CosEU session with 6 presentations – Cosmetics Europe – The Personal Care Association (CosEU) has been the voice of Europe’s cosmetic, toiletry and perfumery industry since 1962 and represents the interests of more than 4000 companies. Consumer safety is always highly prioritised and CosEU has a long track record of successful developments of alternative approaches to animal safety testing. The cosmetics industry is the only sector completely affected by an animal testing ban (EC, 2009) requiring only the use of full replacement methods. This ban was introduced despite the fact that replacement methods were not available for the most complex endpoints and the area for which replacement can only be expected within a reasonable time frame is for skin sensitisation (Adler et al., 2011). The CosEU Science Strategy focuses on the development of alternatives for replacement. The program applies the OECD Adverse Outcome Pathway (AOP) concept (OECD, 2012) as a regulatory basis for the conception of testing strategies for safety assessment. CosEu will strive towards leaner and faster validation approaches and regulatory acceptance at EU level and internationally. The industry is committed to find replacement approaches in order to maintain its licence to operate and innovate.

References
EC (2009), Regulation No 1233/2009.

II-8-084
Systemic toxicity alternatives (SEURAT-1 and beyond)

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Cosmetics Europe has contributed 50% funding to the SEURAT-1 programme together with the European Commission. The purpose of Seurat-1 is Safety Evaluation Ultimately Replacing Animal Testing, where the focus has been to advance the scientific knowledge and technology building blocks towards a mechanistic understanding of changes in biological processes that lead to adverse effects in humans. A conceptual risk assessment approach will be discussed, as well as the importance of the nine Seurat-1 case studies in aligning solutions for safety assessment without animals. A mapping exercise for global activities in the area of systemic toxicity alternatives will be presented and demonstrates the challenges of harnessing the emerging science for safety decision making. The remaining challenges that the Cosmetic Industry faces in achieving our ambition of being able to make robust safety decisions without relying on animal test data are discussed – not least of which is a thorough toolbox that enables evaluation of dose response in human-relevant systems and the ability to link the changes seen to adverse outcomes.

II-8-404
The Cosmetics Europe animal-free strategy for genotoxicity testing

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The Cosmetics Europe Genotoxicity Task Force has led 3 projects to help improve the predictive capacity of current in vitro genotoxicity assays and develop new in vitro models as follow-up alternatives to positive outcomes in the initial test battery:
- The completed “False Positives” project optimized the in vitro micronucleus assay by selecting more relevant cells (e.g., human lymphocytes) and more sensitive toxicity measures and thereby reduced the percentage of “false positive” results.
- The “3D skin model” project focuses on developing and validating human reconstructed skin (RS) models for genotoxicity testing. The RS Comet and micronucleus assays have demonstrated good inter- and intra-laboratory reproducibility and we are now in the final validation phase, exploring the predictive capacity of these models.
- The “Metabolism” project evaluated the enzyme capacities of human skin and RS models and showed them to have comparable
metabolic capacities, confirming the usefulness of RS models for testing dermally-exposed compounds.

The program has already helped reduce animal use beyond cosmetics by improving the initial test batteries’ predictivity. The RS genotoxicity project has provided support for the use of skin models as a follow-up test for dermally exposed chemicals and therefore shows promise as a direct replacement for in vivo studies.

Il-8-575

An overview of the Cosmetics Europe skin bioavailability and metabolism project

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The Cosmetics Europe Skin Bioavailability and Metabolism Task Force was set up to improve the measurement and prediction of the bioavailability of dermally-exposed compounds. Our aim is to use in vitro models to generate quality data relevant to the determination of the fate of 50 different compounds (e.g., sensitizers, genotoxins) after application to the skin. Parameters include: solubility, partition/diffusion coefficients in different skin layers and lipids, as well as the kinetics of covalent protein binding, stability in skin S9 fractions, and dermal penetration and metabolism in ex vivo human skin. These data will be used to build a database for dermal bioavailability properties of the selected chemicals. Until now, there have been no other studies that have generated such a comprehensive database of a large number of chemicals using standardized protocols. Current databases rely on single reports from researchers which may differ widely in their methodologies and measurements. This database will provide a comprehensive profile of selected chemicals that will help interpret outcomes from in vitro toxicity assays, such as the 3D skin genotoxicity or sensitization assays and to refine toxicological potency, as well as provide vital measured data to improve or establish new in silico models for dermal absorption.

Il-8-659

Continued developments in the Cosmetics Europe eye irritation task force strategy and programme for prediction and assessment of ocular irritancy

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The Cosmetics Europe Eye Irritation Task Force programme is focused on optimisation/evaluation of in vitro test methods for eye irritation that can be used alone or in combination in testing strategies/approaches for evaluation of eye irritation potential of cosmetic ingredients. Fundamental to this approach is identification of improved in vitro test systems/endpoints/methods that use understanding of mechanisms of eye injury/recovery to better predict human ocular responses to chemical exposure. The approach to achieve this is based on three focus areas: 1) method evaluation through optimisation/refinement of existing in vitro assays e.g., validation study of Reconstructed Human Tissue (RHT) methods with EU-RL-ECVAM (Freeman et al., 2010); 2) use of HPLC/UPLC for endpoint detection of strong colorants in RHT test methods; 3) data integration that includes understanding drivers of irritation for accurate assessment of the performance of test methods for possible contribution to sequential testing approaches (Adriaens et al., 2014) and 4) development of testing strategies.
Session II-9: Poster presentation

II-8-796

**In vitro model for assessing impact of mechanical and chemical skin injuries**

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The challenge is to mimic skin damages currently observed in dermocosmetical field and analyze in vitro exposition of xenobiotic. For this purpose, transepidermal water loss (TEWL) measurements were performed, using Franz cells with full-thickness porcine skin to evaluate the injuries effect on skin barrier.

Session II-9: Exposure

Co-chairs
Robert Landsiedel, BASF, Germany
John Wambaugh, EPA, USA

Session II-9: Oral presentations

II-9-345

**Computational PBTK workflow to assess toxicity from in vivo dermal exposure**

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Within the context of 21st century toxicology, there is the need to develop toxicity prediction and risk assessment methods that rely on the ADME processes to predict the concentration/dose leading to toxicity at the target site. Dermal exposure has generally received less interest than oral exposure, despite the prevalence of skin contact in various occupational and industrial settings. To address this gap, we developed a computational workflow connecting a dynamic skin permeation model with a generic whole-body physiologically-based toxicokinetic model. The workflow enables the calculation of transient target site concentration profiles from a variety of exposure scenarios and application conditions. Next, the workflow was evaluated against a set of human in vivo skin permeation data. Finally, we used it in a quantitative in vitro- dermal in vivo extrapolation scheme to estimate dermal safe dose. We illustrate calculations using in vitro CALUX assay to assess safe levels of 4 chemicals in the context of endocrine disruption. Depending on exposure features (occlusion, removal from application site, vehicle) the dermal safe dose varies by an order of magnitude. Due to its flexibility and realism this approach provides refinement over single dermal fractional absorptions.

II-9-811

**A probabilistic model of human variability in physiology with application to QIVIVE**

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Microneedles are used to obtain temporary mechanical skin disruption for transdermal delivering drugs. Recently, this device was introduced in cosmetic domain to treat scars, wrinkles and stretch marks. Microneedles disposed on a roller were studied with respect to the efficiency of skin perforation. Moreover, a gel containing salicylic acid was used to simulate a daily routine skin scrub with a risk of assaulting. A rotating brush, normally used for face cleansing, was applied during five minutes on the biopsy with and without the cosmetic gel. For removing hair, two methods were studied, hot wax was applied on skin and the effect was compared to the application of a cream containing potassium thioglycolate. Besides, tape-stripping a widely used method was performed to obtain model of skin injuries. TEWL results were compared with all mechanical and chemical damages, and the different biopsies were also observed histologically by microscopy (optical, electronic).

Supported by ANSM (French Health Agency): TroughSKIN Project.
The risk assessment of environmental chemicals and drugs is moving towards a paradigm shift in approach which seeks the full replacement of animal testing with high throughput, mechanistic, in vitro systems. This new vision will be reliant on the measurement in vitro, of concentration-dependent responses where prolonged excessive perturbations of specific biochemical pathways are likely to lead to adverse health effects in an intact organism. Such an approach requires a framework, into which disparate data generated using in vitro, in silico and in chemico systems, can be integrated and utilized for quantitative in vitro-to-in vivo extrapolation (QIVIVE), ultimately to the human population level. Physiologically based pharmacokinetic (PBPK) models are ideally suited for this and are obligatory in order to translate in vitro concentration-response relationships to an exposure or dose, route and duration regime in human populations. An understanding of human variability is critical. In this work we describe the PopGen software for generating anatomically realistic healthy human populations for use in forward dosimetry modelling. We also discuss recent research that translates PopGen output into gender, age and ethnicity dependent probability distributions that may be used as prior distributions in reverse dosimetry (QIVIVE) applications.

Forecasting exposure in order to use high throughput hazard data in a risk-based context

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The ToxCast program and Tox21 consortium have evaluated over 8000 chemicals using in vitro high-throughput screening (HTS) to identify potential hazards. Complementary exposure science is needed to assess risk, and the U.S. Environmental Protection Agency (EPA)’s ExpoCast initiative has been developing mechanistic and heuristic models for high-throughput exposure (THE). Coupled with hazard-related HTS, HTE modeling can move risk-based evaluation earlier in chemical management decisions. Chemicals where the putative human dose of concern from hazard HTS is comparable to doses predicted by HTE become targets for further investigation. We used Bayesian analysis to infer ranges of exposures consistent with biomarkers measured in urine samples by the U.S. Centers for Disease Control National Health and Nutrition Examination Survey. We used linear regression models on chemical descriptors gleaned from databases and chemical structure-based calculators. Separate calibrations allow for demographic-specific prioritization of exposure. For all groups the same five heuristics are able to explain half of the variance in the inferred exposures; including children aged 6-11 and women of child bearing age. Those chemicals with properties and uses that are most like the chemicals to which people are known to be highly exposed are targets for further.

Extrapolation of systemic availability assessing skin absorption and epidermal and hepatic metabolism of aromatic amine hair dyes in vitro

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Approaches to assess the role of absorption, metabolism and excretion (AME) of cosmetic ingredients that are based on the integration of different in vitro data are important for their safety assessment without animals. In order to estimate systemic exposure (AUC) to aromatic amine hair dyes under use conditions, firstly, the systemically available parent fraction was estimated using in vitro skin penetration, human keratinocyte (HaCaT) metabolism studies. Using measured skin penetration/skin metabolism rates together with standard toxicokinetic skin parameters the dose of the parent compound that can reach the system after the passages was estimated for six hair dyes. In each case the estimation was in the same order of magnitude as measured values from experiments using viable human skin from surgeries. Secondly, the AUC was estimated from the systemically available parent fraction by additionally calculating scaled hepatic clearance values utilizing measured in vitro hepatocyte metabolism rates together with standard toxicokinetic liver parameters. Finally, for the hair dye p-phenylenediamine, the predicted parent AUC data were found to be in the same order of magnitude as those published for human volunteers, indicating that appropriate toxicokinetic information can be generated based solely on non-animal data.
stratum corneum (SC), and in vitro skin penetration using pig and human skin with radioisotopically labeled and cold chemicals.

The protocols for Ksc and Dsc included: SC concentration-depth profile determined by tape-stripping with infinite doses and infinite dose diffusion through isolated SC fitted with non-linear regression. Both methods produced similar Ksc and Dsc values for resorcinol, caffeine and 7-ethoxycoumarin. Incubations with dried SC were conducted as a simpler alternative to measure Ksc.

For comparing human and pig skin absorption a finite dose was applied to skin discs for 24 h. The distribution of the 3 chemicals was similar, indicating pig skin (a waste by-product of the meat industry) can be used when human skin availability (acquired with donor consent) is limited. For these stable and non-reactive chemicals, the use of chemicals did not significantly affect their penetration.

These initial studies suggest that there is some flexibility in how the main studies (with 50 chemicals) can be carried out, without compromising on the quality of the data.

II-9-102

Standardized experimental set-up for dermal absorption studies in vitro

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Skin absorption is an in vitro method described in OECD guideline 428 and the technical guidance GD 28. Since the study design and experimental parameters can be varied, we performed systematic investigations under defined experimental conditions to understand influencing parameters and develop an optimized testing protocol for dermal absorption studies in vitro.

Dermal absorption studies were performed with skin preparations of rats, pigs and humans in Franz-like diffusion cells. We used different model compounds, e.g., testosterone or caffeine which could be analyzed by liquid scintillation counting, HPLC or photometry. Different model compounds, donor vehicle and receptor fluids. Furthermore, several freezing-cycles, static versus flow through, concentration of test chemicals did not significantly affect their penetration.

Results: Irritant chemicals except H2O2 could be determined through cell viability below 50% at 90 min exposure time. Moreover, the end point at 50 pg/ml could be an acceptable parameter for IL-1α profile. Consequently, the combination of two cut-off values, CV 50 and 50 pg/ml of IL-1α could be considered as criteria to identify the irritation potential of oral care ingredients.

Conclusion: This in vitro oral tissue model based on two parameters, CV 50 and 50 pg/ml of IL-1α, is expected to be a good methodology for evaluating mucosal irritation potential of oral care ingredients.

II-9-385

Development of exposure systems for the in vitro assessment of “heat-not-burn” tobacco and electronic nicotine delivery devices

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Traditionally, cigarette smoke generated for in vitro product comparison studies is generated at defined regimes such as ISO (35 ml puff over 2 seconds every 60 seconds (35/2/60), 3308:2000) and Health Canada Intense (HCl, 55/2/30). With the emergence of novel “Heat-Not-Burn” (HnB) tobacco products and e-cigarettes, traditional smoking machine engineering and smoking regimes may not be suitable for product comparison purposes.

We report preliminary work undertaken to adapt a Borgwaldt RM20S smoking machine to deliver aerosols generated from a variety of heated tobacco systems and a commercially available e-cigarette. Aerosol delivery was quantified as particulate mass using quartz crystal microbalances fitted into exposure chambers, traditionally utilised for cell-based cigarette smoke exposure studies. Preliminary data indicate deposited particulate mass could be detected from low dilutions of the generated aerosols from 10 minute exposures of low-temperature (40-80 ng/cm²; at 55°C using a 100/2/30 regime) and high-temperature glycerol-based HnB tobacco (~35,000 ng/cm²; at 180°C using the HCl regime) and e-cigarettes (~50,000 ng/cm² using the HCl regime).

Adaptations made to the smoking machine facilitated the delivery of quantifiable particulate matter from novel products. Further work is required to ensure aerosol delivery is reproducible prior to in vitro product assessment.

II-9-338

The methodology for evaluating mucosal irritation on human oral tissue model

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Objective: To develop a robust alternative method for determination of mucosal irritation potential of oral care ingredients.

Methods: To set up acceptable criteria, reconstructed human oral models, EpiOral™ were exposed to positive substance (Triton X-100) and oral care ingredients, 3 irritants (SLS, H2O2 and ethanol) and 1 non-irritant (chlorhexidine) for 20, 90 and 180 min, respectively. And then cell viability (CV) was measured via MTT assay. In addition, cytokine profile and its histological patterns were monitored via ELISA and H&E staining, respectively.

Results: Irritant chemicals except H2O2 could be determined through cell viability below 50% at 90 min exposure time. Moreover, the end point at 50 pg/ml could be an acceptable parameter for IL-1α profile. Consequently, the combination of two cut-off values, CV 50 and 50 pg/ml of IL-1α could be considered as criteria to identify the irritation potential of oral care ingredients.

Conclusion: This in vitro oral tissue model based on two parameters, CV 50 and 50 pg/ml of IL-1α, is expected to be a good methodology for evaluating mucosal irritation potential of oral care ingredients.
Assessing the skin irritation potential of rinse off personal care products using a reconstructed human epidermis model: exposure time and product dilution impact

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The assessment of personal care products, specifically rinse off products (due to their chemistry), relies on the evaluation of adequate exposure methods for avoiding under or over estimation of skin irritation potential.

12 commercial products were evaluated under eight different conditions: 2, 4, 15 and 20 hours contact periods plus 2% and 5% product dilutions, using an in-house 3D human reconstructed epidermis model “VitroDerm” and comparing to human patch test reference classification.

A two way ANOVA analysis indicates significant statistical differences for dilution and exposure time factors and for their interaction (p<0.001). Further T test analysis showed significant differences (p<0.05) for both periods against 2 and 4 hours at both dilutions and against 15 hours for the 5% dilution; also significant differences (p<0.05) between both dilutions at all exposure times. Comparison of in vitro results against the human patch test classification showed the lowest specificity values (75%) for the 5% dilution at 15 and 20 hours exposure times.

The identification of the impact of exposure conditions on the prediction of skin irritation using a reconstructed human epidermis model will allow future refinement of the test method for rinse off products.

Bovine serum albumin binding and toxicity of cationic chemicals in in vitro cell assays

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The effectiveness of in vitro in vivo dose extrapolations depend on what dose metric is used to determine effect concentrations in in vitro toxicity assays. The traditionally used nominal concentration may underestimate toxicity when the chemical binds to serum constituents such as bovine serum albumin (BSA). This binding reduces the freely available concentration to cells and consequently the observed effect. Binding affinities of ionic chemicals to BSA remains poorly understood. Therefore, this study characterized BSA binding for several cationic chemicals by using solid phase microextraction (SPME).

Both primary (partly charged at pH 7.4) and quaternary amines (fully charged at pH 7.4) were included in the study. Binding affinities of primary amines were significantly lower than those of quaternary amines when normalized against their LogKow values, indicating that octanol water partition coefficients insufficiently predict albumin binding for cationic chemicals as one group. Furthermore, binding strength correlates well with observed LC50 values in the fish RT-Gill W1 cell line. This work provides a relationship between amines, protein binding and free observed toxicity based on their hydrophobicity parameters. However, more work has to be done in this area to fully comprehend binding for cationic chemicals and estimate free concentrations in vitro adequately.

AH OH exposure induced oxidative stress in Caco-2 cells

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Alternariol (AOH) is a mycotoxin produced by Alternaria sp. often found in fruits and vegetables. In order to determine if the oxidative pathway could be implicated in AOH’s toxicity, the cytotoxicity of AOH, the generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) were investigated in Caco-2 cells. Subsequently, the induction of oxidative stress by the antioxidant defenses imbalance related to glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were also evaluated.

Cytotoxicity of AOH (from 3.125 to 100 μM) was determined during 24, 48 and 72 h by the MTT assay. Decreasing cell viability was observed, but no IC50 values were obtained. To determine oxidative stress, AOH at sub-cytotoxic concentrations (15, 30 and 60 μM) was assayed. Early ROS production (1.2 folds of control) by H2DCFDA fluorescence probe after 120 min was observed. LPO generation ranged from 50% to 145% compared to the control by TBARS method after 24 h of AOH exposure. AOH oxidative stress was corroborated by alteration of GSH levels and the antioxidant activities. The GPx and CAT activities and totally GSH levels decreased at 60 μM of AOH exposure. However, significant increase in SOD was observed at all concentrations assayed.

SDF-1 induced migration of THP-1 cells through a monolayer of human coronary artery endothelial cells promotes differentiation of monocytes to an adhesive phenotype

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Humoral and direct cell-to-cell interactions occurring between endothelial cells and monocytes represent critical steps in atherogenesis. We investigated the transendothelial migration of monocytes using an electrical impedance-based system able to capture cell migration and differentiation in real-time. Cell invasion and migration (CIM) inserts were seeded with human coronary artery endothelial cells (HCAEC). In the presence of a confluent HCAEC monolayer on the CIM insert and stromal cell-derived factor 1 (SDF-1) in the bottom chamber (1-100 ng/ml), THP-1 cells migrated through the cell monolayer. After traversing the CIM insert membrane, cells adhered to its lower side as revealed by increased impedance. A proportion of migrating THP-1 cells also adhered to the bottom of the CIM plate. Experiments with THP-1 cells in the absence of HCAEC resulted in a potent dose-dependent induction of migration by SDF-1 (1-100 ng/ml) without cell adhesion to any surface. Our experiments indicate that both, SDF-1 and a functional monolayer of HCAEC are required for monocytes to differentiate and...
acquire an adhesive phenotype. We are currently establishing additional assays to better recreate the transformation of monocytes into macrophages. We believe this approach could reduce the number of in vivo models used to conduct research on vascular inflammation.

II-9-692

**Overcoming the barriers to the uptake of microsampling techniques in regulatory safety studies**

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Presented on behalf of the NC3Rs microsampling working group.

Toxicokinetic (TK) data are an essential component of non-clinical safety studies and are used to correlate drug exposure with pathology or functional effects. International guidelines indicate that TK information should provide proof of exposure, but do not dictate how exposure is measured, allowing for technological innovations in bioanalysis. Improved sensitivity of bioanalytical techniques allows analysis with small volume samples, around 25 to 30 µl; termed “microsamples”. The use of microsampling enables blood sampling from main study animals which represents a significant opportunity to reduce rodent use in toxicology studies. Research co-ordinated by the NC3Rs identified widespread use of microsampling in discovery, dose finding and pharmacokinetic studies. An NC3Rs workshop with 80 delegates from 33 companies established that the major perceived barrier to TK sampling from main study animals was the potential effect on parameters routinely measured during regulatory safety assessment.

An international working group has been sharing data on current practise and the impact of blood sampling on pathology endpoints in toxicology studies. This evidence will feed into the revision of the ICH guidelines.

**II-9-850**

**Retrospective analysis on the correlation between cytokine release from a 3D keratinocytes tissue model and clinical human ocular response to mild surfactant systems**

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57 unique mild surfactant formulations have been studied in both the *in vitro* epithelial irritation test and the clinical human ocular test. Tissue viability and IL-1α release from 3D human skin equivalents (MatTek Corp., Ashland, MA) were determined following exposure to mild surfactant based formulations. In the clinical evaluation, the formulations and a water control were instilled into each subject’s eyes and then the bulbar conjunctivitis, palpebral conjunctivitis, and lacrimation were clinically graded at 30 s, 15 min and 60 min. The inflammatory cytokine release and cell viability parameters were used to evaluate the impact on the physiological inflammatory redness response investigated in the clinical human ocular study. Our data showed that across the 57 unique formulations tested, the IL-1α release well correlated with the human ocular bulbar conjunctivitis evaluated in the clinical study at 15 min. We have found that the epithelial irritation test can be used to predict the human ocular redness response for mild surfactant based formulations. Further investigations are needed to evaluate if this *in vitro*-clinical correlation concept can be applied to other classes of final formulations intended for human use.

**Session II-10a: Topical toxicity – Skin**

**Co-chairs**

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**Session II-10a: Oral presentations**

**II-10a-244**

**Use of HPLC/UPLC-photometry for detection of formazan in *in vitro* Reconstructed human Tissue (Rht)-based test methods to expand their applicability to strongly coloured test substances**

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*In vitro* skin corrosion/irritation test methods using Reconstructed human Tissue (Rht) are accepted by regulatory authorities, e.g., OECD. For serious eye damage/eye irritation, two Rht test methods were recently evaluated in a prospective EURL ECVAM/Cosmetics...
Europe validation study to discriminate irritants/corrosives from non-irritants. Irritation potential is determined by measuring cell viability in treated tissues using the colorimetric MTT reduction assay after topical application of a chemical onto the tissue surface. A known limitation of the MTT assay is possible interference with Optical Density (OD) measurement of reduced MTT (formazan) for strongly coloured chemicals. To address this, Cosmetics Europe evaluated the use of HPLC/UPLC-photometry as an alternative formazan detection system applicable for all RhT methods. This presentation covers; 1) qualification, based on an FDA guideline (FDA, 2001), of three HPLC/UPLC systems; 2) reproducibility of formazan detection in three independent laboratories; 3) results and conclusions on 26 substances, balanced for colour/non-colour, tested in eye/skin irritation and skin corrosion RhT methods. Results demonstrate high between laboratory reproducibility and ability to determine viability when OD cannot be measured. As such, HPLC/UPLC-photometry has been demonstrated to extend applicability to strongly coloured chemicals irrespective of test system/toxicity endpoints. Scientific recognition through EURL-ECVAM and OECD is in progress.

Reference


An immature human reconstructed epidermis model to assess baby skin irritancy

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Baby skin is not fully matured at birth and differs in structure, function and composition from that of adults. Therefore, the risk assessment of products for babies cannot be extrapolated from human adult data but must be considered as a specific approach with adapted models. In order to respect the properties of baby skin, an “immature” human reconstructed epidermis has been developed to assess skin irritation for baby products.

72 infant formulations were tested on both mature and immature epidermis. 10 formulations decreased the cell viability to below 50% with the immature epidermis only, allowing the identification of potentially irritating formulations. A statistically significant difference (t test; p<0.05) was found when mature and immature models were compared. This difference became more relevant when analysis was made between rinse off and leave on products. Human patch test data enabled the adjustment of the method for rinse off and the inflammatory markers expression as end-point was added to improve the accuracy of the model.

The refinement in the prediction of irritation with this new model allows a better risk assessment during the development of baby products and can be used as a tool to select the most appropriate ingredients during formulation.

II-10a-377

Human experimental data – the ultimate confirmation of in vitro methods relevance for safety and efficacy testing

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The EU ban on animal testing requires to verify the predictive capacity of available in vitro methods for safety and efficacy evaluation of cosmetics. Comparison of experimental in vitro data with human skin tests is essential for reliable prediction of human skin irritation hazard.

The aim of the presented studies included evaluation of skin irritation hazard of preservatives, protective effects of herbal ingredients against detergents in cleaning products and phototoxicity of essential oils used as perfumes and active ingredients. The battery of in vitro tests comprised 3T3 Balb/c cytotoxicity/phototoxicity assays and skin irritation/phototoxicity tests using reconstructed human epidermis model EpiDerm. The data generated in vitro were confirmed in a group of volunteers.

Excellent concordance of results obtained in vitro and in vivo was confirmed in the studies on preservatives and herbal ingredients. In the phototoxicity study, sporadic phototoxic reactions has been recorded in the human photopatch test, if the highest non-phototoxic concentrations determined in the EpiDerm assay were applied. The human skin model test seems to be a useful tool for consideration of initial concentrations for confirmatory human photopatch tests to prove product safety, however, a safety factor of 10 might be considered for extrapolation.

II-10a-862

Formulation testing in the 21st century

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When developing formulated plant protection products (PPP), typically an acute toxicity battery is conducted in support of registration. This includes acute oral, dermal, and inhalation toxicity, skin and eye irritation, and sensitization tests. Consistent with our commitment to the 3Rs, a tiered in vitro strategy for assessing eye and skin irritation potential as well as skin sensitization has been developed. In this approach, the neutral red release (NRR) assay is first utilized to identify strong eye irritants and the EpiOcular assay is then used to differentiate mild to moderate irritants. This strategy allows for accurate prediction across the spectrum of eye irritants where a single assay may produce less reliable data. To address skin irritation potential, we implemented OECD guideline approaches utilizing reconstructed human skin models. Finally, we have demonstrated the utility of the Keratinosens assay for addressing skin sensitization potential of PPP formulations with high concordance with in vivo results. Additional approaches, including quantitative structure activity relationships (QSAR) are also employed to support a weight of evidence conclusion on sensitization potential. Successful application of these in vitro assays for formulated PPPs demonstrates their potential utility to address data requirements in a reliable manner while greatly reducing animal use.

II-10a-519
Session II-10b: Oral presentations

II-10b-046
EURO ECVAM – Cosmetics Europe prospective validation study of Reconstructed human Tissue-based test methods for serious eye damage/eye irritation testing

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A prospective validation study of two Reconstructed human Tissue-based in vitro test methods (EpiOcular™ EIT and SkinEthic™ HCE) was conducted by EURO ECVAM and Cosmetics Europe to evaluate their usefulness to identify chemicals as either not classified for serious eye damage/eye irritation (No Category) or as classified (Category 1 or Category 2) within UN GHS, in the framework of a Bottom-Up/Top-Down test strategy (Scott et al., 2010). The study assessed the validity of two EpiOcular™ EIT protocols for liquids and solids, two independent SkinEthic™ HCE protocols based on short-time (SE) and long-time (LE) exposures and a test strategy combining SE and LE. The results and conclusions of this study will be presented.

Briefly, over 100 chemicals were tested and both methods showed high reproducibility (>90%). The EpiOcular™ EIT liquids protocol met all the study acceptance criteria for predictive capacity (Adriaens et al., 2014), but not all of these criteria were met by the solids protocol nor by any of the SkinEthic™ HCE protocols/strategy. This led to optimisation of the EpiOcular™ EIT solids protocol and further validation being conducted. With final sensitivity of 96%, specificity of 63% and accuracy of 80%, the EpiOcular™ EIT met all the study acceptance criteria and is considered valid for the proposed study objective.

References

II-10b-142
Retrospective analysis of the Draize test for serious eye damage/eye irritation: importance of understanding the in vivo endpoints under UN GHS / EU CLP for the development and evaluation of in vitro test methods

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For more than two decades scientists have been trying to replace the regulatory in vivo Draize eye test by in vitro methods but so far only resulting in partial replacement. To better understand the reasons for this, historical rabbit data were analysed in detail to i) reveal which in vivo endpoints are most important in driving UN GHS/EU CLP classification for serious eye damage/eye irritation and ii) evaluate the within-test variability for proposing acceptable and justifiable target values of sensitivity/specificity for alternative methods. Amongst the Cat1 chemicals evaluated, 36-65% (database dependent) were classified based only on persistence of effects (remainder mostly based on severe corneal effects). The most important endpoints driving Cat2 classification are conjunctiva redness (75-81%) and corneal opacity (54-75%). Resampling analyses demonstrated an overall probability of at least 11% that chemicals classified as Cat1 by the Draize test could be equally identified as Cat2 and about 12% of Cat2 chemicals to be equally identified as NoCat. The over-classification error for NoCat and Cat2 was negligible (<1%). For successful replacement of the Draize eye test in vitro tools are needed that address the most important in vivo endpoints and the uncertainties of the Draize test should be recognized.
Pre-validation study of Vitrigel-EIT (Eye Irritancy Test) method


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A collagen vitrigel membrane (CVM) composed of high density collagen fibrils was previously developed by Takezawa et al. (2004). A three-dimensional human corneal epithelium (HCE) model with excellent barrier function was easily fabricated on the CVM (Takezawa et al., 2011). Taking this advantage, Yamaguchi et al. established a new eye irritancy test (EIT) method: “Vitrigel-EIT method”. Here, the Vitrigel-EIT method provides an excellent estimation of widespread eye irritancy merely by analyzing the time-dependent relative changes of transepithelial electrical resistance for 3 minutes after exposing chemicals to the HCE model (Yamaguchi et al., 2013). To evaluate reliability and relevance of this test method, the pre-validation study started last year. In association with the International Collaboration on Alternative Test Method (ICATM) the International Validation Management Team was organized and pre-validation study was performed to evaluate transferability by three Japanese laboratories.

The experimental trial was conducted with the protocol developed by Yamaguchi et al. using non-coded five test substances distributed by Japanese Center for the Validation of Alternative Methods (JaCVM). The obtained data were analyzed by a biostatistician.

As a result of the pre-validation study, the high transferability of this test method was confirmed. The next pre-validation study to evaluate within- and between-reproducibility is on-going.

Pre-validation study of Vitrigel-EIT (Eye Irritancy Test) method

References


The predictive capacity and inter-laboratory reproducibility of the Short Time Exposure (STE) test for assessing eye irritation

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The STE test is an alternative eye irritation test using SIRC (rabbit corneal cell line) cells following a 5 minute treatment. Cell viability, following exposure to a 5% concentration, was used as an indicator of eye irritation potential, where a material is considered a UN GHS No Category (NC) if the viability was more than 70% and an Irritant (I: UN GHS category 2 and 1) if the viability was less than 70%.

We compared the in vivo data of 99 chemicals to the result of the STE test. The accuracy for classifying as either NC/I was 84.8%. Moreover, the prediction rate could reach to 92.2% when with applicability domain by except high vapor pressure liquid chemicals etc. The false negative chemicals also were decreased from 9 to 1.

To evaluate inter-laboratory reproducibility, first we assessed one lab by using 5 chemicals (e.g., sodium lauryl sulfate, calcium thioglycolate). This lab had same result compare to the data of leader lab.

Development of EYEIRR-IS®, an toxicogenomic assay using the Skinethic HCE model for evaluating chemical ocular irritation potency

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Although for eye irritation potency classification, assays using 3D reconstructed tissues and MTT measurement have been developed, their ability to evaluate irritation potency is limited. For formulation but also to address new and future regulatory demands, the ability to measure and quantify the eye irritation potential of chemicals and mixtures without animals is of high importance.

We developed IRR-IS, a new method, based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed human corneal epithelium. The selection of biomarkers was done by analysis expression profiles in 3D reconstructed corneal epithelium with several irritants. Test chemicals were applied for 15 mn then washed and the tissues further incubated for 6 hrs. Tissues were teased, total RNA purified with Trizol and expression of genes measured by quantitative PCR after reverse transcription. We selected 20 biomarkers and developed a algorithm based on analysis of magnitude of gene expression.

We will present here the preliminary results of these studies and will show the quantitative capacity of this approach on a set of 8 chemicals.
**Session II-10c: Topical toxicity – Phototox**

**Co-chairs**
Abigail Jacobs, USFDA, USA
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**II-10c-260**

**Development of an in vitro tiered approach to evaluate phototoxic potential**

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In order to predict the phototoxic potential of chemicals, the in vitro 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU-PT) was adopted by OECD as TG432. Even though this method is now widely used, hydrophobic chemicals cannot be evaluated accurately due to their poor solubility. To overcome this limitation, yeast growth inhibition (YGI) assay and test methods with reconstructed human epidermis (RhE) have been developed (Daniels, 1965; Portes et al., 2002; Jirová, 2005). The objective of this study was to develop a tiered approach for evaluation of phototoxic potential of various chemicals.

We adopted multiple methods based on solubility of chemicals. Following reactive oxygen species (ROS) assay, which is an indicator that compounds may trigger phototoxicity (Onoue, 2006), the 3T3 NRU-PT, YGI assay or the RhE-based method was employed. We used the 3T3 NRU-PT for hydrophilic compounds and the YGI assay for hydrophobic compounds dissolved in some organic solvents, e.g., dimethylsulfoxide, ethanol or acetone. The RhE-based method was suitable for compounds less soluble in the solvents above. Furthermore, we refined the experiment conditions of the RhE-based method and modified the criteria.

We succeeded in developing a tiered approach that included four in vitro methods. This strategy enables us to evaluate phototoxic potential of various chemicals more systematically.

**References**


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**II-10c-715**

**Antioxidant potential and phototoxicity of new UV-filters candidates based on marine natural compounds**


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Many studies have been performed all over the world in order to find new photoprotective substances; however, there is almost no safety information regarding their use in cosmetic formulations, leading to enhanced consumer exposure to unpredictable risks. Therefore, the aim of the present study is to evaluate the antioxidant activity and phototoxicity of mycophenolic acid, a compound isolated from the red algal genus *Bostrychia* and associated fungi, as well as its derivatives.

Solutions of the compounds were analyzed by UV spectrophotometry to obtain the ratio of the mean UV A to the mean UVB absorbances. In vitro phototoxicity was evaluated by using 3T3 monolayer fibroblast culture for the determination cell viability in the presence and absence of UVA radiation, according to OECD TG 432. Results showed that mycophenolic acid had high absorption in the UVB band, showing similar profile to some mycosporine-like amino acids, which...
have been studied as candidates for biological UV-filters. It also could be considered a good antioxidant. In the phototoxicity studies, it was observed that mycophenolic acid and its derivatives did not present any phototoxicity potential. In conclusion the studied compounds could be good candidates for new UV-filters.

Support: FAPESP

Il-10c-719

The ROS in vitro phototoxicity assay for ICH

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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 and the Ros assay validation management team developed the recommended protocol of this assay in accordance with the panel’s proposal. Based on the validation reports and peer review report, this assay has described at the ICH S10 Guideline on Photosafety Evaluation.

Il-10c-935

Update on ICHS10-photosafety testing without animals

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Advances in understanding of photochemistry, photoreactivity, and phototoxicity of pharmaceuticals offer the opportunity to evaluate the potential for photosafety risk in humans while minimizing or eliminating testing in animals. Generally, compounds that absorb light in the visible and UV ranges and generate reactive oxygen species (ROS) pose a potential risk for phototoxicity. Photoreactivity tests to detect generation of ROS and in vitro bioassays to detect phototoxicity (3T3-NRU and 3D-skin assays) offer high sensitivity but lower selectivity with which to predict phototoxicity potential in humans. It is most important that assays show high sensitivity, because negative assay results are usually conclusive and do not warrant further photosafety evaluation. Thus, it is not essential that positive assay results always predict a clinically relevant phototoxic response. The fact that these assays measure entirely different endpoints lowers the probability of multiple “false positive” findings, such that one negative finding with other positives is generally sufficient to indicate negligible photosafety risk. Finally, compounds that absorb in the relevant spectrum and test positive in chemical and/or in vitro assays can be evaluated for photosafety in clinical trials with proper precautions, without intermediate in vitro or animal testing if the sponsor chooses.

Session II-10: Topical toxicity – Poster presentations

Il-10-068

Digging between photoallergenic or photoirritant behaviour with the RBC-PT

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Objective: There is an obvious need for a phototoxic screening strategy based on complementary in vitro tests. The Red Blood Cell Photo Test (RBC-PT) is regarded as useful and important adjunct test to overcome the limitations of the 3T3 NRU PT, and has proven be useful for studying the mechanism underlying phototoxicity potential of chemicals. Thus, the aim of the present study is to elucidate the potential use of RBC-PT as in vitro assay to discriminate between photoirritants and photoallergens, because a decrease in phototoxic activity was observed only in the case of photoallergen products. Further investigation increasing the number of chemicals assayed is mandatory.

Il-10-099

Additional joint research on eye irritation alternative method with human corneal model; LabCyte CORNEA-MODEL24


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Results and conclusion: all chemicals tested exhibited photohemolytic activity being photooxidative only those with photoallergenic properties. The use of scavengers opens the possibility to use the RBC-PT to discriminate between photoirritants and photoallergens, without animals.
Aims/Objectives: In order to confirm the technical transferability of the eye irritation test (EIT) method using the reconstructed human corneal epithelium, LabCyte CORNEA-MODEL24, twenty four laboratories carried out the joint research sponsored by the planning committee in the JSAAE. As a result, we revealed that this EIT method easily caused false positive. Because it could be considered that washing method of a solid or a viscous material caused these results, we examined the improvement of this EIT protocol. Then, in order to confirm the technical transferability, within- and between-laboratory reproducibility of the improved EIT protocol, we carried out the additional joint research by five laboratories.

Methods: We examined whether the result of the improved EIT protocol can achieve proficiency level of preset criteria.

Results and Conclusion: The results of additional joint research, we confirmed that the technical transferability of the improved EIT protocol was easy. Furthermore, both within- and between-laboratory reproducibility of the improved EIT protocol were in agreement with all tests. However, some laboratories pointed out that large dispersion of the cell viability sometimes occurred. In order to improve this dispersion, we are studying improvement of the EIT protocol again.

Virtual skin model – a software tool for detailed computation of skin penetration

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Aims/Objectives: To develop a user friendly software tool for detailed computation of skin penetration which allows skin scientists to conduct simulations even without detailed expertise in numerics.

Methods/Software: The software tool is based on the simulation system UG4 and a framework for visual programming, VRL (Vogel et al., 2014; Hoffer et al., 2014). By providing a modern user interface it enables the interactive configuration of the simulation. Visual components give access to problem specific properties. Once defined, a workflow can be saved. Additionally, it is possible to hide unimportant parameters and to customize and to reduce the interface complexity.

Results: Recently, a two-dimensional computer model for calculating finite dose skin penetration has been developed (Naegel et al., 2011; Selzer et al., 2013; Naegel et al., 2013). The results from in vitro experiments and simulation were in good agreement, indicating a high predictive quality of the mathematical model. This computer model is based on the complex software environment UG which is not simple enough for a broad application in the experimental community. We demonstrate how to apply a user friendly software tool in finite dose and infinite dose skin absorption studies (Naegel et al., 2011; Selzer et al., 2013).

Conclusions: A user friendly software tool for detailed computation of skin penetration after infinite and finite dosing has been developed.

References
Assessment of in vitro eye irritation – testing strategy

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Since the number of chemicals to be assessed for eye irritation solely based on in vitro data (for e.g., REACH or cosmetics) will strongly rise in the next years, it is urgent to establish a reliable in vitro eye irritation testing and assessment strategy. Several OECD accepted in vitro tests for the detection of severe eye irritants are available. However, no test is available for the detection of moderate skin irritants, which renders the development of such a strategy currently difficult. Here we present our rationale for an in vitro eye irritation testing strategy, based on sequential testing in the BCOP (OECD 437), HET-CAM (Hen’s egg test), and HCE (human cornea epithelial cell) test. This test battery was developed to reflect the different tissue types of the eye in vivo, e.g., cornea, blood vessels, mucous membranes, and in addition different strengths of effects (severe eye damage, moderate eye irritation, no eye irritation). First results following this testing strategy will be shown.

More knowledge on the applicability domain and on limitations of each in vitro test system and more experience with the testing strategy is necessary to come to an overall recommendation.

Further refinements of IRR-IS®, an Episkin® based model for quantifying chemical irritation potency

F. Cottrez, E. Boitel and H. Groux

Quantification of the skin irritation potential is of high importance not only for transport labeling and occupational health but also for the toxicological risk evaluation. There is need for further refinement of alternative skin irritation measurement assays so they could address the question of intermediate irritation levels (mild-irritants) in line with new and future regulatory demands.

We developed IRR-IS, a new method, based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®), thus providing a possible way to come closer to potency assessment. The selection of biomarkers was done by analysis expression profiles in 3D reconstructed epidermis with several irritants.

Long noncoding RNAs (IncRNAs) are transcripts that have no apparent protein-coding capacity; however, many IncRNAs have been found to play a major biological role in human physiology. Their deregulation is implicated in many human diseases, but their exact roles are only beginning to be elucidated. Nevertheless, IncRNAs are extensively studied as a novel source of biomarkers. Using a set of 40 selected chemicals we further refine the IRR-IS assay by incorporating into the analyzed biomarkers long non coding RNAs (IncRNA). The refinements resulting from incorporating these biomarkers will be discussed.

New evaluation method for skin irritation of oil-soluble chemicals with monolayer culture system

N. Imai, Y. Goto, S. Nomura and T. Masunaga

In vitro skin irritation test with reconstructed human epidermis (RHE) was adopted by OECD as TG439. This is the alternative method to TG404, on the purpose of the hazard identification of chemicals in accordance with 4 hr exposure skin irritation. However, evaluation of 24 hr exposure testing is required for safety assessment of chemicals and products such as cosmetics, quasi-drugs and other topical skin products. Hence, we assessed the predictivity of TG439 to the 24 hr exposure irritation test. As a result, TG439 was not necessarily suitable for prediction of the 24 hr skin irritation, especially for oily substances. To develop a new high-predictive method for evaluating the skin irritation of oily substances, we employed the same technique as used in STE, in which mineral oil was applied as the solvent for oil-soluble chemicals to monolayer culture system. By conducting experiments with varying exposure time, concentration of substance and cut-off value of cell viability, we succeed in obtaining good predictivity to the 24 hr skin irritation. Those results suggest that our new test method, called Direct Exposure Skin Irritation Test (DESIT), is effective for prediction of 24 hr skin irritation of oil-soluble chemicals.
In vitro skin corrosion and irritation assessment of ingredients using EpiSkin methods: sequential top-down and bottom-up approaches

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The OECD has proposed an Integrated Approach on Testing and Assessment (IATA) for skin corrosion and irritation, in view of replacing the existing testing and evaluation strategy. The aim of this study is to provide key performance characteristics when integrating all information using accepted OECD TG431 and TG439 EpiSkin methods.

Concordant classification between runs has been demonstrated for both skin corrosion and irritation toxicological endpoints on 87 chemicals. None of the skin corrosives was predicted as non-irritants, independently of top-down and bottom-up approaches. 63 non-classified substances were overpredicted as either irritants or corrosives. High accuracy (90%) was obtained when discriminating corrosives versus irritants versus non-classified substances. As such, EpiSkin method 1/ allows sub-categorisation of corrosives into Cat.1A or Cat.1B-and-1C, 2/ is able to distinguish Cat.2 and No cat., and thus serves to determine the final classification for non-corrosives and non-irritants.

Assessment of in vitro skin corrosion and irritation results for the 87 commercially available substances using EpiSkin methods in Top-Down and Bottom-Up approaches shows that a combination of Guidelines (irritation and corrosion) allows the determination of the potential hazard of tested chemicals.

This comprehensive evaluation should facilitate regulatory acceptance decision on the methods, and it proposed uses in IATA.

Integrated approach for skin corrosion and skin irritation using in vitro SkinEthic RHE test methods

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Acute dermal integrated approach on testing and assessment (IATA) include adopted in vitro skin corrosion and irritation test methods. As such, the SkinEthic Reconstructed Human Epidermis (RHE) test method is considered in the current OECD guidance document.

Illustration of IATA is provided by combining and evaluating both SkinEthic RHE skin corrosion and irritation methods in the stepwise top-down and bottom-up approaches.

Concordant classification between runs has been demonstrated for both toxicological endpoints amongst the 83 tested substances. Only 1/83 false negative was defined (consistently false negative in all in vitro tests). High accuracy (90%) was obtained when discriminating corrosives versus irritants versus non-classified substances. Furthermore, similar classifications were defined by discriminating the UN GHS subcategories Cat.1A, versus Cat.1B-and-1C versus irritants (Cat.2) versus not-classified (NC) substances applying both the top-down and bottom-up approaches.

In conclusion, assessment of in vitro skin corrosion and irritation results for 83 commercially available substances using SkinEthic RHE methods in stepwise approaches shows that a combination of Guidelines (irritation and corrosion) allows the determination of the potential hazard of tested substances. The sequence of the prospective testing in either a top-down or a bottom-up approach should be guided by all available information of the substance.

Development of a testing strategy to evaluate 24 h-exposure skin irritation using OECD TG439 and new monolayer culture system

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In vitro skin irritation test using reconstructed human epidermis has been adopted as OECD Test Guideline (TG) 439, which predicts skin irritation potential at the 4 hour exposure (4 h-exposure skin irritation). Regardless less irritant materials such as cosmetic ingredients, it is important to evaluate 24-h exposure skin irritation, so alternative methods are required. Therefore, we assessed the predictive performance of TG439 for 24 h-exposure skin irritation. Furthermore, to predict 24 h-exposure skin irritation, especially for oil-soluble materials, newly developed test method using monolayer culture system (Direct Exposure Skin Irritation Test; DESIT) was also assessed. As a result, TG439 showed good predictive performance in the assessment of water-soluble materials, but less sensitivity in oil-soluble materials.

On the other hand, evaluation of oil-soluble materials using DESIT resulted in a good accuracy and sensitivity. In conclusion, we proposed a testing strategy to evaluate 24 h-exposure skin irritation for both water-soluble and oil-soluble materials using TG439 and DESIT, respectively. The established strategy enables us to classify a cosmetic ingredient as non-irritant and contributes to full replacement of animal tests for 24 h-exposure skin irritation of cosmetic ingredients.

The multi-center study (MCS) of an alternative method by Chinese CAIL/CIQ laboratories: the validation of in vitro skin irritation testing method based on EpiSkin model

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The European Cosmetic Directive 1223/2009/EC came into effective in 2013 for total ban of animal testing. To take into consideration the change of the international regulation, and initiate the practical application of the international technical standards of alternative cosmetic testing method in China, four affiliated laboratories directed by AQSIQ of China and the laboratory of L’Oréal R&I China performed...
Modification of 3T3 NRU photo-toxicity test conditions for the evaluation of poorly water-soluble substances

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In the 3T3 NRU phototoxicity test method in the OECD TG432, oil droplets or precipitations occur during preparation or addition process of poorly water-soluble substances, thus the accurate evaluation is difficult. In this study, five minutes pre-incubation time with dimethyl sulfoxide (DMSO) as a sample preparation solvent at the final concentration of 10% has been set. With this experimental condition, the phototoxicity of thirty compounds were evaluated and compared with the results obtained under the conventional experimental condition.

High concordance between the results obtained under two experimental conditions about the positive/negative compounds has been derived. The calculation of IC₅₀ for 2-Octylmethacrylate, one of the poorly water-soluble substances, has been impossible under the conventional condition. However, due to the improvement of solubility, its reliable IC₅₀ value was obtained under the new condition. Furthermore, the variation of IC₅₀ for Bithionol, a weak phototoxic agent, showed a narrow range.

From the results obtained, we suggest that 3T3 NRU photo-toxicity testing with modified experimental conditions can produce similar results with those obtained by the conventional test method, and can serve as an appropriate method for the evaluation of poorly water-soluble substances. The results obtained with the combination of ROS assay will also be reported.

Integrated CAMVA and BCOP methods to predict eye irritation of cosmetics

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Considering no single in vitro test was found to be capable of completely replacing the in vivo Draize eye test. Integrated methods with different applicability and purpose to complement and/or partly overlapping toxicology adverse reactive is an effective way. The purpose of this study was to integrate two in vitro methods to predict eye irritation of cosmetics. 60 kinds of cosmetic on the market in China were surveyed to determine the predictive of ocular irritation potential by Choriosallantoic Membrane Vascular Assay (CAMVA), Bovine Cornea Opacity and Permeability (BCOP) and Draize Rabbit Eye Test. The result showed that CAMVA method can distinguish 41 samples with non-irritation and 18 samples with irritation. 35 samples of non-irritation, 21 samples of light-moderate irritation and 4 samples of severe irritation were predicted by BCOP assay. Combination CAMVA and BCOP methods can obviously improve the identification ability of different irritation and classification consistency with Draize testing reach to 98.3%. The integrated test strategy combined with BCOP and CAMVA can be appropriate to predicting ocular irritation of cosmetics and predicted range covering non irritation to severe irritation of the samples.

Cytotoxicity, phototoxicity and genotoxicity evaluation of sucupira oil: a potential new cosmetic ingredient

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From the results obtained, we suggest that 3T3 NRU photo-toxicity testing with modified experimental conditions can produce similar results with those obtained by the conventional test method, and can serve as an appropriate method for the evaluation of poorly water-soluble substances. The results obtained with the combination of ROS assay will also be reported.
Comparison of mouse primary hepatocyte spheroids formation on Cell-able™ plates with various feeder cells

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It is difficult but important to reconstruct three-dimensional (3D) tissue structures, which are expected to retain the original function, in in vitro. Spheroids with primary hepatocytes are reported to keep some original functions and various methods have been used to form them. Here we used commercialized 96-well plates (Cell-able™, Transparent) for spheroid formation and compared the 3D form to assess the hepatic functions. Eight cell lines were used as feeder cells for spheroids forming. Then, gene and protein expressions were examined to evaluate by quantitative RT-PCR and immunohistochemistry [albumin (Alb) and o-fetoprotein (Afp)] with three cell lines as feeder cells (Balb/c 3T3, 3T3-Swiss and HH cells). When Balb/c 3T3 cells were used as feeder cells, the spheroids showed the finest form. However, in Alb and Afp, similar expressions were observed in the spheroids on all three feeder cells by both methods. It is suggested that the simple shape of the spheroids did not reflect the hepatic features. When in vitro culture is used to examine the original function of the tissue, it is expected to function stably, and these functions should be easily identified. It is important to estimate the hepatic functions with adequate detection systems.

Predicting eye stinging using the novel NociOcular assay

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Although several in vitro eye irritation models exist, none have demonstrated the ability to predict eye stinging. The NociOcular assay, a novel neuronal model based on activation of the Transient Receptor Potential Vanilloid type 1 (TRPV1) channel, has been shown to distinguish stinging from non-stinging baby bath products. In this study we sought to evaluate surfactant-based products, ophthalmic solutions, and sunscreens in the NociOcular assay. The TRPV1 expressing SH-SYSY neuroblasts were exposed to test substance and TRPV1 channel activation was measured by acute increases in the intracellular free Ca2+. The shampoo products demonstrated a range of responses in the assay and were classified as either stinging or non-stinging. Over the counter ophthalmic solutions were tested at higher concentrations to model direct instillation of neat solution. However, even at the highest concentrations there was no appreciable Ca2+ influx and the products were classified as non-stinging. During evaluation of sunscreen formulations, several technical challenges arose including insolubility of the products with the assay buffers and issues with pipetting the viscous solutions. Further research will be focused on evaluation of target ingredients in sunscreens and modifications of the assay to assess final sunscreen formulations.

Observations on the use of the bovine corneal opacity and permeability (BCOP) assay to evaluate the eye irritation potential of prototype cosmetic formulations containing salicylic acid, glycolic acid and ethanol

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Prototype cosmetic formulations containing either ≤2% salicylic acid (SA) or ethanol (10% or 50%) were classified as having minimal to mild eye irritation potential in the BCOP assay. In contrast, three formulations containing 2% SA and ethanol (10%, 15% or 30%) resulted in a classification of moderate eye irritation potential (opacity scores were higher and histopathological injury more pronounced than 100% ethanol control). The eye irritation potential of formulations containing glycolic acid (GA) is low (Epiderm™ ET50 = 55-55 minutes). Inclusion of ethanol (5%, 15% or 30%) in formulations containing 3.2-3.9% GA had minimal impact on BCOP opacity or permeability scores (each classified as having minimal eye irritation potential); however, formulations containing either 20% or 30% ethanol, 3.9% GA and 2% SA resulted in a classification of severe eye irritation potential. In both cases opacity and permeability scores were significant-


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Evaluation of the eye irritation potential of ingredients used in cosmetics products is a fundamental part of the overall safety assessment for the finished product to address ocular exposure through intended use or accidental exposure. A number of eye irritation in vitro test methods have now been evaluated/validated and externally/regulatory accepted for specific applicability in testing strategies (Scott et al., 2010) for evaluation of chemicals. Furthermore, a Cosmetics Europe expert meeting held in 2008 developed decision trees for safety assessment of cosmetics products and their ingredients in which tiered testing strategies and the use of a Weight-of-Evidence (WoE) approach are considered fundamental principles (McNamee et al., 2009). The application of such a tiered approach to evaluation of eye irritation for cosmetic ingredients is demonstrated here using the hair dye material 3-amino-2,6-dimethylphenol. Results of: 1) physicochemical properties analysis; 2) read-across from historical in vivo Draize test data generated on structurally related chemicals and 3) in vitro data the Isolated Chicken Eye (ICE) test data produced on 3-amino-2,6-dimethylphenol and structurally related chemicals are presented and placed in the context of a tiered testing and WoE approach to determine the EU CLP classification for eye irritation of 3-amino-2,6-dimethylphenol as a neat material and at relevant on-head concentrations.

References


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The importance of understanding drivers of irritation in vivo for selection of chemicals used in the development and evaluation of in vitro serious eye damage/eye irritation assays: Cosmetics Europe analysis

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Cosmetics Europe’s Task Force Eye Irritation is actively involved in the development and evaluation of in vitro methods to assess serious eye damage/eye irritation potential of cosmetic ingredients. Selecting
Chemicals based on proper understanding of what drives irritation in classification of ocular effects in the in vivo Draize eye test is a critical element that enables identification and evaluation of predictive capacity/applicability at an early stage of in vitro methods development (Adriaens et al., 2014). In this context, Cosmetics Europe undertook an in-depth analysis of data from external databases containing Draize data for chemicals (>500 independent studies). This analysis was based on availability of good quality in vivo data allowing clear understanding of the different ocular tissue effects that drive classification, such as degree of severity and/or persistence of corneal opacity, iritis, conjunctiva redness and/or conjunctiva chemosis. All chemicals were screened for commercial availability, to cover the whole irritation range and represent relevant chemical classes and physical states. This analysis of the whole dataset demonstrates that the majority of chemicals are classified by a few key drivers, e.g., high involvement of corneal effects and low prevalence of iritis driving classification and importance of conjunctiva effects in classification of GHS Cat 2A versus 2B.

**Reference**


II-10-714

**Skin irritation potential of UV-filters and vitamin A using a full-thickness reconstructed skin model**

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The present study aimed to assess the skin irritation potential of different UV-filters and vitamin A using a full-thickness reconstructed skin model. Skin models were developed according to Brohem et al. (2010), in which keratinocytes were cultured onto a dermal equivalent compartment and exposed to an air-liquid interface for 2 weeks. UV-filters (avobenzone or benzophenone-3) vitamin A (retinol or vitamin A palmitate) solutions, vehicle and also irritant (SDS) and sensitizer (dinitrochlorobenzene – DNCB) positive controls were applied topically to the skin models and incubated for 1h. The supernatants IL-1α were determined by ELISA and MTT was performed to assess epidermal metabolism/viability. Data were analyzed by ANOVA. Results showed that SDS treated skin models showed statistically higher amounts of IL-1α than DNCB, which are in line with similar studies using commercial skin models which show that skin irritants provokes a higher release of IL-1α than allergens. In the studied conditions, vitamins and UV-filters did not promote skin irritation. These results are very important, once it reveals a possible three-dimensional irritation skin model use of a full-thickness reconstructed skin, which are more important, once it reveals a possible three-dimensional irritation skin model use of a full-thickness reconstructed skin, which are more valuable for predicting skin irritation in vivo.

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**Reference**


II-10-774

**Exploring of new alternative test method for phototoxicity in ophthalmic agent using SIRC cell lines and 3D human reconstituted cornea models**


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Many types of chemicals have been reported to induce phototoxic effects. Their common feature is their ability to absorb light energy within the sunlight range. So far, to identify the phototoxic potential of a test substance induced by the excited chemical after exposure to ultraviolet (UV) irradiation, the in vitro 3T3 NRU phototoxicity test(PT) has been used in 3T3 mouse skin fibroblast. However, when taking into consideration that the phototoxicity occurs in ocular, development of more suitable test method using cornea-derived cells is necessary. In this study, we tried to develop a new in vitro PT method using rabbit corneal cell lines, SIRC, with ophthalmic agent. The phototoxicity of five ophthalmic agent were evaluated by measuring cytoxicity and performed in vitro phototoxicity test. In the results from 3D human cornea models, the UV-induced eye tissue toxicity with test substances were corresponded well with in vitro phototoxicity test. Meanwhile, the results from 3D PT for ciprofloxacin, norfloxacin and tetracycline in the 3D human cornea model (HEC) were partially comparable. Consequently, we suggested the new phototoxicity test method with SIRC cell lines, but also sequential testing strategy, such as 3D PT was proposed to have good relevant human information for eye topical agent.

II-10-868

**Use of ECVAM validated EpiDerm skin corrosion test (EpiDerm SCT) for sub-categorization according to the UN GHS**

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Skin corrosion refers to the production of irreversible damage to the skin.OECD adopted four reconstructed human skin model assays for predicting skin corrosion in vitro (OECD TG 431).The guideline, however, does not yet fully satisfy international labeling guidelines for transport of dangerous goods.

The UN-GHS utilizes 3 corrosion sub-categories (1A-very dangerous, 1B-medium danger and 1C-minor danger). Labeling a chemical as 1A has important consequences for transport and animal tests are still utilized for assessing the packaging subclasses.An in vitro method that could discriminate at least between the 1A vs 1B/1C classes would therefore have a substantial impact on reducing animal tests for this purpose.

The current study evaluates prediction of the subclasses using the EpiDerm SCT and 80 chemicals selected by the OECD expert group.
for skin irritation and corrosion. Using tiered classification strategy, sensitivity for class 1A was 86% using 3 min exposure time-point. None of 1A chemicals were under-predicted as NC. Specificity for NC chemicals was 74%. As demonstrated, the 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 a 3 min endpoint into the validated EpiDerm SCT and the OECD TG 431 will allow identification of severely corrosive substances without use of animals.

II-10-869
Identifying the appropriate protocol for testing surfactants and surfactant-based formulations in the bovine corneal opacity and permeability assay

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The Bovine Corneal Opacity and Permeability (BCOP) assay is an ex vivo test for predicting ocular irritation. OECD Test Guideline (TG) 437 specifies that liquid and solid surfactants are tested as 10% aqueous dilutions for 10 minutes (alternate dilutions and exposure times may be conducted with scientific rationale). Guidance Document (GD) No. 160 suggests that solid and concentrated liquid surfactants may be diluted to 10% for testing, and thus surfactant solids should not be tested using the solid chemical protocol. GD No. 160 further directs that surfactant-based formulations are usually tested neat, but could be diluted with justification, imparting some confusion in identifying the most appropriate test methods. In the absence of clear guidance, we present on the testing of a few common surfactant ingredients (sodium lauryl sulfate, Triton X-100, and benzalkonium chloride), and surfactant-based liquid and solid formulations in BCOP using standard and modified dilutions and exposures to evaluate the impact of these variables. Histopathology was performed to confirm corneal changes. We found that surfactants may not exhibit dose-related effects at high concentrations, and opacity and permeability changes should be evaluated individually in a hazard assessment. Accordingly, a framework for testing surfactants and surfactant-based formulations is proposed.

II-10-874
Performance of the chorion-allantoic membrane stained by trypan blue (CAM-TBS) of different concentrations of surfactant sodium dodecyl sulfate (SDS) for assessing the eye irritation potential

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The aim of this study was to determine the performance of CAM-TBS for detecting the eye irritation potential. In vivo results were obtained from a routine data base. Ten dilutions of SDS (from 0.03 to 15% w/v) were tested using 4 SPF Leghorn 10 days eggs for each concentration. In vivo SDS data classified as “non-irritant” (0.5, 1 and 2%), “mild irritant” (4%) and “moderate irritant” (8%) were used. CAM-TBS presented linear range between 0.03 and 1.1% concentrations. The coefficient of determination (R2) was 0.709 for this stretch of the curve and it was improved to 0.754 when the amount of dye was expressed per gram of CAM. Above 1% the curve reached a plateau and a kind of desquamation was observed. Histological studies are being performed but, maybe it be explained due to the higher concentration of surfactant applied to the membrane which produces a greater interaction with lipids and other molecules causing its degradation. These preliminary results suggest that, in this linear range, CAM-TBS may be used for identifying non-irritant samples.

II-10-936
Reconstructed human epidermis test methods for skin corrosion endpoint: how can changes in prediction models improve final predictions?

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Classification of corrosive chemicals is based on UN GHS sub-categories and addressed by OECD test guideline No. 431 (TG 431) for test methods using reconstructed human epidermis tissues. In this guideline, prediction types are “corrosive category 1A” versus “corrosive category 1BC” versus “non-corrosive” chemicals. These predictions are based on prediction models (PMs) using cell viabilities at 3 minutes (v3), 60 minutes (v60) for EpiDerm™, SkinEthic™, epitCys® and EpiSkin™ and also at 240 minutes (v240) for EpiSkin™. These PMs lead to high over-prediction rates of 1BC chemicals, except for EpiSkin™. It is possible to develop new PMs consisting in two alternate variations, PMvar1 and PMvar2 that provide increased correct classifications of 1BC chemicals as well as increased overall accuracy. PMvar1 is based on changes of cutoff in cell viabilities values through a two-step approach, whereas PMvar2 is based on a single composite indicator of cell viability. In both variations, overall accuracy values were increased, and although category 1A chemicals were less correctly predicted, category 1BC chemicals were much more correctly predicted. For a majority of methods, results were better with PMvar2. Additionally, PMvar2 helps performing a ROC analysis in an easy manner.

II-10-951
NativeSkin – a standardized ex vivo skin system to predict human skin responses

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In the search of a suitable ex vivo skin model that closely mimics as possible the responses of human skin in vivo, we have developed a
A quantitative model of systemic toxicity using ToxCast and ToxRefDB

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EPA’s ToxCast program profiles the bioactivity of chemicals in a diverse set of ~700 high throughput screening (HTS) assays. In collaboration with L’Oréal, a quantitative model of systemic toxicity was developed using lowest effect levels (LEL) from ToxRefDB for 633 chemicals with HTS data, and chemical fingerprints. Floor and ceiling performance baselines (95% Confidence Intervals) were 4.6 and 2.8 orders of magnitude uncertainty (OMU), respectively based on historical LEL distributions and reproducibility across study type and species. An initial read-across model was developed using chemical fingerprints to identify structurally similar neighbor LEL values resulting in 3.9 OMU, a 1/5th reduction in model uncertainty based on our performance baselines. HTS data was then incorporated into the model using 74 groups of assays based on biology, technology annotation, and assay confounders. For each assay grouping, a mean activity value was computed and adjusted for confounders. Incorporating HTS data with read-across resulted in a 3.7 OMU, a total reduction in model uncertainty of 2/5th. Herein, we have identified a model that incorporates HTS (dynamics), and read-across (chemistry) to predict systemic LEL harnessing and incorporating the power of both new and existing data.

This abstract does not necessarily represent EPA policy.

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Cheminformatic approaches for analog-based toxicity assessments

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Read-across from well-studied chemicals to structurally similar compounds is an effective approach to toxicity assessment. The search for analogs with toxicity data can be facilitated by the use of toxicology databases that are searchable by chemical structure/substructure, such as DSSTox database. Searches return a number of potential analogs, which must be assessed for suitability using established rules (Wu et al., 2010). These rules have been evaluated in blinded case study assessments (Blackburn et al., 2011) and shown to be valid. Another use of cheminformatics is the development of decision trees to determine whether there are any precedents in the literature for a chemical with the same structural feature to be toxic. We have developed such a decision tree for developmental and reproductive toxicity (DART) based on literature data for over 800 chemicals (Wu et al., 2013). Chemical features associated with DART can be grouped into 25 categories, many of which can be tied to a putative mode of toxicity. This decision tree could be used as the foundation for a mode of action ontology, to serve as an organizing framework for adverse outcome pathways. Read-across can also be supported by showing comparable biological activity/mode of action.

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Repeated dose systemic toxicity: which predictive methods?

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Different research initiatives in Europe and overseas are currently addressing repeated dose systemic toxicity. This topic is a major research challenge: a state of the art review published in 2011 by Adler et al., concluded that a full replacement of the animal tests used for repeated dose/reproductive and developmental toxicity testing is not available and the timeframe for a full replacement cannot be clearly estimated.

Nonetheless, several possibilities are being explored in the area of toxicodynamics and toxicokinetics. In the toxicodynamics area, the adverse outcome pathway approach is paving the way to a mechanistic based approach. Regarding the toxicokinetics, different models are being investigated and the results obtained can be used to derive in vitro to in vivo information.

While some of the information is derived from biochemical and two dimensional in vitro models, chemoinformatics tools and more complex organotypic models are also key elements in the development of predictive approaches for repeated dose systemic toxicity.

This session will cover some of the key components that will contribute to establishing predictive methods for repeated dose systemic toxicity.

Reference

Alternative model for repeated dose inhalation toxicity using precision-cut lung slices

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Testing inhalable substances regarding respiratory adverse effects in accordance with the three R principles requires appropriate alternatives for animal inhalation studies. Precision-cut lung slices (PCLS) are an alternative widely used for testing of adverse outcomes for the respiratory tract. Yet, PCLS have been used mostly for acute respiratory toxicity questions. To assess PCLS suitability for long-term toxicity studies, e.g., testing slowly metabolized inhalable chemicals, PCLS were long-term cultivated (≥14 d). For repetitive exposure rat PCLS were treated on three consecutive days with different concentrations of a model compound (TritonX-100) and compared to single and double exposure. LDH and WST-1 assay were used to assess viability. Additionally, structural and functional variations were analysed by histopathology and bronchoconstriction over the 14-days period. Vitality remained constant over time with slight decreases towards day 14. Histopathology showed no fibroblasts proliferation, but preservation of alveolar structure, bronchiolar epithelium and smooth musculature. Bronchoconstriction was measureable for 14 days, with slightly decreased sensitivity (EC50_1d=8.22×10^(-6)M vs. EC50_15d=4.70×10^(-4) M methacholine). Repetitive exposure did not influence the sensitivity of the lung tissue (EC50_1d=76 µM to EC50_15d=80 µM). Overall these results showed that repeated chemical exposure of PCLS is possible with constant vitality.

The added value of the 90-day repeated dose toxicity test for low toxicity chemicals

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Many regulatory regimes ask for both a 28-day and a 90-day rodent study to determine the effect of repeated oral dosing of the substance. In the interests of avoiding unnecessary animal tests, in cases where the 28-day study exists and gives a NOAEL of 1000 mg/kg bw/d or greater. However, for 20 of these the NOAEL was also 1000 mg/kg bw/d or greater in the 90-day study.
In HeMiBio, we are generating a liver-simulating device mimicking the complex structure and function of the human liver. The device should maintain hepatocytes and non-parenchymal liver cells for over 1 month in vitro to test the effects of repeated exposure to chemicals. The hypothesis for the successful creation of a 3D liver-simulating device suitable to test repeated dose toxicity is that: hepatocytes and non-parenchymal cells are combined; both homotypic and heterotypic cellular interactions between the different components are required to maintain the functional, differentiated and quiescent cell state; oxygenation and nutrient transport are optimised to support long-term maintenance of hepatocyte and non-parenchymal cell function; the system is built such that repeated on-line assessment of cellular integrity, metabolic and transport function, and physiology of the different cellular components is possible. We incorporate molecular sensors and electro-chemical sensors, to allow assessment of function and cell integrity. As human livers are in general unavailable for studies in the cosmetic and pharmaceutical industry, we are in the process of isolating the cellular components from pluripotent cells. Alternatively we use cells isolated from livers that can be expanded by genetic manipulation using UpCyte® technology, without loss of mature function.

This analysis strongly suggests that the 90-day test adds little to risk assessment of substances that can already fit a “low toxicity profile” with high quality data. Although this low toxicity profile appears to be of low prevalence within industrial chemicals (10-15%), serious consideration should be given to the need to conduct a 90-day study for such substances.

**Session II-11: Poster presentations**

**II-11-270**

**Pharmacokinetics of serum honokiol with oral administration Magnolia ethanolic extract in Sprague-Dawley rats**

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Honokiol, a pharmacological bioactive constituent of Magnolia plants, has shown anti-tumor effect in a variety of human cancer xenograft models through the induction of apoptosis. Additionally, honokiol could enhance the cytotoxicity in colon cancer cells with combination of oxaliplatin. The pharmacokinetic profiles of honokiol were detected with a single dose administration and repeated dosed administration of Magnolia ethanolic extract. After 7-consecutive daily doses, blood was also subjected to analysis of ALT, AST and BUN. In single dose pharmacokinetics of 0.823 g/kg Magnolia ethanolic extract that was normalized to a dose of 40 mg/kg honokiol, the results showed that the elimination half time, Cmax, mean residence time (MRT), and bioavailability (BA) of honokiol were 12.07 ±5.36 hours, 07.64 ±11.73 ng/ml, 17.34 ±8.75 hours, and 2.14 ±1.02%, respectively. In conclusion, the pharmacokinetics demonstrated that the pharmacokinetic profile of honokiol was improved by micronization of Magnolia ethanolic extract in the elimination half time.
Do we still need the 2-year rat carcinogenicity study?

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Objective: To prove that for non-genotoxic compounds, safe exposure levels can reliably be established without a 2-year rat carcinogenicity study by identification of the No-Observed-Adverse-Effect-Level (NOAEL) from a sub-chronic (3-6 months) repeated dose toxicity study.

Materials and methods: Data have been collected from EFSA and Tox Ref EPA databases. Histopathological findings considered as early risk factors for rat neoplasia, observed in sub-chronic studies with chemicals, were correlated with the tumour outcomes in rat 2-year carcinogenicity studies.

Results: True negative compounds: 134 (65%). True positive compounds: 8 (4%). False positive compounds: 16 (8%), preneoplastic lesions in stomach, bladder, thyroid and spleen in the sub-chronic studies, but no tumours in carcinogenicity studies. False negative compounds: 47 (23%), no preneoplastic lesions in sub-chronic studies, but 24 compounds inducing benign and 23 compounds malignant tumours in carcinogenicity studies.

Conclusion: The absence of histopathological evidence of putative preneoplastic lesions in rat 3-months study using a whole animal approach is reliable for predicting negative tumour outcome in the 2-year carcinogenicity study, provided that i) the compound is non-genotoxic; ii) the compound caused no hormonal perturbation and iii) an additional assessment factor of 10 (to be confirmed) is applied.

Developing an integrative approach for predicting acute oral toxicity: lessons learned

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The 7th Amendment of the Cosmetics Directive prohibits in Europe the marketing of cosmetic products containing ingredients or combinations of ingredients which were tested, among other toxicity endpoint, in in vivo acute toxicity studies after March 2009. At L’Oréal, acute oral toxicity studies were conducted to support the development and to ensure the safety of new ingredients for early efficacy testing.

Acute toxicity data are still required for both regulatory and safety assessment purposes. As such, the need for predictive methods has lead L’Oréal to enter a new phase in its innovation process. Efforts have been made to pursue the directions and recommendations laid out. Central to this strategy is the reliance on in vitro data and in silico approaches to model the complexity of the biological processes involved.

In the frame of a pluriannual research program with different partners (academic and private), an integrated testing strategy was constructed in order to cover, as appropriate, hypothetical mechanisms of action as well as the complexity and diversity of our chemical space. The predictive capacity of individual tiers and examples of case studies illustrating the use of such a strategy will be presented.

An integrative approach for the prediction of acute oral toxicity: past and future

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The model presented assesses results of mechanism-based in vitro assays and relevant physical-chemical properties to predict an LD50 value, the standard measurement of acute oral toxicity. The uniqueness of such a model is that it can serve multiple purposes, at a multidisciplinary level: to rank-order compounds and guide the design of hits, to identify hypothetical mechanisms of actions, and to provide an estimate of acute oral toxicity for labeling purposes. The predictive performance of our model was assessed using a set of proprietary compounds. Chemicals were defined as toxic (T) or non-toxic (NT) on the basis of an in vivo LD50 threshold of 500 mg/kg, a value that could be acceptable for early efficacy testing. The model remained highly predictive only for the set of 39 chemicals categorized as non reactive (sensitivity = 83%, specificity = 94%) while for the 43 reactive compounds, the model could not be applied. This study is a clear demonstration that one way to address acute systemic toxicity was to combine multiple, mechanism-based parameters and key chemical properties. However, further efforts need to be made to fully handle the complexity of cosmetic ingredients in terms of chemical reactivity and classes of solubility profiles.
Progress in the implementation of the EURL ECVAM strategy on skin sensitisation

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In 2013 EURL ECVAM released a strategy paper outlining its planned activities for the short, medium and long term for contributing to the replacement of animal testing for skin sensitisation hazard identification and classification (available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-strategy-papers/strat-skin-sensitisation). To this end, EURL ECVAM is undertaking the development of integrated approaches, underpinned by physico-chemical properties, in silico predictions and data from mechanistically relevant in chemico and in vitro methods. Such approaches are envisaged to have the capability not only to discriminate between sensitisers and non-sensitisers but also to categorise sensitisers into sub-categories 1A and 1B of the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS) to fulfill information requirements under the 2018 REACH registration deadline. In addition, in order to facilitate a globally harmonised approach for skin sensitisation assessment, EURL ECVAM is playing a leading role at the OECD in the development of in vitro Test Guidelines on validated test methods addressing key events of the skin sensitisation Adverse Outcome Pathway (AOP) and in the drafting of a guidance document on Integrated Approaches to Testing and Assessment (IATA) for skin sensitisation. An overview of the progress made so far with these activities will be provided.

The beauty (and accuracy) of simplicity: the 2 of 3’s of testing for skin sensitisation hazard identification and beyond

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Evaluation of the sensitization potential of a chemical, the result of a complex multifactorial sequence of events, is one of the principal end-points in both hazard and risk assessments. The key steps of the pathways involved are relatively well characterized and can be structured into an adverse outcome pathway (AOP) which describes the key steps culminating in the adverse effect. Nonanimal test methods addressing three key events in this process are currently well developed have gone through the validation process at ECVAM, and additional tests are also available. They are not considered to be sufficient as a stand-alone method but should be used within an integrated testing strategy (ITS). Regulatory acceptance and the use of nonanimal tests for safety assessments is dependent on the predictivity of a method or an ITS. This in turn is supported by the availability of data allowing the strengths and limitations of the methods or ITS to be defined, as this helps to build confidence to use the ITS. Results of a “2 of 3” approach, in which two concordant results drives the prediction of the sensitization hazard potential, compellingly verify the applicability of this beautifully simplistic ITS for sensitization testing for many chemicals.
Bayesian Integrated Testing Strategy for skin sensitization potency – ITS-3 the next generation

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To maximize practical benefit, ITSs need to follow evolution of 1) assays available to assess the endpoint; 2) mechanistic knowledge, 3) trends in risk assessment towards quantitative. To this end we developed Bayesian network ITS-3 with DPRA, KeratinoSens and h-CLAT assays generating a probabilistic hypothesis for pEC3=log (molecular weight/eC3). A novel data set was compiled containing 168 chemicals. Bioavailability inputs were reformulated to reflect degree of ionization, volatility in addition to previously used kinetic inputs. In the ITS-3 all variables are expressed in molar unit that leads to improved, compared to ITS-2, prediction accuracy for weak and moderate sensitizers. Practical use of the ITS-3 will be discussed including assays’ applicability domain as well as the refined decision rules for full and incomplete records.
prediction model(s) for skin sensitization potential based on integrated testing strategy (ITS) concept. In order to build a hazard characterization model by combination of these methods, we attempted to make a prediction model using artificial neural network (ANN).

**Methods:** About 100 chemicals assessed by LLNA, h-CLAT, DPRA and KeratinoSens™, we investigated a relationship among LLNA thresholds (EC3/highest tested doses of non-sensitizers) and indicators obtained from these in vitro tests. Next, the ANN analysis of in vitro measurements and LLNA were performed.

**Results and Discussion:** We confirmed that each characteristic value of h-CLAT, DPRA and KeratinoSens™ was correlated with LLNA threshold. The ANN model built with these in vitro tests showed a good correlation with LLNA thresholds (r=0.86, RMS error=0.54, N=94). Furthermore, when we evaluated 18 chemicals not listed as learning dataset of this model, this prediction performance was maintained (r=0.85, N=18). These results suggested that the ANN model based on ITS concept could be useful for evaluation of skin sensitization potential.

**References**
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**Il-12b-399**

**Assessment of SENS-IS®, a 3D reconstituted epidermis based model for quantifying chemical sensitization potency: results of an inter-laboratories study**

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A range of different in vitro chemistry-based (DPRA, GSH reactivity) and cell-based methods (MUSST, hCLAT, KeratinoSens) have been developed and some of them are currently evaluated for their applicability to cosmetic ingredients. Although these assays appear to be promising for hazard identification, potency assessment is still limited. Possible limitations may be linked to the metabolism, the bioavailability and the danger signal that may be different in monolayers as compared to a natural tridimensional microenvironment.

We have developed SENS-IS, a method, based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®), thus providing a possible way to encompass these limitations and come closer to potency assessment. The aim of this study was to confirm the transferability and reproducibility of the SENS-IS protocol and its ability to correctly classified sensitizing potency in 5 classes similarly to the LLNA. Three laboratories participated in this SENS-IS ring study using nineteen chemicals tested blindly. All chemicals were similarly classified by the 3 laboratories with was exception using HCA that was overestimated by one laboratory. Analysis of predictive capacity (with 5 class) was 84% as compare to LLNA and 100% with one class difference. These results will be discussed.

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**Il-12b-533**

**Integrating cosmetic industrial needs in skin sensitization**

**Integrated Approaches to Testing and Assessment (IATA)**

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Skin sensitization remains a major environmental and occupational health hazard. If different mechanical approaches, including structure-activity, skin metabolism determination and biological comprehension of contact sensitization have been developed over the last decades, till recently, only animal tests were accepted by regulations. Since March 2013, the 7th Amendment of the Cosmetics Directive prohibits in EU the marketing of cosmetic products containing ingredients which were tested on animal-based assays and prompted the implementation of Integrated Approaches to Testing and Assessment (IATA) for Skin Sensitization.

While there is a common understanding of the Adverse Outcome Pathways (AOP’s) leading to skin sensitization, as well as a wide appropriation of a core battery of assays addressing these AOP key
events, the ways of integrating such data to allow risk assessment of new ingredients is still in its early experimental phase. Like others, we have developed our own integrated testing strategies. Based on this experience on cosmetic case studies and through a comparative review of different approaches that were published, we will present the opportunities and remaining challenges to support the ongoing OECD IATA initiative to reach the final goal of safety evaluation and risk assessment of new ingredients.

**Session II-12: Poster presentations**

**II-12b-817**

**Cosmetics Europe’s strategy for skin sensitisation safety assessment without animal testing: the toolbox**

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The mechanisms of Allergic Contact Dermatitis were recently mapped as an Adverse Outcome Pathway (AOP). Led by this, Cosmetics Europe Skin Tolerance Task Force (STTF) compiled a toolbox of non-animal test methods with the aim to measure the impact of substances on specific AOP key events and to finally identify sensitisers. The initial events of the AOP covering dermal bioavailability and metabolism are addressed by Schepky et al. Sixteen test methods that focus on haptenation and keratinocyte and dendritic cell activation were subjected to a systematic evaluation using data on a common set of ten substances and compilation of information including the level of standardisation, existing data, throughput, transferability and accessibility in cooperation with the test method developers. The presented analysis forms a comprehensive review of the results obtained, which informed the selection of test methods for the next evaluation phases. To cover the later stages of the AOP, the STTF is supporting the development of methods addressing T cell activation and proliferation.

This toolbox of methods is envisaged to allow the establishment of a data integration approach for skin sensitisation safety assessment of cosmetic ingredients which will be presented by Hoffmann et al.

**II-12-027**

**Development of a new local lymph node assay that evaluates the elicitation response**

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Aims: We aimed to develop a new local lymph node assay (LLNA) that evaluates the elicitation response to discriminate the true skin sensitizers from the other chemicals classified as borderline positive by LLNA modified by Daicel based on ATP content (LLNA-DA) and to evaluate cross sensitization potential (Yamashita et al., 2005; Idehara et al., 2008).

Method: Using the new method, we tested 25 kinds of chemicals. Test mice were treated with the chemicals on the dorsum of their right ear on days 1, 2, and 3, and on the dorsum of both ears on day 10. Control mice were treated with the chemicals on the dorsum of the left ear on day 10. Lymph nodes were excised and weighed on day 12. If the difference in weights of the lymph nodes from the left ear between both groups was statistically significant, then we classified the tested chemical as a skin sensitizer. We tested 4 chemical pairs for evaluating the potential of this new method for cross sensitization testing (Yamashita et al., 2014).

Results: Our results for all 25 chemicals, except nickel chloride, were consistent with those from LLNA. In addition, two of the four pairs showed clear cross sensitizing potential.

Conclusion: The new assay has potential for stand-alone skin sensitization testing.

References


An inter-laboratory validation study of IL-8 Luc assay using a stable THP-1-derived IL-8 reporter cell line, THP-G8

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We developed IL-8 Luc assay as a new alternative method for predicting skin sensitization using THP-1 cells transfected with an IL-8 luciferase reporter gene (Takahashi et al., 2011). We performed a validation study of IL-8 Luc assay to examine the transferability and predictivity, we are currently performing a Phase II study.

We assessed 10 coded chemicals 3 times. In the Phase IIb and IIc studies, the results demonstrated intra-laboratory and inter-laboratory reproducibility based on Criterion 1 were 0.78 and 0.89, those based on Criterion 2 were 0.79 and 1, and those based on Criterion 3 were 78%, 86% and 61%, respectively. These data could reveal high sensitivity as a characteristic of IL-8 Luc assay and more favorable results by Criterion 2.

References

A dataset on 80 chemicals tested by IL-8 Luc assay

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We previously reported a dataset on 35 chemicals covering the ECVAM list evaluated by IL-8 Luc assay (Takahashi et al., 2011), in which the effects of chemicals on the IL-8 promoter activity were evaluated by THP-1 cells transfected with IL-8 luciferase reporter gene. To further clarify the performance of IL-8 Luc assay, we created another dataset on 80 chemicals (56 sensitizers, 24 non-sensitizers). According to the suggestions from the VMT meetings, we revised the protocol as follows. Modification-1: We changed the incubation time from 5 hours to 16 hours. Modification-2: We deleted the criteria regarding the suppression of IL-8 promoter activity by N-acetylcysteine. Modification-3: We tentatively used the following three criteria to judge chemicals as sensitizers. Criterion 1: FlnSLO-LA³1.4, Criterion 2: the lower limit of the 95% confidence interval of FlnSLO-LA³1.0, Criterion 3: the intersection of criteria 1 and 2. The results demonstrated that the accuracy, sensitivity and specificity judged by Criterion 1 were 78%, 85% and 62%, those by Criterion 2 were 83%, 94% and 57%, those by Criterion 3 were 78%, 86% and 61%, respectively. These data could reveal high sensitivity as a characteristic of IL-8 Luc assay and more favorable results by Criterion 2.

References

Investigation of the use of THP-1 cells and IL-8 Luc assay to assess water-insoluble chemicals with the Short Time Exposure test method

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Aims/Objectives: The difficulties of accurately assessing skin sensitization induced by water-insoluble chemicals by means of in vitro assays such as the human Cell Line Activation Test (h-CLAT) or the IL (Interleukin)-8 Luc assay have been documented (Ashikaga et al., 2010). A high incidence of false-negatives due to exposure to the maximum allowable limit of DMSO as solvent is a significant issue when assessing water-insoluble chemicals. The aim of this investigation was to establish methods for water-insoluble chemicals that would reduce false-negatives.

Methods: We measured viability of THP-1 cells and IL-8 release for a variety of parameters (i.e., short time exposure, using mineral oil or DMSO, etc.) for a number of water-insoluble chemicals. Results: Citral, which has been reported to release IL-8 after 24 hours of exposure, also released IL-8 after a short-term exposure of five minutes. In contrast, Thiohelic anhydride, which has been reported not to release IL-8 after 24 hours of exposure (Nukada et al., 2008), did release IL-8 after a short-term exposure to a high concentration in mineral oil.

Conclusion: Future investigations will use this technique to examine a wide range of water-insoluble chemicals for application to other existing test methods.

References
EURL ECVAM validation study on skin sensitisation test methods

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Non-animal test methods for skin sensitisation testing that model key events of the sensitisation adverse outcome pathway may contribute to the replacement of current animal tests. Between 2009 and 2011 EURL ECVAM, the European Union Reference Laboratory for Alternatives to Animal Testing, coordinated a validation study on test methods addressing the key mechanisms of haptenation and dendritic cell activation. The study was designed to generate information on the transferability and within and between laboratory reproducibility of the assessed test methods. Evaluation of the individual predictive capacity was outside the scope of the validation study since the test methods are not proposed as stand-alone methods, but rather they are considered to be useful information sources within integrated approaches for skin sensitisation hazard assessment. Amongst the evaluated test methods, the Direct Peptide Reactivity Assay (DPRA) and the human Cell Line Activation Test (h-CLAT) successfully completed the validation study and underwent peer reviewed by the EURL ECVAM scientific advisory committee (ESAC). An overview of the study results will be provided together with the validation management group conclusions on the study outcome and recommendations on future activities.

II-12-110

LLNA variability: an essential ingredient for a comprehensive assessment of non-animal skin sensitisation test methods and strategies

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The development of non-animal skin sensitisation test methods and strategies is progressing. Either individually or in combination, the predictive capacity is usually described in comparison to local lymph node assay (LLNA) results. In this process the important learning from other endpoints, such as skin or eye irritation, to account for variability reference test results – here the LLNA – is widely disregarded.

In order to provide assessors as well as method and strategy developers with appropriate estimates, we investigated the variability of EC3 values from repeated substance testing using the publicly available NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods) LLNA database.

Repeat experiments for more than 60 substances were analysed – once taking the vehicle into account and once combining data over all vehicles. For LLNA sensitiser the variability of EC3 values was evaluated, while for substances that in at least some experiments are LLNA negative the concordance of results was used. Finally, the impact of the variability on LLNA potency classes has been analysed.

With our analysis we stress the importance of considering the LLNA variability in the assessment of skin sensitisation test methods and strategies and provide estimates thereof.

II-12-112

Inter-laboratory validation of an in vitro method to classify skin sensitizers

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The development of nonanimal methods to assess skin sensitization is a priority due to the EU cosmetics testing ban, the 2018 REACH deadline, and the goal of reducing animal use. Cyprotex’s in vitro SenCeeTox® assay was tested at CeeTox (subsequently purchased by Cyprotex) and at the Flemish Institute for Technological Research (VITO). In this assay, MatTek’s three-dimensional human skin model, EpiDerm, was treated in triplicate with six concentrations of each test article. Test articles were run in a blinded manner and included: metol, isoeugenol, 2,3-butanedione, 2-mercaptoethanol, eugenol, 1,3-chloro-2,4-dinitrobenzene, glycerol, 2-hydroxyethylacrylate, and lactic acid. Following exposure, cytotoxicity, glutathione depletion, and gene expression levels of several target genes controlled by the Nrf2/Keap1/ARE or AhR/ARNT/XRE signaling pathway were examined. An algorithm was used to analyze the results and to predict each chemical’s likelihood of causing sensitization.

SenCeeTox® predicted sensitization as well as or better than the LLNA for the compounds tested and correctly predicted the potency within one potency category of the LLNA. The results also show that SenCeeTox® is transferrable between laboratories. Further validation of this assay is ongoing, after which all results will be submitted to the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM).

II-12-132

Predicting skin sensitization using 21st century toxicology

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Allergic contact dermatitis (ACD) is an adverse health effect from repeated exposure to skin-sensitizing chemicals. Regulatory authorities require tests, like the murine local lymph node assay (LLNA), to identify potential skin sensitizers. The Organisation for Economic Co-operation and Development (OECD) established an Adverse Outcome Pathway (AOP) for skin sensitization (OECD, 2012). To reduce or replace animal use, OECD is using the AOP as a framework for developing integrated testing strategies with novel in vitro and in silico approaches. The Tox21 and ToxCast projects include high-throughput screening (HTS) assays that map to key events in the AOP (e.g., oxidative stress, cytokines) (Kavlock et al., 2012; Tice et al., 2013). We
built a cross-validated random forest model using ToxCast Phase II data and a balanced training set of 60 chemicals with LLNA data. The model predicted LLNA results with 80% accuracy. The assays with highest variable importance included known AOP targets (e.g., Nrf2, T-cell proliferation) and targets outside the current AOP (e.g., Coll III, PPAR, PXR, ER). Well-characterized AOPs like skin sensitization provide opportunities to use HTS data to develop efficient testing strategies that minimize animal use in regulatory testing.

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References

ll-12-134

**Development of an open-source integrated testing strategy for skin sensitization potency**

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Regulatory authorities require testing to identify substances with the potential to cause allergic contact dermatitis. To reduce or eliminate animal use in testing, integrated testing strategies (ITS) that combine data from *in silico* and *in vitro* test methods have been proposed. Scientists at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and Procter and Gamble (P&G) have developed an open-source version of a previously published ITS for skin sensitization (Jaworska et al., 2011, 2013). The original ITS was developed with a Bayesian network using a commercial software package. NICEATM and P&G developed the open-source model utilizing R software for building and performing exact inference with a Bayesian network. The open-source and the commercial models had identical overall classification accuracies. Two case studies of representative substances, chlorobenzene and 2-mercaptobenzothiazole will be presented. The open-source model provides availability and transparency, and represents a major step in allowing the ITS to be reproduced and tested, which is essential for use in a regulatory framework. The model is available on the NTP website (http://ntp.niehs.nih.gov/go/its).

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References

ll-12-177

**THP-1 assay predicts allergic potential of chemicals in accordance with ratings given by LLNA and patch tests**

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The SENS-IT-IV project had shown that myelomonocytic cell lines, working as antigen presenting cells in *in vitro* assays, may discriminate sensitizer from non-sensitizer chemical agents. Based on that, our group established a THP-1 activation assay. For this, naïve THP-1 cells were incubated for 24 h with chemical agents at the concentration responsible for 75% cell viability (CV75). Interleukin (IL)-1beta, -6, -12 and TNF-alpha secretion as well as CD54 and CD86 activation marker expression were evaluated. When incubated with THP-1, 21 out of 23 sensitizer chemicals – classified by LLNA (dos Santos et al., 2009; Takenouchi et al., 2013) or patch tests (Zuq et al., 2009) – led to detectable IL-8 levels in the culture supernatants and/or CD86 expression on the THP-1 surface. In addition, from eight non-sensitizer chemicals tested neither IL-8 level nor CD86 expression were observed. The RNA expression of IL-8, CD54 and CD86 were also evaluated, but those analyses did not add relevant information. In the same way, IL-1beta, -6, 12 and TNF-alpha secretion and CD54 expression did not allow to discriminate sensitizer from non-sensitizer chemicals. In our conditions, when the results of CD86 expression and IL-8 release analyses were combined, it was possible to discriminate sensitizers from non-sensitizers.

References

ll-12-180

**Modelling the skin sensitisation Adverse Outcome Pathway (AOP) for risk assessment**


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Current understanding of the key events that drive the induction of skin sensitisation has recently been documented as an AOP (OECD, 2012). Our aim is to apply this knowledge to human risk assessment, i.e., assessing the likelihood of sensitisation following a defined chemical exposure (dose per unit area of skin exposed). Central to our approach is mathematical modelling of the response and evaluation of model output against available clinical data on sensitisation (MacKay et al., 2013; Maxwell et al., 2014).

Our current model outputs naïve CD8+ T cell activation as a surrogate measure for sensitisation induction in humans. Ordinary differential equations are used to model key events of the AOP: skin pen-
Skin sensitisation EPAA/CEFIC LRI activities progressing non-animal testing strategies

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Under the umbrella of a cross sector EPAA project team, EPAA members and Cefic LRI have joined forces since 2010 and organised a number of joint initiatives in the area of skin sensitisation. The objective is to share industry experience on the latest available methods and approaches, their applicability and/or limitations with respect to addressing regulatory safety requirements. Expert workshops were organised in 2011 and 2013 focusing on the opportunities to make use of non-animal test data for REACH dossiers. Attention has been given to the early engagement of regulators (ECHA and national experts), validating bodies (EURL ECVAM) and international authorities (OECD). The project will further examine how the information produced from non-animal assays can be used in regulatory decision-making, not only for hazard identification (classification and labelling decisions) but also for risk assessment decisions (potency evaluation). Results and recommendations from workshops and current activities will be presented.

Improving dendritic cell-based skin sensitization assays by integrating tolerogenic markers

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The skin sensitization potential of chemicals can be assessed in vitro by assays addressing specific events of the adverse outcome pathway. Several assays are based on inflammatory responses of dendritic cells (DCs) or DC-like cell lines. However, DCs can acquire either immunogenic or tolerogenic phenotypes and are essential for the induction of peripheral tolerance (Shortman and Liu, 2002). Hence, the focus on the analysis of cell activation may miss biologically-relevant information. Here, we evaluated the capability of tolerance-associated markers CD273, CD274 and indoleamine 2,3-dioxygenase (IDO) to improve the discrimination between sensitizing and non-sensitizing chemicals in the primary DC-based PBMD (Reuter et al., 2011) assay. We show that sensitizers (DNCB and 7-hydroxycitronellal), but not non-sensitizers, repress the expression of CD273 and CD274 in primary human DCs. Moreover, concomitant induction of CD273, CD274 and CD16 characterized the effect of several compounds that are anti-inflammatory in vivo but nevertheless produce false positive results in vitro. In contrast to the specific response of CD273 and CD273, the tolerogenic marker IDO was non-specifically repressed by skin sensitizers and non-sensitizers. We conclude that the implementation of tolerogenic markers in existing DC-based in vitro assays may improve the specificity of DC-based skin sensitization assays by distinguishing tolerogenic from immunogenic cell activation.

Skin sensitization prediction: transferability and reproducibility of the MUSST

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The MUSST test method addresses one of the key events in the Adverse Outcome Pathway for skin sensitization: like the dendritic cells, upon contact with sensitizers, U937 human myeloid cells induce CD86 expression as they are activated. The prediction model is defined by a stimulation index above 150 with a dose response relationship in at least 2 concordant experiments.

The aim of this study was to demonstrate the transferability and the reproducibility of the MUSST in 4 laboratories and define its predictivity for identifying sensitizers (S) versus non-sensitizers (NS).

A high intra-laboratory reproducibility of 95% was obtained with 21 chemicals tested in 3 independent experiments within L’Oréal. The 4 laboratories similarly classified 11/14 substances, resulting in an inter-lab reproducibility with a pair-wise comparison comprised between 86-93%.

On a wide range set of 123 substances, an accurate predictive capacity (86.2%) of the MUSST was achieved with a sensitivity of 86.1% and a specificity of 86.4%, respectively.

In conclusion, the MUSST was demonstrated as an efficient transferable, reproducible and reliable assay for the skin sensitization hazard characterization. Therefore, the MUSST has been identified as one essential piece in an integrated approach for risk assessment.
Langerhans cells (LCs) are immature dendritic cells located in the epidermis and surface epithelium that play a central role in T-lymphocyte mediated skin immunity (Tchou et al., 2003; Pena-Cruz et al., 2001). In order to achieve the biological compartment of immature LCs regarding their reaction with allergens and cosmetics we tested different protocols to extract and cryopreserve fresh epidermal immature LC that where tested for phenotypic and functional characteristics. All the LCs were isolated from skin epidermis obtained from plastic surgery and the four methods were tested in parallel: (a) the role skin was processed with the mechanic disruption obtaining a single cell suspension, that were seeded in culture flasks to collecting the supernatant containing the non adherent cells or purified with a density gradient system (Ficoll Paque Plus®); (b) using a single cell suspension from the epidermis and applied a Ficoll flotation process; (c) using a homemade manual skin graft mesher and the LC mechanism of migration. The (b) and (c) systems were equally efficient (78.1 ± 7.3%) but method (c) is better due to reduction of time in processing, delaying the maturation process, showed by flow cytometer analyses. The (a) system was less efficient being left out after the results obtained in (b) and (c).

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References

Evaluation of the sensitizing potential of chemicals based on their reactivity: use of reconstructed human epidermis
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Allergic contact dermatitis is generally induced by low molecular weight electrophilic chemicals which trigger reactive species (RS) production and provoke an electrophilic stress leading to the accumulation of the transcription factor nuclear-related factor 2 (Nrf2) in dendritic cells and cell lines such as THP-1. We investigated if a correlation exists between the reactivity of chemical sensitizers toward amino-acids (lysine, cysteine and arginine) with the generation of RS, the expression of Nrf2-related genes and the presence of the Nrf2 protein. Indeed not all sensitizing chemicals are reacting with the same amino acids even if cysteine and lysine are the most often considered. Up-to now the chemical reactivity was investigated using model peptides such as in the DPRA (Direct Peptide Reactivity Assay) but little is known on the chemical reactivity in the epidermis. To validate data obtained in the DPRA assay (Direct Peptide Reactivity Assay) but little is known on the chemical reactivity in the epidermis, we investigated the reactivity of two chemicals, namely the methylisothiazoline (MI) and the phthalic anhydride (PA) in a reconstructed human epidermis (RHe) using high resolution at magic angle spinning (HRMAS) NMR. Our data confirmed that the epidermis is reacting only with cysteine residues while PA was reacting only with lysine residues validating the DPRA data.

Evaluation of the sensitizing potential of chemicals based on their reactivity: activation of the Nrf2 pathway
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Allergic contact dermatitis is generally induced by low molecular weight electrophilic chemicals which trigger reactive species (RS) production and provoke an electrophilic stress leading to the accumulation of the transcription factor nuclear-related factor 2 (Nrf2) in dendritic cells and cell lines such as THP-1. We investigated if a correlation exists between the reactivity of chemical sensitizers toward amino-acids (lysine, cysteine and arginine) with the generation of RS, the expression of Nrf2-related genes and the presence of the Nrf2 protein. Chemical reactivity was determined with candidate peptides and adducts were characterized using 13C-NMR. This work was conducted in THP-1 cells, the cell line used in the hCLAT assay, as a surrogate for dendritic cells, 29 molecules with specificity for cysteine, lysine/ cysteine, lysine and arginine were selected according to the literature and results obtained in the DPRA assay (Direct Peptide Reactivity Assay). Cysteine and cysteine/lysine reactive chemicals were able to induce both gene and Nrf2 protein expression in THP1 cells. All the lysine-reactive and arginine-reactive chemicals with the exception of two were not able to induce the expression of more than one Nrf2-dependent gene and the expression of the Nrf2 proteins.

International ring trial of the epidermal equivalent sensitizer potency assay: reproducibility and predictive-capacity
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This study describes the international ring trial of the epidermal-equivalent (EE) sensitizer potency assay. This assay does not distinguish a sensitizer from a non-sensitizer, but may classify known skin sensitizers according to their potency. It assesses the chemical concentration resulting in 50% cytotoxicity (EE-EC50) or the 2-fold increase in IL-1α (IL-1α2x). Four laboratories received 13 coded sensitizers. Reproducible results were obtained in each laboratory. A binary prediction model, EC50 ≥7 mg/ml = weak to moderate sensitizer and EC90 <7 mg/ml = strong to extreme sensitizer had an accuracy of 77%. A superior EE (EC50 and IL-1α2x) correlation was observed with human in vivo DSA05 data compared to LLNA-EC3 data. Human in vitro NOEL and LLNA-EC3 data correlated to a similar extent to in vitro EE data. Our results indicate that this easily transferable EE potency assay is suitable for testing chemical allergens of unknown potencies and may now be ready for further validation, providing complementary potency information to other assays already undergoing validation for assessing skin sensitization potential.

**Evaluation of the direct peptide reactivity assay (DPRA) in identifying epoxy resins with skin sensitizing potential**

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Allergic contact dermatitis (ACD) develops as a result of the hypersensitivity response of human skin upon repeated exposure to contact allergens. The direct peptide reactivity assay (DPRA) is an in chemico method that has been developed to potentially replace the current “method of choice” the murine local lymph node assay (LLNA). To date, the DPRA has been used primarily to assess the skin sensitizing potential of chemicals utilized in cosmetics and toiletries. The aim of this study was to optimize the DPRA for evaluating the skin sensitizing potential of a different class of chemicals namely epoxy resins (ER), using three heptapeptides (Corl-C420, cysteine and lysine) under a range of assay conditions. Our preliminary data using five ER with the three heptapeptides, showed an accuracy of 80% for each heptapeptide. An integrated approach combining the use of these three heptapeptides for assessing the skin sensitizing potential of 22 chemicals showed a high correlation between the DPRA and the data generated using a quantitative structural-activity relationship (QSAR) prediction tool. Our data show for the first time that the heptapeptide, Corl-C420, may be used to extend the DPRA non-animal testing method, for identification of potential skin sensitizers from the epoxy resin chemical class.

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**Photo-safety assessment using multiple in vitro tests**

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Phototoxicity and photopotentiation are essential endpoints in safety evaluation for cosmetic ingredients, and recent attention has been drawn to development of efficacious in vitro screening systems for photosafety. This study aimed to develop an in vitro photo-safety system that consists of multiple tests to examine biochemical and biological properties related with phototoxicity. In this study, over 30 test chemicals were examined. In the first step of the evaluation, photochemical property of test chemicals was assessed using both UV/VIS spectral analysis and the Reactive Oxygen species (ROS) assay. Then, photo-irritation test, 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) (OECD TG 432) was conducted. Finally, two in vitro photo sensitization tests were done. Photo-SH/ NH2 test, one of the photosensitization tests, can detect changes of thiols and amines expressed on cells (we used THP-1, human monocyte cell line) after UV-irradiation in the presence of test chemicals. On the other hand, the Photo-ARE (Antioxidant Response Element) assay can detect augmentation of ARE-dependent luciferase activity of a reporter cell. After having examined chemicals with these assays, we have built a decision tree, which is composed of UV/VIS spectral analysis, ROS assay, 3T3 NRU PT, photo-SH/NH2 test and photo-ARE assay.

**Development of skin sensitization test method using THP-1 cells cultured on a collagen vitrigel membrane chamber for oily materials**

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**Introduction:** In order to develop a suitable exposure method of oily chemicals in the skin sensitization test using THP-1 cells cultured on the collagen vitrigel membrane (CVM) chambers, the usage of 10% DMSO in culture medium was attempted as an application vehicle of test chemicals.

**Methods:** THP-1 cells in 10% FBS-RPMI1640 medium were seeded on a CVM chamber placed in 12-well culture plate. Various concentrations of test chemical in 10% DMSO-medium solution were applied on the bottom side of CVM chamber for 10 or 30 min. Test chemical solution was then exchanged by culture medium, and incubated. After 48 hr, viability of the cell and IL-8 concentration in supernatant was measured.
Results and Discussion: Exposure to 10% DMSO-medium from the bottom side of CVM did not affect the viability of the cells. The IL-8 production of cells those were treated with sensitizers such as 2,4-dinitrochlorobenzene, nickel sulfate and resorcinol was higher than that of vehicle-treated cells, and increased in a concentration-dependent manner. Non sensitizers such as methyl salicylate did not induce significant IL-8 production at any tested concentration. These results suggested that this method using 10% DMSO-medium might be a suitable for the assessment of the skin sensitization potentials of oily test chemicals.

II-12-358

Relationship of contact allergen potency and size of the T cell pool and T cell receptor repertoire

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The mouse Local Lymph Node Assay (LLNA) is used for the identification of chemical contact allergens and for the assessment of their allergenic potency. None of the current in vitro assays to replace the LLNA can faithfully measure potency. We have used previously established human T cell priming assay (hTCPA) (Dietz et al., 2010; Richter et al., 2013) to test whether allergenic potency of contact allergens correlates with the size of the T cell pool and the diversity of the T cell receptor (TCR) repertoire. For this purpose naïve human T cells are primed and re-stimulated with autologous dendritic cells (DC) and test chemicals. Cytokine production is used as readout. RNA is isolated from chemical-specific T cells and TCR Vb spectratyping (DNB, TNBC, Oxazolone) allowing detection of all TCR Vb family members while for the moderate and weak contact allergens (tNBS; tNCB, Oxazolone) allowing detection of strong and moderate/weak allergens is in progress to rule out that tCR repertoire appears more restricted. Intra-individual comparison of hapten-specific T cells. As a result of recent research projects (EU program) aiming at replacement of animal testing for contact allergen identification, human T Cell Priming Assays (hTCPA) demonstrate the feasibility of hapten-specific T cell priming in vitro. These assays use chemical-loaded monocyte-derived dendritic cells (MDDCs) as antigen-presenting cells (APC) and autologous human peripheral blood leukocytes (PBL) depleted of several immunomodulatory cell types or purified naïve T cells as responding cells. We have now demonstrated a sensitivity of 67% and specificity of 100% in a blinded study conducted on 16 chemicals (including reference- and non-sensitizers) (Vocanson et al., unpublished results) to shorten the duration of the assay we are currently testing mature fast-derived MDDCs as APC. To improve its sensitivity we are tracking T cell proliferation (CFSE or Ki67+) and functions (IFN-g, TNF-a, IL-17a, IL-4 or Granzyme B expression) by multi-parametric flow cytometry. Targeting inhibitory receptors (PDL-1/ PD-1) on the surface of MDDCs or T cells might further increase T cell priming and the detection of contact sensizers.

References

II-12-366

Application of the reconstructed human epidermis model, Keraskin™, for identifying skin sensitizer

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Recent changes in social recognition on animal-tested cosmetics have accelerated the development of in vitro test to identify the potential of skin sensizers. In this study, we tried to develop an in vitro skin sensitization assay using reconstructed human epidermis made of keratinocytes, Keraskin™ (MCTT Inc., Korea). Chemicals were applied to the epidermal surface for 24 hours and culture supernatant was used to investigate cytokine profile by ELISA or cytokine array. Representative pro-inflammatory cytokine secretion from keratinocyte -IL-1α, IL-6, IL-8 and IL-18- was measured by spectrophotometric method and IL-18 was comparatively identified as skin sensitizer. In order to explore additional marker, we performed cytokine array and the data showed that DNCB increased the secretion of RANTES, GM-CSF, IP-10 and sTNF R I. And we confirmed that IL-18 and sTNF R I were increased above 1.5 fold when several sensizers were treated. In conclusion, our data suggests that sTNF R I could be a valid marker in company with IL-18 for distinguishing sensizer from non-sensitizer.

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II-12-380

Optimisation of the human T Cell Priming Assay for the identification of chemical allergens

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The key event of Allergic Contact Dermatitis (ACD) is the priming of hapten-specific T cells. As a result of recent research projects (EU “Sens-it-iv” consortium; Cosmetics Europe “Alternative methods” program) aiming at replacement of animal testing for contact allergen identification, human T Cell Priming Assays (hTCPA) demonstrate the feasibility of hapten-specific T cell priming in vitro. These assays use chemical-loaded monocyte-derived dendritic cells (MDDCs) as antigen-presenting cells (APC) and autologous human peripheral blood leukocytes (PBL) depleted of several immunomodulatory cell types or purified naïve T cells as responding cells. We have now demonstrated a sensitivity of 67% and specificity of 100% in a blinded study conducted on 16 chemicals (including reference- and non-sensitizers) (Vocanson et al., unpublished results). To shorten the duration of the assay we are currently testing mature fast-derived MDDCs as APC. To improve its sensitivity we are tracking T cell proliferation (CFSE or Ki67+) and functions (IFN-g, TNF-a, IL-17a, IL-4 or Granzyme B expression) by multi-parametric flow cytometry. Targeting inhibitory receptors (PDL-1/ PD-1) on the surface of MDDCs or T cells might further increase T cell priming and the detection of contact sensizers.
obtained when integrating the DPRA/OeCD QSAR toolbox in this study. The accuracy of the predictions was 80/81% or 89/86% when compared to llNA or human data were obtained. Accuracies of evidence” (Woe) approach that has previously been published to assess the skin sensitizing potentials of the test substances. Accuracies of 79 and 73%, respectively.

An alternative approach is the analysis of structural characteristics as associated with protein-binding capacities performed in chemico. Allergic contact dermatitis is typically induced by small-sized electrostatic chemicals which bind to cutaneous proteins to form complete allergens found in occupational and consumer products. In the interests of consumer and worker safety, the skin sensitization potential of a substance must be assessed. Several in vitro/in silico methods have been described in the literature, only few are close to pass the formal European validation. Among the initial cellular event is the activation of keratinocytes. So far only KeratinoSens™ has been validated to address this cellular event. Herein, we report on the LuSens assay, that uses a human keratinocyte cell line harboring a reporter gene construct composed of the rat antioxidant response element (ARE) of the gene of the NAPDH:quinone oxidoreductase 1 and the luciferase gene. The assay was validated in house using 74 substances including the LLNA performance standards. The predictivity of LuSens assay for sensitization hazard identification was comparable to other non-animal methods. In particular, since LuSens provides the same readout as KeratinoSens™ and also yields a comparable predictivity, both methods could be used equivalently. When used as part of a testing battery based on the OECD adverse outcome pathway for sensitization, LuSens assay, DPRA and dendritic cell line activation test attained predictivity similar to that of LLNA.

Analysis of in chemico and in silico methods to predict skin sensitization potentials


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Allergic contact dermatitis can develop following exposure to allergenic substances found in occupational and consumer products. In the interests of consumer and worker safety, the skin sensitization potential of a substance must be assessed. Several in vitro/in silico methods have been described in the literature, only few are close to pass the formal European validation. Among the initial cellular event is the activation of keratinocytes. So far only KeratinoSens™ has been validated to address this cellular event. Herein, we report on the LuSens assay, that uses a human keratinocyte cell line harboring a reporter gene construct composed of the rat antioxidant response element (ARE) of the gene of the NAPDH:quinone oxidoreductase 1 and the luciferase gene. The assay was validated in house using 74 substances including the LLNA performance standards. The predictivity of LuSens assay for sensitization hazard identification was comparable to other non-animal methods. In particular, since LuSens provides the same readout as KeratinoSens™ and also yields a comparable predictivity, both methods could be used equivalently. When used as part of a testing battery based on the OECD adverse outcome pathway for sensitization, LuSens assay, DPRA and dendritic cell line activation test attained predictivity similar to that of LLNA.

References


LuSens: a novel reporter gene-cell line to identify skin sensitizers

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Allergic contact dermatitis can develop following exposure to allergenic substances found in occupational and consumer products. In the interests of consumer and worker safety, the skin sensitization potential of a substance must be assessed. Several in vitro/in silico methods have been described in the literature, only few are close to pass the formal European validation. Among the initial cellular event is the activation of keratinocytes. So far only KeratinoSens™ has been validated to address this cellular event. Herein, we report on the LuSens assay, that uses a human keratinocyte cell line harboring a reporter gene construct composed of the rat antioxidant response element (ARE) of the gene of the NAPDH:quinone oxidoreductase 1 and the luciferase gene. The assay was validated in house using 74 substances including the LLNA performance standards. The predictivity of LuSens assay for sensitization hazard identification was comparable to other non-animal methods. In particular, since LuSens provides the same readout as KeratinoSens™ and also yields a comparable predictivity, both methods could be used equivalently. When used as part of a testing battery based on the OECD adverse outcome pathway for sensitization, LuSens assay, DPRA and dendritic cell line activation test attained predictivity similar to that of LLNA.

Role of ROS and HMGB1 in contact allergen-induced IL-18 production in human keratinocytes

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Keratinocytes play a key role in all phases of allergic contact dermatitis. We have recently identified the possibility to use IL-18 production for the in vitro identification of contact allergens. The purpose of this study was to characterize the molecular mechanisms underlying allergen-induced IL-18 production, in order to identify the cellular source of ROS and the danger signals involved. The NCTC254 was exposed to three contact allergens, namely PPD, 2,4-dinitrochlorobenzene and citral in the presence or absence of diphenyle ylidion (DPI),
allopurinol and rotenone to identify the source of ROS, and to anti-TLR4 antibody and glycyrrizic acid to characterize the DAMPs. In the case of PPD, the induction of IL-18 can be modulated by rotenone, allopurinol and DPI. In the case of DNCB, rotenone completely prevents the induction of IL-18 while for citral DPI completely prevents the induction of IL-18. We demonstrated the ability of all allergens tested to induce the release of HMGB1 (high-mobility group protein B1). Its sequestrant by glycyrrizic acid significantly modulate PPD-induced IL-18 production and completely prevents DNCB and citral-induced IL-18. We found that different intracellular source of ROS are triggered by contact allergens and an important role for HMGB1 in chemical allergen-induced IL-18 production was demonstrated.

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II-12-559

Variability in data from skin sensitization and reactivity testings

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Until March 2013, skin sensitization potency of chemicals was evaluated through animal tests, e.g., local-lymph-node-assay (LLNA). Skin sensitization depends, amongst others, on chemicals’ ability to covalently bind with skin proteins. Thus, in chemico approaches called direct-peptide-reactivity-assays (DPRA) could be alternatives to animal testing. To build in silico potency models for skin sensitization knowing the experimental data’s reliability is key.

In this variability study LLNA EC3% data from 26 chemicals with minimum five values were used. For EC3 values from 0.0 to 6.0%, a linear relationship between the variance and mean EC3 values was observed. In the 6.0-20.0% range the variance of EC3 data remained constant.

For the variability analysis of DPRA % reactivity data minimum four different reactivity data for 29 chemicals with cysteine peptide (Cys) and 27 chemicals with lysine peptide (Lys) were used. These data revealed a parabolic relationship between the variance of Cys or Lys and their mean values of % reactivity.

Diverse confidence intervals were constructed, allowing categorising chemicals from LLNA and DPRA data sets at levels of confidence from 95% to 75%. They could also be used for a more robust classification of chemicals, according to their skin sensitization and reactivity potencies determined by different sources.

II-12-594

Epidermal Sensitization Assay (EpiSensA) with reconstructed human epidermis for detecting sensitizing potential of lipophilic chemicals

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For the replacement of animal tests, we observed a good accuracy (81%, N=139) when utilizing the testing strategy composed of the human Cell Line Activation Test (h-CLAT), an in vitro skin sensitization assay we developed, and the Direct Peptide Reactivity Assay (DPRA). However, lipophilic chemicals (Log Kow ≥3.5) could not be correctly evaluated by this strategy. To overcome the limitation of water-solubility, we developed the Epidermal Sensitization Assay (EpiSensA) based on expression of stress-related genes in a reconstructed human epidermis. In the EpiSensA study, we showed a high predictivity of the EpiSensA using the ECVM-referenced chemicals (Log Kow <3.5).

In this study, to develop a testing strategy for detecting the sensitization potential of various lipophilic chemicals, we evaluated several lipophilic chemicals using the EpiSensA. As a result, lipophilic chemicals (undec-10-enal, benzyl salicylate, dibutyl aniline, amyl cinnamal, citronellol, damascene, and limonene) induced a 10-fold increase of stress-related genes compared to vehicle.

These data suggest that we can evaluate various chemicals by using the combination of EpiSensA and the Direct Peptide Reactivity Assay for lipophilic chemicals (log Kow <3.5) and the EpiSensA for lipophilic chemicals (log Kow ≥3.5).

II-12-680

Estimation of the skin sensitizing potency based on peptide reactivity, dendritic cell activation and read-across using the hair dye 2-methoxyethylparaphenyleneediamine as example

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Evaluation of the skin sensitizing potency of ingredients is a key part of the safety assessment for topically applied products to assess the likelihood to induce contact allergy. Well established in vitro assays, i.e., for peptide reactivity and the dendritic cell activation were applied to assess the skin sensitization potential of the new hair dye molecule 2-methoxy-methyl-p-phenylenediamine (ME-PPD) that is structurally closely related to the current key hair dye precursors p-phenylenediamine (PPD) and p-toluylenediamine (PTD). Protein reactivity and dendritic cell activation data for ME-PPD indicated an attenuated innate immune response compared to PPD and PTD. The reduced skin sensitization potential observed in vitro was in line with historical data generated in the local lymph node assay (LLNA), i.e., the concentration of ME-PPD needed to induce lymphocyte proliferation 3-fold above background (EC3 value) was 4.3. This indicated a moderate skin sensitizing potency, whereas EC3 values of 0.1 and 0.17% for PPD and PTD, respectively, corresponded with a strong potency. Our results indicate that the in vitro assessment of ME-PPD in comparison to PPD and PTD together with read-across from the historical in vivo (LLNA) data for PPD and PTD can be applied to estimate the skin sensitizing potency of ME-PPD.
II-12-710

In vitro skin sensitization methods: evaluating the available reference datasets

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The evaluation of skin sensitizers requires both a measure of hazard and relative potency. In vivo methods, particularly the LLNA, currently provide both pieces of information. With respect to non-animal alternatives, there have been significant advances in our mechanistic understanding of allergic responses. This has resulted in a variety of in vitro/in silico test methods that are designed to model key biological and chemical events. While there are clearly areas for refinement, the current non-animal alternatives all provide a degree of hazard identification. However, the science of potency estimation remains a significant need and is the focus of numerous research efforts to develop integrated testing strategies (ITS). A key challenge affecting potency estimation is the fact that the available reference data, based on the LLNA and used to benchmark ITS, was not selected based on a systematic understanding of the underlying chemistry and biology. Rather, these in vivo data were based on practical availability in the open literature and proprietary data generated by other purposes (fulfilling regulatory requirements, R&D). This presentation will review the available references datasets with the aim of identifying areas for improvement that can speed the development of ITS and move towards increased human relevance.

II-12-716

Development of improved skin sensitization assays based on 3D reconstructed human epidermal equivalents and secreted luciferase reporter activity

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Determination of skin sensitizing potential (SSP) is an important concern for consumer products and cosmetic ingredients. However, recent legislative and societal concerns against animal testing necessitate development of in vitro methods for predicting SSP. When testing lipophilic ingredients/complex mixtures, in vitro models that reproduce the 3D structure/function of native human skin are highly desirable. We previously developed a PCR-based SSP assay (epiSensA), based on expression of antioxidant response element (ARE)-regulated genes in an in vitro reconstructed human skin model (EpiDerm™). We also engineered the model to express an ARE-regulated luciferase reporter gene. Testing of the luciferase reporter model revealed promising results, but further optimization was necessary. The goal of the current work was to develop an improved high-throughput SSP assay based on new promoter sequences coupled to a secreted Cypridina luciferase. Testing of the new reporters in human keratinocytes revealed promising candidates that responded to skin sensitizers and produced low background. An additional advantage of secreted luciferase reporters is the ability to sample reporter activity at multiple timepoints from the same sample. 3D reconstructed human epidermal models containing the new secreted luciferase reporter were also developed. These models are being tested for determining SSP of lipophilic ingredients/complex mixtures.

II-12-725

Evaluation of SENS-IS®, an Episkin® based model for identifying chemical sensitizers of fragrance ingredients

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In the context of the 2013 ban given by EU Cosmetics Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance for the cosmetic industry. A range of different in vitro chemistry-based and cell-based methods have been developed and we are currently evaluating some of them for their applicability to cosmetic ingredients and their physicochemical diversity. Although these assays appear to be promising for hazard identification, potency assessment is still limited.

Immunosearch has developed SENS-IS, a new method, based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermies (Episkin®). This new assay provides a possible way to encompass the limitations of monolayer culture models (lack of skin bioavailability properties, different metabolism of the models compared to skin, inability to test water insoluble chemicals) and might therefore allow a better assessment of the sensitization potency of cosmetic ingredients. With the aim to evaluate the predictive capacity of this approach on a panel of cosmetic ingredients, L’Oréal challenges this method with a blinded-set of 20 fragrance ingredients. We present here the result of this study and will analyze the genomic signature of those volatile weak/moderate sensitizers.

II-12-742

IL-18 secretion as a marker for identification of contact sensitizers in the EpiDerm™ in vitro human skin model

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Assessment of the allergic potential of chemicals has traditionally been conducted in animal models. However, recent legislation has prohibited the use of animals for conducting such tests on cosmetics or cosmetic ingredients. Thus, animal alternative tests for contact sensitization are urgently needed. Interluekin-18 (IL-18) secretion has been identified as a useful endpoint for determination of contact sensitization potential in keratinocyte monolayer cultures. Because sensitization is dependent on chemical penetration and metabolism in the skin, 3D organotypic skin models that possess in vivo-like barrier properties and metabolizing capabilities may provide significant benefits over monolayer culture
models. Thus, IL-18 was evaluated as an endpoint for sensitization potential using the EpiDerm™ in vitro human skin model. Eleven contact sensitizers and 4 non-sensitizer chemicals were tested. A protocol was developed using aqueous or ethanol based vehicles for topical application. EpiDerm™ tissue viability and IL-18 secretion by ELISA were evaluated 18-24 hours following topical application of test chemicals. 10/11 contact sensitizers were correctly identified by the assay (90.9% sensitivity) with no false positive results (100% specificity). Thus, the EpiDerm IL-18 assay appears to be a promising tool for in vitro determination of contact sensitization potential.

II-12-779
Refinement of the peroxidase peptide reactivity assay (PPRA) and prediction model
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We have previously developed a mechanistic in-chemico peptide reactivity assay allowing for quantitative analysis of a chemical’s reactivity for screening its skin sensitization potential. A horseradish peroxidase-hydrogen peroxide (HRP/P) oxidation system has been incorporated into the assay for characterizing reactivity of hapten and pro-/pre-hapten sensitizers. Although a predictive accuracy of 83% (relative to the LLNA) was achieved, apparent false positives were attributed to cysteine depletion at high concentrations and for some chemicals expected to react with the NH₂-group of lysine, little/no (relative to the LLNA) was achieved, apparent false positives were incorporated into the assay for characterizing reactivity of hapten and activity for screening its skin sensitization potential. A horseradish activity assay allowing for quantitative analysis of a chemical’s reactivity for characterizing reactivity of hapten and activity for screening its skin sensitization potential.

II-12-801
A highly differentiated 3D epidermal skin model to characterize skin sensitizers
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To develop an in vitro assay to identify and characterize skin sensitizers, we adapted the keratinocyte interleukin-18 (IL-18) response assay developed by Corsini and colleagues for use with epiCS®, a primary human keratinocyte 3D skin model. The assay measures release of IL-18 into culture medium of test substance-treated tissues over 24 hours, by ELISA. Results are expressed as Stimulation Index (SI) compared to vehicle; an SI>1.5 was considered a positive sensitizer response. Four vehicles and three sensitizers were compared. Ethanol:DMSO 4:1 yielded the highest SI values for the sensitizers. The basal amount of IL-18 release for all vehicles was 1.1-17 pg/ml. DNBC at 0.15% produced highest SI values with the vehicles, and a dose-response at 0.018%-0.3%. A dose-response was caused by nitrobenzyl bromide (0.025%-0.2%) and p-phenylenediamine (PPD, 0.1%-2.0%) in ethanol. Cinnamic alcohol and resorcinol were negative in ethanol. Cinnamic alcohol and resorcinol were negative in ethanol, however, resorcinol was clearly positive (SI = 2.7) in AOO 4:1. Irritant/nonsensitizers 2% phenol and glycerol were negative (SI<1.5). Hair dyes containing PPD were tested neat on the epiCS® tissues. An increase in IL-18 over controls (13 pg/ml) was observed in dyes from 71 pg/ml to 76 pg/ml to 85 pg/ml PPD (SI 5.7, 6.0 and 6.8).

II-12-802
Implementation of the RHE / IL-18 sensitization assay – accuracy, sensitivity and specificity evaluation
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Safety testing of cosmetic raw materials and finished products using alternative methods to animal use, has become a major challenge. Among critical endpoints to be addressed, sensitization potential has been the subject of extensive research during the last decade. As a result, several assays are already at the pre-validation stage at the ECVM.

In anticipation of high demands for sensitization testing, we have implemented one of the most promising models, namely the RHE/
IL-18 assay performed on the SkinEthic RHE model. This assay was designed to first discriminate sensitizers from non-sensitizers and subsequently to classify the identified sensitizers according to their respective potency, into appropriate groups. The implementation was conducted in partnership with an Industrial Sponsor, along with the support of an academic expert.

18 substances, of which the sensitization potential had been previously characterized, were screened using this assay. Results showed that the RHE/IL-18 assay allows an accurate identification of sensitizers with high degree of sensitivity and specificity. However, detailed analysis of the results pointed out some limitations which may be attributed to production variations or handling of the test system for this very purpose. These latter observations have to be taken into account to insure robustness of the method.

Co-culture assay for the identification and investigation of dermal sensitizers (epi-dc)

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to investigate the mechanism of dermal sensitisation, a co-culture model of human keratinocytes and dendritic cells was developed and optimized. This model (Epi-DC) comprises EpiDerm™ tissue grown atop a suspension culture of plasmacytoid dendritic cells. IL-18 release and activation of surface markers CD86 and CD54 on DC were measured. Chemicals were dosed topically on EpiDerm™ for 24 hours. Criteria for a positive sensitizer included: (1) increase of IL-18 of >1.5-fold over vehicle, or (2) increase in percentage of DC surface markers CD86+ or CD54+ of >1.5-fold over vehicle, measured by flow cytometry. Dinitrochlorobenzene (DNCB; 0.0003% to 0.1%) and cinnamaldehyde (CA; 0.0003% to 0.006%) were tested diluted in the ethanol vehicle.

Treatment of Epi-DC with DNCB yielded IL-18 concentrations that peaked at 0.1% DNCB, resulting in 1.9-fold increase over vehicle. A dose-dependent increase of CD86+ DC was shown with treatment of DNCB from 0.003% to 0.1%, peaking at 2.1 fold. At 0.006% Cinnamonaldehyde, increases of 1.6-fold over control were observed in CD86 expression. No increases in CD54+ DC were seen at any of the test substance treatments. In this initial evaluation, the Epi-DC co-culture model is a useful and predictive tool for identifying dermal sensitizers without the use of animals.

Probabilistic hazard assessment for skin sensitization potency using machine learning to design integrated testing strategies

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Integrated Testing Strategies (ITS) aim to combine various information streams to hazard prediction. They are fueled by the increasing understanding of Adverse Outcome Pathways (AOP), i.e., mechanistic understanding and the development of tests reflecting these mechanisms. However, simple addition of further information bears the danger of adding noise and over-fitting. The problem is further amplified when potency information (dose/response) of hazard shall be estimated by these ITS. We curated such a dataset and combined a recursive variable selection algorithm to evaluate the information available through in silico, in chemico, and in vitro assays. Chemical similarity alone could not cluster chemical’s sensitizing potency, and in vitro models consistently ranked high in recursive feature elimination approaches. This allows to reduce the number of tests included in an ITS. Next we performed analysis with a Hidden Markov model that takes advantage of an intrinsic inter-relationship amongst the LLNA classes – that is, the monotonous connection between LLNA and dose. The Dose-informed Random Forest/Hidden Markov Model outperformed significantly the Dose-naive Random Forest model on all data sets. Although from the standpoint of balanced accuracy the improvement may seem small, this obscures the actual improvement in mis-classifications.

Session II-13: Endocrine disruption

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Session II-13: Oral presentations

High throughput chemical screening for endocrine disrupting potential

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The US Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) uses a two-tiered approach to determine if a chemical may pose a risk to human health or the environment due to potential interactions with the estrogen, androgen, and thyroid hormone signaling pathways in humans and wildlife. Requirements for testing, data review, and weight of evidence determination require substantial temporal, financial, animal, and human resources, with costs out weighing the initial benefits. Recognizing that over ten thousand chemicals are in use and hundreds of chemicals are introduced into the market every year, the current pace of screening must
Inter-laboratory validation of the yeast estrogen and androgen screens for identification of endocrine active substances

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Endocrine disruptor compounds (EDCs) are group of natural or synthetic compounds that potentially can 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 potential impact that this interaction could have on human health, there is 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. Herein, we report on the inter-laboratory validation of two robust models that address agonistic and antagonist effect at the human hormone receptor, the Yeast Estrogen and Androgen Screen (YES, YAS, respectively). Both assays are non-animal alternatives to the estrogen/androgen receptor binding assays regulatory accepted. The results from the inter-laboratory validation demonstrate a high reproducibility (>85%) for both methods among the different participating laboratories. Most importantly, the assessment of the predictivity towards in vivo data from the literature shows that the YES and YAS resulted in an accuracy of 84% and 93%, respectively. In conclusion, the methods have been successfully transferred to naïve testing laboratories and they exhibit a high accuracy to identify EDCs that interact with sex-hormone receptors.

Risk-based chemical prioritization and screening for endocrine disruption

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The US Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) uses a two-tiered approach to determine a chemical’s potential to interact with estrogen, androgen, and thyroid (EAT) hormone pathways in humans and/or wildlife. Requirements for testing, data review, and weight of evidence determination require
substantial temporal, financial, animal, and human resources. Currently, more than 10,000 chemicals are in use and 10s of new chemicals are introduced every year, necessitating a more rapid method for screening the “chemical universe”. To address this need, EPA is leveraging in vitro and in silico computational tools to identify potential interactions with EAT pathways. These data will be evaluated in a hypothesis-driven, adverse outcome framework, proposed to the OECD Adverse Outcome Pathway Workgroup as a harmonized conceptual approach. Resulting hazard identification data will be coupled with estimates of physiologically and environmentally relevant exposure data to produce a risk-based method of prioritizing chemicals for EDSP screening. This approach facilitates rapid identification of endocrine disrupting chemicals that are likely to have the greatest potency and pose the greatest risk to human and environmental health.

This abstract does not necessarily reflect EPA policy.

Session II-13: Poster presentations

II-13-128
Performance of the BG1Luc and ER beta-lactamase estrogen receptor transactivation assays in Tox21 compound screening

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The BG1Luc and ER-beta-Lactamase (ER-Bla) estrogen receptor transactivation (ER TA) assays were adapted for use in the U.S. Tox21 program. Each assay was used to screen ~10,000 chemicals for ER agonist and antagonist activity. Concentration-response data (N=15) were analyzed to evaluate assay performance. Data quality was high for both assays as indicated by acceptable signal to background ratio (2.5 to 8), coefficient of variation (<10.5%), reproducibility (outcome mismatches across triplicate runs ≤0.5%), and Z’ factor (≥0.4). Results for both assays were compared to the ICCVAM ER TA performance standards (42 agonist, 25 antagonist compounds) (ICCVAM, 2011). Agonist assay accuracy, sensitivity, and specificity were 97%, 96%, and 100% for BG1Luc assay and 90%, 87%, 100% for ER-Bla assay. Antagonist accuracy, sensitivity, and specificity were 100% for both assays. EC50 reference standard values for estradiol were 30 pM (BG1Luc) and 275 pM (ER-Bla), and IC50 reference standard values for hydroxytamoxifen were 71 nM (BG1Luc) and 6 nM (ER-Bla). Understanding the differences in performance of these assays is critical to their acceptance and utilization by both regulators and industry.

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Reference

II-13-129
Development of reverse toxicokinetic models to correlate in vitro and in vivo estrogen receptor activity

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High-throughput screening (HTS) assays provide an efficient way to identify endocrine-active chemicals. However, nominal in vitro assay concentrations of a chemical may not accurately reflect doses that cause in vivo effects, mostly due to differences in bioavailability and clearance between the two systems. Therefore, we developed reverse toxicokinetic (TK) models to more accurately correlate in vitro concentrations with effective in vivo doses for potential endocrine-active chemicals. Our TK models estimate the daily oral equivalent doses (OEDs) in laboratory animals and humans that would result in steady-state blood concentrations equivalent to the point of departure (POD) values identified from the Tox21 HTS in vitro estrogen receptor transactivation assay, BG1Luc. For most of the chemicals tested, OEDs estimated from POD values are lower than the lowest effective doses for rodent uterotrophic assays, suggesting that BG1Luc HTS provides a more conservative hazard estimate for use in risk assessment. In addition, we performed sensitivity analyses to evaluate the impact of different pharmacokinetic parameters on OED estimation. This modeling approach highlights the importance of PK considerations in ranking endocrine-active chemicals based on in vitro HTS assays.

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II-13-136
Targeted testing of FXR Tox21 active compounds

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The farnesoid-X-receptor (FXR), a nuclear hormone receptor, plays an integral role in bile acid homeostasis. Once considered an orphan receptor with no known endogenous ligand, it was discovered that endogenous bile acids effectively induce FXR activity at physiological concentrations, thus establishing the role of FXR as a bile acid receptor. FXR also regulates lipid and glucose metabolism. To characterize FXR activity, a mammalian one-hybrid assay using a beta-lactamase reporter gene was used to screen the Tox21 10K chemical library. Approximately 1.5% of the chemicals displayed FXR agonist activity, while 2.7% showed some level of antagonism. Twenty-one of these potential FXR-active chemicals, representing the range of agonist and antagonist responses and including established actives and novel compounds, were tested in confirmatory in vitro assays using human or Medaka FXR constructs transfected into CV-1 African green monkey-derived kidney cells. Chemicals were tested at concentrations from 10 nM to 100 mM and 24 hr post-exposure luciferase activity was determined. These in vitro results will be confirmed by monitoring alterations in gene expression and liver histopathology in a Medaka, Oryzias latipes, eluteroembryo model.

An Inter-laboratory validation study of an androgen receptor stably transfected transcriptional activation (AR STTA) assay for a new OECD test guideline


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Phase 2, five coded chemicals were tested for agonistic and antagonistic effects in order to evaluate inter-laboratory reproducibility and accuracy of the protocols. In the agonist assay, qualitative and quantitative results were reproducible in all laboratories. In the antagonist assay, an inconsistent result of one chemical was reported from one laboratory due to a difference in the maximum concentration tested, determined from solubility tests. The results indicated that the AR STTA assay is robust and reproducible both qualitatively as well as quantitatively within and between laboratories, making it appropriate for an OECD test guideline. However, it is necessary to state how to determine the maximum concentration, more clearly in the protocol.

New biomarkers for endocrine disruption evaluation on microplate using a human placental cell line

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Endocrine disruptors (ED) are omnipresent and can have adverse effects on human health. Some plasticizers, such as mono(2-ethylhexyl) phthalate (MEHP) and bisphenol A (BPA), are ED but little is known about new plasticizers. There are few in vitro models to study endocrine disruption, so our aim is to develop a simple and fast cell model with known EDs to screen new plasticizers.

MEHP and BPA were incubated on the human placental cell line for 72 h. P2X7 cell receptor activation was assessed performing the cytofluorometric YO-PRO-1 assay, and the release of two placentals hormones, the human placental lactogen (hPL) and the human chorionic gonadotropin (HCG), was determined by ELISA.

After 72 h MEHP and BPA induced no loss of cell viability and both activated P2X7 receptor. Furthermore, any of them altered HCG level but both increased hPL release. As expected, paracetamol, which is not an ED, had no effect on the release of these hormones.

Human placental cell line secretes placental hormones and high-lights endocrine disruption induced by plasticizers after a short time exposure and P2X7 activation. Therefore, our in vitro cell model seems to reveal chronic endocrine toxicity and could be proposed in a regulatory study to evaluate new plasticizers or ED.

Estimation of estrogen receptor binding affinity by QSAR approach

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Chemicals may interact with proteins such as the estrogen receptor (ER) initiating a cascade of biological effects and perturbing the endogenous hormone system. Despite the complexity of the endpoints for reproductive impairment, it has been long appreciated that chemical binding to the ER is one of the significant mechanisms interfering with process of reproduction. Since testing of reproductive toxicity in vivo is very expensive, alternatives are being developed.
The ER is nonspecific enough to permit binding with a diverse array of chemical structures. There are three primary ER binding subpockets, each with different requirements for hydrogen bonding. Steroidal compounds usually interact at two points within the ER using two hydrogen-bonding groups. However, there are also chemicals with one hydrogen-bonding group that bind to the ER and cause subsequent gene activation.

The aim of the work is to characterize the groups of chemicals with potential to bind to ER and cause adverse effect in the organism. Over fifty compounds used in food contact materials were analyzed using QSAR and computational chemistry.

**References**


**Animal-free toxicology: the use of human tissue to replace the use of animals – examples from human biomonitoring and human placental transport studies**

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Human data on exposure and adverse effects are the most appropriate for human risk assessment, and modern toxicology focuses on human pathway analysis and the development of human biomarkers. Human biomonitoring and human placental transport studies provide necessary information for human risk assessment, in accordance with various chemical, medicine and food safety legislation. Placental transport data in three types of test system, i.e., in vivo in humans around birth, ex vivo by placental transfer studies, and in vitro by BeWo cell studies can be compared. By combining data from actual measurements of concentrations in umbilical cord blood and maternal blood at the time of birth with the experimental data from our test systems, we will be able to rank and compare the transport of different classes of substances. Toxicology studies based on human mechanistic and exposure information can replace animal studies. These animal-free approaches can be further supplemented by new in silico methods and chemical structure-activity relationships. The inclusion of replacement expertise in the international Three Rs centres, the ongoing exploration of alternatives to animal research, and the improvement of conditions for research animals, all imply the beginning of a paradigm shift in toxicology research toward the use of human data.

**Embryonic stem cell test (EST) is one of the most promising tests for embryotoxicity, which takes advantage of mouse embryonic stem (ES) cell differentiation into cardiomyocytes (Spielmann et al., 1997). We have previously reported that Hand1 (heart and neural crest derivatives expressed transcript 1) gene was a quantitative molecular endpoint for predicting embryotoxicity during cardiomyocyte differentiation of ES cells (Suzuki et al., 2011a), and a novel short term test was developed using an engineered ES cell line for detecting expression of Hand1 gene with luminescence analysis (Suzuki et al., 2011b). Predictive power and validity were evaluated by analysis of well-known test chemicals and the test offered high predictability and accuracy with a reduced test duration and manpower compared to the original EST. Now, we are carrying out some modifications of the test and we also developed new engineered ES cell lines to analyze embryotoxicity during ES cell differentiation into neural cells.

Here we introduce the progress of our approaches to developing novel, convenient and sensitive in vitro tests for embryotoxicity using engineered ES cell lines with luminescence analysis.

This study is supported by a research grant from the Ministry of Economy, Trade and Industry (METI) of Japan.

**An exposure-based validation list for developmental toxicity assays**

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Validation of alternative assays requires comparison of the responses to toxicants in the alternative with in vivo responses. Chemicals have
been classified as “positive” or “negative” in vivo, despite the fact that developmental toxicity is conditional on magnitude of exposure. We have developed a list of positive and negative developmental exposures based on maternal plasma Cmax, selected based on mode of action and relevance to in vitro dosing paradigms. We selected a series of 20 chemicals that caused developmental toxicity in rats and had toxicokinetic data. Where possible, we used the same chemical for both positive and negative exposures, the positive being the Cmax at a dose level that produced significant teratogenicity or embryolethality, the negative being the Cmax at a dose level not causing developmental toxicity. It was not possible to find toxicokinetic data at the no-effect level for all positive compounds, so the negative exposure list contains Cmax values for some compounds that do not have developmental toxicity up to the dose level tested. Assay performance should be evaluated against biological domain of assays. This exposure-based reference list represents a fundamentally different approach to the evaluation of alternatives and is a step towards their application in quantitative risk assessment.

II-14-656

**Evaluations of in vitro embryotoxicity tests for Chinese herbal medicines**

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**Background:** Chinese herbal medicines like other pharmaceuticals may do harm to pregnancy, embryo-fetal development and prenatal and postnatal growth. In this study, we evaluated in vitro embryotoxicity test methods to assess the developmental toxicity of Chinese herbal medicines.

**Methods:** 14 Chinese herbal medicines with known in vivo developmental toxicity were selected. Three validated in vitro embryotoxicity tests, embryonic stem cell culture (EST), micromass culture (MM) and whole embryo culture (WEC), were conducted. Embryotoxic potentials of the Chinese herbal medicines were classified by corresponding linear discriminated prediction models (PM). Accuracy, predictivity and precision and of the in vitro embryotoxicity tests for Chinese herbal medicines were compared.

**Results:** Most strongly embryotoxic medicines were accurately classified by MM and WEC PM2, while all non-embryotoxic medicines were accurately classified by EST, MM and WEC PM2. Compared with pharmaceuticals, accuracy of MM and WEC PM2 to predict the embryotoxic potential of Chinese herbal medicines were compatible (70% and 80% both), but accuracy of EST and WEC PM1 were lower (78% vs 70% and 68% vs 40%). EST and WEC PM1 have 100% predictivity while WEC PM2 has 100% precision for strongly embryotoxic medicines. WEC PM1 has 100% precision for weakly embryotoxic medicines. EST and MM have 100% precision while WEC PM2 has 100% predictivity for non-embryotoxic medicines.

**Conclusions:** MM and WEC PM2 tests are superior to EST and WEC PM1 tests in identifying embryotoxic potential of Chinese herbal medicines. Further optimization and specific prediction models may require to assess embryotoxic potential of Chinese herbal medicines.

**Session II-14:** Poster presentations

II-14-038

**In vitro cytotoxicity assessment of a trichotene mycotoxin T-2 toxin on SerW3 cells**

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**Aims:** T-2 toxin is produced by Fusarium species on grains and grain products is known to cause decreases in sperm number, however the exact mechanism is not known (Agag, 2005; Yang et al., 2010). The purpose of the study is evaluating the effects of T-2 toxin on Sertoli cells supporting the developing germ cells mechanically.

**Methods:** SerW3 cells, which is a rat Sertoli cell line, were exposed to T-2 toxin at doses of 0.025, 0.25 and 2.5 nM for 24 and 48 hours. Cytotoxicity of T-2 toxin was evaluated by trypan blue staining, MTT test and measurement of lactate dehydrogenase activity (LDH) in the cells.

**Results:** Cytotoxicity of SerW3 cells increased in dose and time dependent manner according to trypan blue staining, MTT and LDH assays. The sensitive assay seemed to be LDH among other assays in response to T-2 toxin.

**Conclusion:** T-2 toxin was cytotoxic in SerW3 cells at nanomolar concentrations and SerW3 cells were sensitive to T-2 toxin. This study is crucial because of being the first study evaluating the cytotoxicity of T-2 toxin on SerW3 cells.

**References**


II-14-164

**Development of alternative tests for reproductive and developmental toxicity: whole embryo culture in humanized CYP3A (CYP3A-HAC) mice**

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**Purpose:** Mice and rats are insusceptible to teratogenic effects of thalidomide. However, species differences in the teratogenic mechanism

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of action are not completely understood. This study explored species differences between human CYP3A and mouse Cyp3a by using a whole-embryo culture system in which thalidomide was added to a culture medium containing foetuses with humanized CYP3A.

Methods: Foetuses from CYP3A-human artificial chromosome (HAC) mice, containing the entire human genomic CYP3A locus in which the endogenous mouse Cyp3a genes were deleted, were used. The foetuses (E11.5) were cultured for 24 h in a 100% rat serum medium, to which thalidomide was added.

Results and Discussion: In E11.5, cultured mouse foetuses, there were no differences in crown-rump length, or the total number of somites or protein content, between the CYP3A-HAC-negative and CYP3A-HAC-positive groups. Interestingly, 42.9% (9/21) of the cultured CYP3A-HAC-positive foetuses subjected to thalidomide showed limb abnormalities.

Conclusions: These results provide the first evidence that thalidomide may induce limb abnormalities in CYP3A-HAC mouse foetuses, and that the human CYP3A enzyme may contribute to teratogenicity. These findings suggest that compared to the existing embryo culture methods, those combining a metabolic system may more accurately predict abnormalities due to a chemical and its metabolites.

### II-14-215

**Acrylamide and its metabolite glycidamide can effect antioxidant system and steroidogenesis in Leydig cell in vitro**

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**Aims:** Almost everyone is daily exposed to multiple toxic substances originated from food and environment sources. Acrylamide is formed in vegetable food materials having high-carbohydrate, low-protein foods that have been exposed to high temperatures (Stadler et al., 2002). Glycidamide, much more reactive chemicals than acrylamide, is formed as a result of intracellular metabolism of acrylamide (Pruser and Flynn, 2011). Increasing evidence demonstrated that acrylamide intake from foods has genotoxic, neurotoxic, carcinogenic, and mutagenic effects (Friedmann, 2003). In addition, it has been shown that acrylamide and its metabolite glycidamide can cause infertility due to its toxic effect on the male reproductive system (Yang et al., 2005). The purpose of present study was to investigate the effects of acrylamide and glycidamide on TM3 Leydig cell antioxidant system and steroidogenic enzymes.

**Methods:** TM3 Leydig cells were exposed to acrylamide (10 µM and 1 mM) and glycidamide (1 µM and 0.5 µM) for 24 hours. Following the exposure time, the Leydig cells were evaluated for measurement of antioxidant enzymes, cellular antioxidant and steroidogenic enzymes (3β-HSD and 17β-HSD).

**Results:** The results showed that Leydig cell antioxidant system and steroidogenesis were interrupted in a dose dependent manner.

**Conclusion:** Results suggest that acrylamide consumption may induce impairments on testicular steroidogenesis and antioxidant system in male.

**References**


### II-14-216

**Effects of acrylamide and glycidamide on antioxidant systems and steroidogenic enzymes on TM4 Sertoli cell**

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**Aims:** Acrylamide, which is commonly used in the industrial sector, has been found that is formed in the food products which have been cooked in high temperatures (Stadler et al., 2002). Glycidamide, the ultimate genotoxic metabolite of acrylamide, is generated by CYP4502E1-mediated epoxidation (Sumner et al., 1999). In previous studies about developmental toxicity of acrylamide on reproductive systems, increasing exposure of acrylamide and its metabolite glycidamide on daily basis might have a potential negative impact on male fertility, including spermatogenesis and sperm fertilizing ability (Friedmann, 2003; Wang et al., 2007). The present study was aimed at determining the direct effects of acrylamide and glycidamide on Sertoli cell enzymes involved in steroidogenesis and the antioxidant system in vitro.

**Methods:** In this study, acrylamide (10 µM and 1 mM) and glycidamide (1 µM and 0.5 µM) was exposed to TM4 Sertoli cells for 24 hours. After incubation, the treated cells were used for measurement of antioxidant enzymes, cellular antioxidant and steroidogenic enzymes.

**Results and Conclusion:** The results showed that the activities of steroidogenic enzymes, enzymatic and non-enzymatic antioxidants were significantly diminished in a dose-dependent manner. These findings suggest that direct exposure to acrylamide and glycidamide could induce cytotoxicity and resulting in disruption of antioxidant system and steroidogenesis in Sertoli cells.

**References**


Methods: TM3 Leydig and TM4 Sertoli cells were exposed to acrylamide (10 μM and 1 mM) and glycidamide (1 μM and 0.5 μM) for 24 hours. Following the exposure time, the Leydig and Sertoli cells were evaluated for measurement of cell viability assay, lipid peroxidation assay and propidium iodide-Hoechst stain.

Results: The results showed that Leydig and Sertoli cell viability were reduced whereas; Leydig and Sertoli cell oxidative damage and apoptosis rate were induced in acrylamide and its metabolite glycidamide treatment in a dose dependent manner.

Conclusion: These findings suggest that acrylamide and glycidamide may result in detrimental effects in Sertoli and Leydig cells.

References


II-14-262
Development of in vitro detecting method for developmental toxicity using mouse embryonic stem cells – Hand1-Luc Embryonic Stem cell Test

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Embryonic stem cell test (EST) is a validated in vitro method for prediction embryotoxicity potential of chemicals with inhibition of the differentiation of mouse ES cells into contracting cardiomyocytes (Spielmann et al., 1997). We have previously reported that heart and neural crest derivatives expressed transcript 1 (Hand1) was quantitative and objective molecular endpoint for predicting embryotoxicity, detected at day 6 when ES cells differentiate into cardiomyocytes (Suzuki et al., 2011a) and also reported the establishment of 96 well multi-plate based new EST (Hand1-EST) with luciferase reporter assays using transgenic ES cells (Suzuki et al., 2011b). Extensive investigations were performed to explore predictive power and validity by comparing a set of well-known test chemicals. The Hand1-EST offers high predictability and accuracy with a reduced test duration and manpower compared to the EST. We recognized this test as useful however it would not approved as the test guideline due to lack of evaluation for metabolism and placental transfer of test chemicals in the EST. Therefore, modified test methods named Hand1-Luc EST were currently under development. We are planning to present overview of the method and progress of validation study for the Hand1-Luc EST.

This study was supported by a research grant from the Ministry of Economy, Trade and Industry (METI) of Japan.

References


II-14-357
Express assessment of geno-, cyto- and embryotoxic effects of potential teratogens using an integrated in vitro test-system based on rat whole embryo culture

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The key feature of the proposed approach to assessing the risk of potential teratogens is the possibility of performing a series of tests on the same biomaterial in a relatively short time. To examine the embryotoxicity of tested substances we directly used rat whole embryo culture, one of the three embryotoxicity tests approved by the European Centre for the Validation of Alternative Methods (Piersma et al., 2004). Concurrent use of extraembryonic material (yolk sac endoderm) having high proliferative activity provides an opportunity to complement the basic research with the whole battery of genotoxicity tests (sister chromatid exchange test, micronucleus test, DNA comet assay) and a cytotoxicity test (determination of nuclear division index and cell death rate).

Prospects of the integrated test system:
1. Possibility of adapting to various methods of exposure (direct and indirect administration, introduction of pre-biotransformation);
2. Possibility of adding new tests (related to enzyme activity and inflammatory response to xenobiotics, carcinogenicity assessment);
3. Ability to enhance predictive power of the system by complementing it with analysis of gene expression patterns in exposed embryos (our recent data on 5-fluorouracil and silica nanoparticles is provided).

Reference

II-14-513
Toward validation of a human in vitro assay for developmental toxicity assessment

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Innovative in vitro toxicity screening assays aimed at reducing or replacing the use of animal models are required for the REACH initiative (Europe) and Tox21 initiative (US) to evaluate thousands of chemicals for safety. We have created a predictive, in vitro pluripotent stem cell based developmental toxicity assay that can reduce costs, animal and compound use, and can increase pharmaceutical and chemical safety. The goal of the current study was to assess the assay’s biomarkers of teratogenicity potential across a broader range of chemicals and migrate the method from a high resolution liquid chromatography-mass spectrometry (HR-LC/MS) based analysis to a simpler platform. Induced pluripotent stem (iPS) cells were exposed to 8 concentrations of 71 pharmaceutical, environmental and industrial compounds that have been associated with developmental toxicity or considered free of developmental toxicity. Spent media was collected and analyzed by HR-
LC/MS for biomarker discovery and confirmation. The assay was then migrated to a triple quadrupole (QQQ)-LC/MS platform, enabling targeted biomarker analysis and providing simpler quantification during the assay pre-validation process. The results from initial comparisons of the two LC/MS platforms are highly correlated indicating similar assay performance across a broad series of chemical compounds tested.

Using cellular signals to assess developmental toxicity of compounds in a mouse embryonic stem cell system

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Developmental toxicity testing is an important issue in chemical hazard and risk assessment and large numbers of animals are used in this area. In order to reduce animal testing, alternative in vitro methods are required.

The validated embryonic stem cell test (EST) has potential for developmental toxicity testing but its end point is subjective. Currently, great effort is put into improving the predictive capacity of the EST by increasing background knowledge on biological mechanisms represented in this system.

Toxicogenomics studies in the cardiac and neural EST were performed (Pennings et al., 2011). These studies have shown that mRNA expression of a set of genes can be used to identify embryotoxic compounds. These genes have been shown to play a role in various developmental processes.

Ten of the most predictive genes for developmental toxicity in the cardiac EST were selected from earlier work. Here we show the differential expression of several of the key proteins related to these genes (Neuropilin-1, Foxc1, Mesp-1), upon exposure of embryonic stem cells to embryotoxic compounds (5-fluorouracil, retinoic acid, methotrexate and valproic acid), during early differentiation. Identifying these key proteins will contribute to a more reliable and informative in vitro test system to evaluate this important toxicological end-point.

Reference

Is it possible to predict the exposure of human fetus on the basis of experimental studies?

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Exposure of human fetus to xenobiotics occurs through the early developing placenta. Considerations concerning transplacental transfer are chemical characteristics of the compound, stage of placental development, species differences of placenta, and characteristics of placental transporter proteins. Although in vivo rodent models are useful in studies for fetotoxicity (Anderson, 2004), the fact that placenta is the most variant organ between species should not be overlooked. It is thus feasible that models of human origin have been preferred in placental studies (Myllynen and Vähäkangas, 2013). Human trophoblastic cells or villous explants can be cultured and human placental tissue is more feasible that models of human origin have been preferred in placental studies (Myllynen and Vähäkangas, 2013).

The limitations include availability of placentas, the fact that placenta is the most variant organ between species should not be overlooked. It is thus feasible that models of human origin have been preferred in placental studies (Myllynen and Vähäkangas, 2013). Human trophoblastic cells or villous explants can be cultured and human placental tissue is more feasible than rodent models of development in rodent models of development. Exposure of human fetus to xenobiotics occurs through the early developing placenta.

References
Session II-15: Genotoxicity / Carcinogenicity

Co-chairs
Ralf Fautz, KAO Europe, Germany
David Kirkland, Kirkland Consulting, UK

Session II-15: Oral presentations

II-15-050

Mammalian cell results with Ames-positive chemicals – correlations with presence or absence of in vivo genotoxic or carcinogenic activity

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Positive results in the Ames test tend to correlate well with carcinogenic potential in rodents. However, situations can be envisaged where this may not be the case. Since most chemicals are also tested in mammalian cells, the pattern of mammalian cell results (positive, negative) may be informative regarding potential for in vivo genotoxic or carcinogenic activity. A database of >750 Ames-positive chemicals with in vivo test results has been compiled from multiple sources, and the results in mammalian cell tests for gene mutation and clastogenicity/aneugenicity have been analysed. Ames-positive carcinogens and in vivo genotoxins gave significantly more (85%) positive results in both mammalian cell tests, but this should not be considered definitive since large numbers of chemicals that were negative in vivo (>47%) also gave the same pattern of results. By contrast, negative results in both mammalian cell tests were significantly more frequent for Ames-positive chemicals that were not carcinogenic or genotoxic in vivo, yet such results with carcinogens and in vivo genotoxins were rare. Thus, with an Ames-positive chemical, negative results in 2 mammalian cell tests covering both mutation and clastogenicity/aneugenicity endpoints should be considered an indicator of the absence of in vivo genotoxic or carcinogenic potential.

II-15-063

ToxTracker: a cell-based reporter assay that provides mechanistic insight into the genotoxic properties of chemicals

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The ever-increasing number of chemical compounds that are developed by industry pose a potential threat for human health. These compounds may react with various biomolecules and will activate specialised defence mechanisms that provide protection against the toxic, mutagenic and possibly oncogenic consequences of exposure. Monitoring activation of these specific cellular signalling pathways may therefore allow mechanism-based assessment of their potential (geno)toxic properties (Hendriks et al., 2013). We therefore developed the ToxTracker assay, a panel of mouse embryonic stem (mES) cells that contain different GFP reporter genes (Hendriks et al., 2012). These biomarker genes were identified by extensive whole-genome transcription profiling. The differential responsiveness of the various GFP reporters enables disclosure of the primary reactivity of chemicals. Genotoxic potential of compounds is assessed by reporters that reflect activation of the ATR and NF-kB associated signalling pathways. Oxidative stress-associated (geno)toxicity is detected by Nrf2-dependent and -independent reporters and protein reactivity of chemicals is determined by activation of the unfolded protein response. A p53-associated reporter monitors a broad spectrum of (geno)toxicity responses. ToxTracker is extensively validated using different reference compound libraries. The integrative approach of ToxTracker provides a powerful tool for identification of potentially carcinogenic properties of chemicals by unveiling the cellular signalling pathways that are activated upon exposure.

References

II-15-224 *

Alternative solutions for misleading positive mutagenicity/genotoxicity results of cosmetic substances?

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Mutagenicity/genotoxicity testing is an important endpoint in the safety assessment of cosmetic substances. Although a battery of 3 in vitro tests is currently used for cosmetic substances, its specificity is known to be low and follow-up in vivo tests to overrule a positive outcome are normally performed. This is not achievable anymore for cosmetic substances due to the animal testing and marketing bans in Europe. Consequently, many potentially safe compounds could be lost. Our previous work showed that strategies such as reduction of the number of tests in the standard test battery from 3 to 2 and an optimization of the existing in vitro tests (cell type used, top concentration applied, etc.) might not solve the problem for cosmetic Annex substances. In this study, by using the misleading positive genotoxicity data present in the SCCS opinions, we show that incorporation of transcriptomics
data and QSAR-modeling for mutagenicity could be a possible solution. As such these represent valuable tools in a weight-of-evidence approach to derisk positive in vitro results.

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II-15-237

Cell transformation tests: current status of the SHE and Bhas 42 test system

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The prediction and assessment of carcinogenicity of chemical compounds is an essential step in their development. The 2-year bioassay is the standard method for carcinogen detection which is time and resource intensive. Many short-term test, especially genotoxicity tests have been developed to aid in identification of potential carcinogens. However, the endpoint of these systems is genotoxicity and their concordance between rodent bioassays is only about 60% and a battery of short-term genotoxicity tests can not improve the overall concordance. The presentation will show how results from cell transformation assays can be used in providing information, which in combination with data from other testing methods, are useful for identifying the carcinogenic potential of chemical compounds. The results of these assays in combination with other information such as genotoxicity data, structure activity analysis, in vivo toxicity data and pharmaco-/toxicokinetic information can facilitate a relatively comprehensive assessment of a carcinogenic potential of a chemical. The presentation will include practical examples on how to use these assays for hazard and safety assessment and will additionally give information on the current standings of the OECD guidelines for both, the SHE and Bhas 42 cell transformation assay.

II-15-405

Impact of OECD guideline revisions and availability of reconstructed human skin-based methods on animal use in genotoxicity testing

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Reduction/replacement of animal tests is a long process that involves many players and activities. Two examples from the area of genetic toxicology will be discussed: 1) key changes related to the revision of OECD genotoxicity guidelines and 2) the use of new, 3-dimensional human skin tissue-based genotoxicity assays.

The OECD genotoxicity testing guidelines are currently being revised for all in vivo assays and mammalian cell-based in vitro assays are that are still in regular use. Changes related to the revision of the guidelines will be discussed with an emphasis on impact on animal use. For example, improving the predictivity of the initial in vitro testing battery should help reduce the need of in vivo follow-up testing. Tightening the criteria for the in vivo assays in terms of suitability, e.g., increasing the statistical power, or making sure that an assay is done only if there is target organ exposure, will help eliminate unnecessary studies/repetition. Where follow-up is still needed it is now possible, for dermally exposed chemicals (e.g., cosmetic ingredients), to utilize assays that are based on 3-dimensional human skin models. Skin-based micronucleus and Comet assays are currently being validated and show promise as a direct replacement for in vivo studies.
To assess the genotoxic effects of cosmetic ingredients it is well-established the use of the OECD 487 (OECD, 2010) micronucleous (MN) in vitro assay. This test requires greater time and highly skilled technician; therefore it is necessary to search for alternative methods. The goal of this study was to evaluate the genotoxic potential of Minthostachys setosa (Ms), Pimenta pseudocaryophyllus (Pp) and Drimys brasiliensis (Db) essential oils comparing the classical method to cytometer method. The automated assay was conducted in a similar way to the standard manual in vitro MN test, with the main difference being the scoring of the cells (Diaz et al., 2007). The essential oils were extracted by hydrodistillation and its dispersion was evaluated in culture medium. The test was evaluated using one concentration of each oil, S9 metabolic system and clastogenic and aneugenic controls. The flow cytometer method was adapted from Bryce et al. (Bryce et al., 2007) procedure that incorporates an ethidium monoaizide bromide staining step in order to label the chromatin of necrotic and mid/late stage apoptotic cells. The results obtained in both micronucleus tests using the CHO-K1 cell line were similar and both showed no genotoxic potential for Ms, Pp and Db essential oils.

References

II-15-287 *  
Hydra as a small animal model for environmental toxicity testing: standardization of comet assay  
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Although in vitro cell culture-based assays have become viable approach for risk assessment of synthetic chemicals, they are not much impressive with nanoparticles and, hence, as of now, animal testing becomes unavoidable. There are a few suggestions that animals belonging to the lower level of taxonomic hierarchy may be developed as model organisms for in vivo toxicity testing. One such organism is hydra, which offers tremendous advantage in view of its capacity for regeneration and stemness. Although, a few results have been generated for the toxicity of nanoparticles in hydra, there are no reports pertaining to genotoxic effect of nanoparticles. For the first time, we report the genotoxicity assessment of nanoparticles in hydra adopting comet assay. Comet assay is one of the simple, sensitive and widely accepted methods for assessing the DNA damage. Hydras were exposed to zinc oxide nanoparticles at a concentration of 6 mg l-1 in hydra medium (72 hr LC50 value) for 72 hr to assess the DNA damage at whole animal level. The results suggest that hydra can be utilized as a model to screen nanoparticles for genotoxic effects and this method has potential to be validated for regulatory purposes.
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II-15-320  
The use of SHE cell transformation assay to predict the transforming potential of non genotoxic carcinogens  
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Cell Transformation Assay (CTA) is regarded as a useful tool to highlight the role of chemicals in the carcinogenesis process on the basis of their transforming potential. Syrian Hamster Embryo cells CTA model is currently being used to screen chemicals for their transforming properties. The test is usually performed at pH 6.7 and/or pH 7.0. As it was previously demonstrated, SHE CTA shows a significant level of concordance with carcinogenicity animal bioassay. Besides the ability to screen genotoxic carcinogens, SHE CTA is considered a promising tool to identify non-genotoxic carcinogens.

In the context of the OECD process to develop the test guideline, we analyzed all available information of the genotoxic activity of organic compounds, which had been tested in the SHE CTA. A total of 16 compounds, which had shown transforming properties in the SHE CTA, were classified as not genotoxic. Among them, 11 compounds had been tested at pH 6.7, 2 compounds at pH 7.0, 3 compounds at both pHs. For these compounds the mechanisms of action leading to the carcinogenesis effects in in vitro studies were also considered. All compounds were found to be promoters in the carcinogenesis process. Interestingly, most of them act as endocrine disruptors.

II-15-463  
A new resource for predictive toxicology: EURL ECVAM genotoxicity and carcinogenicity database of positive Ames test chemicals  
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In the recently published EURL ECVAM strategy aimed at avoiding and reducing animal use in genotoxicity testing, the enhancement of
Development of a method to compare genotoxic hazard of water samples

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Various hazardous compounds in water may cause long-term health effects. Measured effects are the result of a mixed toxicity of single chemicals and their degraded products, which can have different biological potency and bioavailability. The aim of our study was to evaluate if the yes/no answer from AmesII bioassay could be expressed more quantitatively. We used 4-NQO to assess the mutation equivalents of one water sample treated without and with ultraviolet light, and concentrated 2,000 to 30,000 times by SPE. The results, expressed as the number of positive wells, were translated to concentrations of 4-NQO, by inversely using a regression model for the dose-response relation. Based on the induction factor, the water sample before UV-treatment was less mutagenic than the one after UV-treatment. In this study we report an estimate of the equivalent 4-NQO concentration and the corresponding upper and lower limits of the 95% confidence interval that takes into account the various uncertainties. Considering the Threshold of Toxicological Concern (TTC) of 4-NQO, our results suggest that extracts of water sample before UV treatment are not genotoxic. Furthermore taking into consideration the various uncertainties, it cannot be excluded that after UV-treatment the extracts are genotoxic.

In vitro cytotoxicity and genotoxicity testing using upcyte hepatocytes

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Novelty: We have developed a technique which causes primary human hepatocytes to proliferate whilst retaining an adult phenotype.

Micronucleus assay: We optimized the assay conditions incorporating upcyte hepatocytes into the micronucleus test. A treatment duration of 96h was optimal for detecting the genotoxicity of the direct-acting, mitomycinC, and the bioactivated compound, cyclophosphamide, whilst negative and “false” positive compounds were correctly identified as negative. The basal MN rate was affected by pre-culture period and medium components. The %MN in control and genotoxin-treated upcyte hepatocytes was similar at different growth stages.

Cytotoxicity assay: The cytotoxicity of 31 compounds was measured using ATP, LDH content and MTS metabolism in upcyte hepatocytes from four donors. The cytotoxicity of the majority of compounds was donor-dependent. There was a good intra- and inter-experimental reproducibility and the predictive capacity of the assay was good such that known non-hepatotoxics were clearly negative and compounds that were associated with hepatotoxicity caused cytoxicity.

In conclusion, these data support the use of upcyte hepatocytes in the MN test, especially since these cells combine proliferation with a sufficient metabolic capacity. Our data also show that upcyte hepato-

References
cytotoxicity screening – combining predictively with a substantial cell source.

II-15-702

In vitro immunotoxicity screening of xenobiotics using human lymphoblastoid cell lines

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Finding the alternative methods to in vivo animal studies is highly desirable and much has been achieved in the field of 3Rs. However, there is still no validated in vitro method to assess the immunotoxicity of xenobiotics. Moreover, evaluation of immunotoxicity might be of great value in the screening for non-genotoxic mechanisms of carcinogenicity. The aim of our study was to evaluate human lymphoblastoid cell lines (LCLs) as a potential in vitro tool for screening of immunomodulatory properties of xenobiotics. The LCLs were generated from peripheral blood B lymphocytes obtained from unrelated healthy individuals. The in vitro test system evaluates cell viability and alteration of cytokine release upon exposure to xenobiotic. Treating LCLs with immunosuppressive compounds resulted in reduced viability. Since cytokine production reflects lymphocytes’ responses to external stimuli, we have evaluated functional responses of LCLs by monitoring their cytokine production. When compared to untreated ionomycin/PMA stimulated cells, a decrease of production of pro-inflammatory cytokines was observed. B cell lines showed no effect on cytokine release. In conclusion, in vitro screening for immunotoxicity of xenobiotics can be performed by using human LCLs.

II-15-767

Investigation of the genotoxic potential of a new Brazilian botanical extract – a case study

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A new botanical extract, obtained from the Brazilian plant Casearia silvestris was developed with the purpose of application in cosmetics. Following a standard approach for development of new cosmetic ingredients, the safety assessment was performed through a huge chemical characterization and a battery of in vitro tests. The aim of this study is to present the strategy defined to investigate an unexpected positive result obtained in mammalian cell in vitro micronucleus test – OECD Test Guideline 487 – during the safety evaluation of the extract. As many other in vitro tests performed in monolayer cell cultures, the micronucleus test is criticized by its high rate of “misleading” positive results. In the present case, the first step in the toxicological investigation was a critical assessment of chemical composition of the plant extract. The most important finding was that the silica, which was present in the extract in concentration of 20%, could cause chromosomal aberrations and/or aneuploidies in V79 cells. After testing the aqueous extract without silica and the silica alone, the hypothesis of interference and misleading positive result was confirmed. This case provides an important learning about the interpretation of in vitro tests results and decision making process in safety assessment.
Session II-16a: Inhalation toxicology – Nano

Co-chairs
Marianne Geiser, University of Bern, Switzerland
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Session II-16a: Oral presentations

II-16a-054

Acute lung toxicity of nanofilm spray product – in vitro versus in vivo correlation
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Nanofilm spray products (NFPs) have caused several incidences of pulmonary injury in consumers. The mechanism of toxicity is not fully known, but we believe that pulmonary surfactant is an important target. The aim of this study was to validate capillary surfactometry (CS), an in vitro method simulating surfactant function in the bronchioles, as a tool for screening of NFP toxicity. Commercially available water soluble products were tested both in the CS and in a mouse bioassay to validate the in vitro testing. The main constituents of the products, the film-forming substance and the solvent, were chemically characterised. The products were tested in vitro to determine the dose-response relationship for surfactant inhibition. To mimic relevant human exposure, we used an acute mouse inhalation model to evaluate the impact of the products on the airway. Disruption of surfactant function in vivo leads to atelectasis and reduced lung function. We compared the effect of ten NFPs both in the in vitro and the in vivo assay. We found that the in vitro assay could predict the in vivo effect very well. The in vitro assay can be used to reduce and refine the need for toxicological testing of NFPs in vivo.

II-16a-094

Nano aerosol chamber for realistic in vitro toxicity studies – NACIVT
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The lungs bear the main burden of occupational, public and ambient exposure to (nano)particles. Inhalation of nanoparticles in industrial processes and consumer products poses an unknown risk (Woodrow Wilson database). Individuals with chronic lung diseases, like cystic fibrosis (CF) are expected to be more vulnerable than normal subjects (Goss et al., 2004). The rapid increase of nano-products urgently requires adequate in vitro test systems for toxicity assessments.

We developed the Nano Aerosol Chamber for In vitro Toxicity (NACIVT) for realistic nanoparticle deposition simultaneously on 24 cell cultures by electrostatic precipitation under controlled conditions (http://www.nacivt.ch) (Jeannet et al., 2014). Air-liquid interface (ALI) cultures of normal and CF human bronchial epithelia (HBE) mimic the target tissue (Fulcher et al., 2005). Acute toxicity resulting from exposure to silver and carbon nanoaerosols was assessed.

NACIVT allows efficient, uniform particle deposition, relevant target-tissue doses, and biocompatibility with short and long-term cell exposures. Silver and carbon nanoparticles had similar effects. Necrosis was significantly higher in CF than normal HBE. Caspase-3 and interleukin-6 were higher in CF than normal HBE, before and after aerosol exposure.

Our data show that NACIVT combined with ALI cell cultures provides an in vitro system for realistic nanoparticle safety testing. Moreover, there is evidence for different responses of CF and normal airways to nanoaerosol exposure.

References

II-16a-429

Next-level in vitro testing strategy to study the effects of carbon nanotube aerosols
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Carbon nanotubes (CNTs) represent one of the most promising nanomaterials, due to their unique properties. During their production, human exposure to CNTs may occur via inhalation. Despite increased investigation into the pulmonary toxicity of CNTs, the impact of CNT exposure upon human lung remains unclear. Therefore, the aim of this study was to mimic inhalation of multi-walled CNTs (MWCNTs) in vitro as realistic as possible by producing MWCNTs aerosols via an Air-Liquid Interface Cell Exposure System (ALICE) in combination with a 3D epithelial airway barrier model of the human lung, cultivated at the air-liquid interface. Single exposure of MWCNTs at deposited concentrations (0.14, 0.20 and 0.39 µg/cm²) did not influence cell viability or morphology over 24hrs. No effect was observed in the release of pro-inflammatory mediators (TNF-α and IL-8) as well as in the intracellular antioxidant glutathione compared to the positive
particle control, DQ12 quartz, which induced a significant pro-inflammatory response. In conclusion, it was possible to realistically mimic the inhalation of MWCNTs by using a combination of an advanced in vitro lung cell model and an air-liquid exposure system. This setup is now also being used to assess the effects of CNTs in a repeated exposure environment.

II-16a-439

Pulmonary toxicity of nanomaterials: a critical review of in vitro and in vivo studies

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Based upon a literature review (Landsiedel et al., 2009) comparing in vitro and in vivo studies on pulmonary effects of metal and metal oxide nanomaterials and multiwalled carbon nanotubes, cellular mechanisms of nanomaterial toxicity are presented. No “nanospecific” toxic effects, attributable to particle size alone, have ever been recorded, but the particle’s biological activity may increase with decreasing size. The in vitro studies encompass a multitude of cell culture conditions, exposure durations, and endpoint detection methods with nanomaterial concentrations ranging from few µg/ml to several mg/ml. Often times, the effective dose, the particle mass reaching the cultured cells, was not addressed (Oberdörster, 2009), and in vitro doses were rarely correlated to lung burdens or aerosol concentrations in inhalation studies. Most frequently, inflammatory and/or cytotoxic reactions were observed in vitro, however at much higher concentrations than can be expected from in vivo exposure (Landsiedel et al., 2014). Scientific shortages are addressed that should be followed up to enable predicting in vivo effects from in vitro data (Donaldson and Poland, 2013). Future research should aim at developing in vitro methods that address toxicity pathways and use relevant concentrations, and both paradigms should be derived from in vivo studies (Oomen et al., 2014).

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References


Session II-16b: Inhalation toxicology – Non-nano

Co-chairs

Michaela Aufderheide, Cultex, Germany

Samuel Constant, Epithelix, Switzerland

Session II-16b: Oral presentations

II-16b-218

Coculture of autologous human macrophages and epithelial cells for investigation of airborne particle safety

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Manuscript: Nanotechnology has entered the market and new drug carriers in the pharmaceutical industry are in development. The possibility of inhaling such particles, even if not intended, raises questions about their safety. Thus advanced in vitro models are needed to monitor inflammatory or cytotoxic effects caused by nanoparticle inhalation. In order to mimic the most prominent barrier functions of the alveolar region, macrophage clearance and epithelial cell barrier, we created a human cell culture model composed of these barriers.
Pre-validation of the ex vivo model precision-cut lung slices (PCLS) for prediction of acute inhalation toxicity

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Tissue models for respiratory toxicity are mainly focused on prediction of toxicological outcomes such as organ injury, sensitization and inflammation. Nevertheless, the regulatory application of alternatives to inhalation toxicity studies has lagged behind other routes of administration. The goal of this project was the standardization and pre-validation of precision-cut lung slices (PCLS) as ex vivo alternative to reduce animal numbers used in inhalation toxicology. The project was conducted in three independent laboratories. In all laboratories, PCLS were exposed to 20 substances. Toxicity was assessed by LDH and WST-1 assay. In addition, protein content and IL-1α were measured. Dose-response curves were fitted and EC50 values were calculated. This presentation shows the final results for all 20 chemicals. More than 900 dose-response curves were analysed. Log10[EC50 (µM)] showed best intra-laboratory consistency for WST-1 and BCA assays. WST-1 and LDH indicated toxic effects for majority of the substances. Some substances induced IL-1α. The reproducibility within the laboratories was acceptably low. The results show (i) that the technique is transferable and (ii) that binary prediction model provides promising results with a specificity of 0.7 and sensitivity of 0.9. The presentation will give an overview about the current use of lung tissue in inhalation toxicity.

Cellular based systems for investigating the toxicity effects of inhalable substances

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In vitro alternative methods are urgently needed and should be developed. However, the in vitro alternative methods have to be relevant and reliable: they should closely resemble and mimic the key events that are known to occur in vivo. Ideally, the in vitro assays should be human cells/tissues-based, with specific readouts/endpoints targeting known signaling pathways. Nevertheless, any relevant in vitro alternative methods should be developed and used (Roggen, 2011). Thus, a survey of the existing models relevant for in vivo inhalation toxicity testing will be presented, including cell lines, primary cells, 3D Air-Liquid Interface tissues, co-cultures models and explants. Bénubié et al. (2009). Pros and cons of each model as appropriate tools for acute and repeated dose inhalation toxicity assessment will be discussed.

Validation requirements for alternative methods in inhalation toxicology – case study of a German two phase prevalidation project


Until now, only animal models are validated and used in OECD test guidelines for inhalation toxicity testing. In order to promote and implement the 3Rs principles, in vitro alternative methods are urgently needed and should be developed. However, the in vitro alternative methods have to be relevant and reliable: they should closely resemble and mimic the key events that are known to occur in vivo. Ideally, the in vitro assays should be human cells/tissues-based, with specific readouts/endpoints targeting known signaling pathways. Nevertheless, any relevant in vitro alternative methods should be developed and used (Roggen, 2011). Thus, a survey of the existing models relevant for in vitro inhalation toxicity testing will be presented, including cell lines, primary cells, 3D Air-Liquid Interface tissues, co-cultures models and explants. Bénubié et al. (2009). Pros and cons of each model as appropriate tools for acute and repeated dose inhalation toxicity assessment will be discussed.

References


In vitro exposure strategies for addressing acute and “chronic” effects of airborne compounds

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Concerning the inhalation toxicology, a variety of in vitro methods are already developed for screening and mechanism studies. Validated OECD Guidance Document 34 will be briefly recapitulated in context of special requirements for validation of alternative methods to predict inhalative toxicity. Selection of promising endpoints, standardization, optimization, assessment of variability within and between laboratories, and over time, as well as the development of a prediction model (PM) can be performed according to GD 34. However, final testing under blind conditions is often hampered by necessary analytics. Moreover, technically challenging analytics may have to be shared between labs in a rotating principle. Since each in vitro exposure experiment only provides one data point of a concentration-response, the number of test items employed has to be significantly lower than in other validation studies. We are reporting about a BMBF-funded study in four laboratories aiming at prevalidation of a methodology exposing human lung epithelial cells (A549) with toxic gases at the air/liquid interface. Using four reference gases in phase1 as a training set, both endpoints (cell viability and Comet genotoxicity) showed promising robustness; and a linear prediction model for acute in vivo toxicity could be developed. In phase2, the data set was enlarged by six gases (one overlap), and both, reproducibility and the PM of the training set could be independently confirmed.
approaches to analyse inhalable gases and particles are currently designed to overcome the difficulties in exposing cells of the respiratory tract directly to airborne compounds at the air-liquid interface. Optimization of the biphasic cell culture and different exposure techniques as well as the availability of human cell models offer new ways to integrate this type of in vitro work into the research strategies for chemical compounds – not only for acute but also for long-term exposure studies. An overview of cellular-based test strategies for epithelial cells of the human respiratory tract will be presented to address the possibilities of using cultivated cells, not only for acute toxicity studies but also under permanent burden conditions in the field of inhalation toxicology. Normal primary epithelial lung cells, for example, can be cultivated and repeatedly exposed to non-toxic concentrations of the test atmosphere at the air-liquid interface for several weeks to establish a permanent stress on the cells, thus initiating changes in the cellular composition of the cell cultures both qualitatively and quantitatively.

Session II-16: Poster presentations

II-16-067
Prototype inhalation risk assessment for biopersistents using adverse outcome pathway (AOP) approaches

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In order to evaluate potential toxicity to the lung of biopersistent ingredients in cosmetic aerosol and spray products, we propose a prototype risk evaluation process based on an understanding of adverse outcome pathways (AOPs) for key events involved in lung toxicity. An initial approach can be based on exposure assessment (Carthew et al., 2002), followed by consideration of waiving of inhalation testing if exposure is below a threshold for an inhalation toxicity concern (Carthew et al., 2009). If this is not appropriate, an in vitro approach is proposed. Several well studied human lung disorders particularly idopathic pulmonary fibrosis (IPF) have complex and well documented pathological. It is possible to identify critical events activated in IPF, compare them to the identified key events seen in existing inhalation toxicology studies, and to use these to define an AOP for lung fibrosis. Such studies have been used to select in vitro systems (e.g., BioMAP® primary human cell cultures) and anchor the in vitro data obtained (e.g., changes to collagen, osteopontin, αSMA and specific genes) to biologically relevant lung responses, i.e., oxidative stress, inflammation, and pulmonary fibrosis. This enables the link between early biomarkers of lung toxicity and disease state to be made without the use of in vivo models.

References

II-16-163
The use of a long shelf-life 3D model of the human airway epithelium (MucilAir™) for acute and repeated dose respiratory toxicity assessment

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In vitro assessment of inhalation toxicity is an emerging and fast growing field since until now, only animal models are used in OECD test guidelines. Most of the in vitro cell models for long term testing suffer of at least two shortcomings: 1. The failure of reproducing the in vivo physiological characteristics of the corresponding tissues. 2. A limited shelf-life. Herein is reported the use of a standardized 3D ALI in vitro cell model of the human airway epithelium (MucilAir™) which is free of these limitations. MucilAir™ is morphologically (presence of tight junctions, cilia, mucus) and functionally differentiated (active mucociliary clearance) and it can be maintained at a homeostatic state for more than one year. Classical airway transporters, ion channels, CypP450s are expressed and functional.

A testing strategy using MucilAir™ will be presented for studying impact of acute and long-term effects of inhaled gasses, vapors, aerosols, fibers, particles or nanoparticles on respiratory tract in vitro including:
– Acute and repeated dose Toxicity testing (first in vitro transposition of OECD T413 will be presented – 90 days repeated dose study) (Huang et al., 2011)
– Trans-epithelial permeability assessment/absorption (Reus et al., 2014)
– Pro-inflammatory effect
– Recent advances in the detection of respiratory sensitizers and irritants (Huang et al., 2013)

References

II-16-273
A technical approach on precise determination of minimal mass of deposited airborne particles

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A novel technique is presented based on a highly sensitive and robust approach to determine the mass of airborne particles deposited, e.g., on a cell layer of an in vitro exposure unit. This device may enable accurate online and in situ measurements in order to gather valid information and data on dose-response-relationships, e.g., in toxicity investigations (Aufderheide et al., 2011).

The measuring principle is based on the physical effect of changing the dielectric medium in-between an electrical condenser resulting in a change of the capacity. There are several advantages in measuring such a change of capacity due to an increase in mass. Firstly, the ca-
Applicability of rat precision-cut lung slices in evaluating nanomaterial cytotoxicity and inflammation

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The applicability of rat precision-cut lung slices (PCLuS) in predicting nanomaterial pulmonary toxicity is presented (Sauer et al., 2014; Klein et al., 2012). Upon 24-hour exposure, the PCLuS system detected early events of nanomaterial toxicity (Nel et al., 2013) assessed by PCLuS total protein contents, reduction in mitochondrial activity, caspase-3/-7 activation, glutathione depletion/increase, cytokine induction, and histopathological evaluation. Ion shedding ZnO and Ag nanomaterials induced severe tissue destruction. Ananase TiO2, and CeO2 nanomaterials and two multi-walled carbon nanotubes (MWCNTs) caused significant cytotoxicity. At non-cytotoxic concentrations, different TiO2, nanomaterials and one MWCNT increased glutathione levels, presumably a defence response to reactive oxygen species. These substances further induced a variety of cytokines. One SiO2 nanomaterial increased caspase-3/-7 activities at non-cytotoxic levels. The highest effective dosages (Teeguarden et al., 2007), however, exceeded those reported for rat short-term inhalation studies (Landsiedel et al., 2012). Overall, data reproducibility was not fully satisfactory in the glutathione or cytokine assays, and PCLuS data were not considered sufficient to predict in vivo toxic potency. Effects were frequently observed in negative controls pointing to tissue slice vulnerability even though prepared and handled with utmost care. Comparisons of the effects observed in PCLuS to in vivo effects reveal some concordances for metal and metal oxide NMs, but less so for the MWCNTs.

References

In vitro assessment of airway effects of key products from ozone-initiated oxidation of limonene and printer exhaust

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In indoor air the reaction of ozone with terpenes may lead to formation of airway irritating gas-phase products. We aimed to perform an in vitro study on possible effects of key products from the ozone-initiated reaction of limonene, in combination with printer exhaust, to represent air mixtures in modern offices.

In vitro air-liquid interface cultures of human bronchial epithelial BEAS-2B cells were exposed for 1 hour to gaseous test atmospheres of limonene (400 µg/m³, 2.8) and ozone (210 ppb, 6.8), combined with printer exhaust generated in an environmental test chamber. Formation of (ultra)fine particles and secondary limonene products were monitored. Relevant cellular endpoints to evaluate the possible induction of acute airway effects were measured: cytotoxicity, apoptosis, oxidative stress, and pro-inflammatory cytokine (IL1b, IL6, IL8, TNFe) expression.

The reaction of limonene with ozone generated a high increase of ultrafine particles (up to 18.000 particles/ml), with a concomit-
The CULTEX® RFS Compact: the next generation of CULTEX® systems for the direct exposure of cells at the air-liquid interface.

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Perspectives on pathway perturbation: focused research to enhance 3R objectives

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In vitro high-throughput screening (HTS) and in silico technologies are emerging as 21st century tools for hazard identification. Computational methods that strategically examine cross-species conservation of protein sequence/structural information for chemical molecular targets (or molecular initiating events [MIEs]) provide a non-destructive means to identify the range of species likely to be adversely affected by chemical perturbation. Libraries of MIEs could be readily identified from U.S. Environmental Protection Agency’s ToxCast HTS data and, when linked to adverse outcome pathways (AOPs), can form a basis for hazard identification. With the development of AOPs, that anchor the chemical-biomolecule interaction (MIE) to key events along the toxicity pathway leading to an adverse outcome at the individual or population level, experiments can be refined to examine earlier upstream molecular events predictive of the apical response, therefore improving animal welfare. With key endpoints elucidated, tools such as the U.S. EPA’s Sequence Alignment to Predict Across Species Susceptibility algorithm (LaLone et al., 2013) can be used to identify likely susceptible species, further focusing testing on specific taxa and potentially reducing the need for testing others. Overall, these methods allow for more efficient and targeted toxicity test designs that support efforts to reduce, refine, and replace animal testing.

Reference

Rainbow trout gut cell line (RTgutGC) as a model for fish intestinal epithelia

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The gut of fish is a multifunctional organ not only involved in absorption of nutrients but also in salt and water homeostasis, gas exchanges, acid-base balance, nitrogen metabolism and endocrine/neuroendocrine functions. Our knowledge and understanding of this organ in fish comes from in vivo studies and ex vivo studies such as the gut sac preparation. The development of an in vitro model for the fish gut has been a desire by the fish physiology and toxicology scientific community for a long time. Recently an intestinal cell line (RTgutGC) has been described (Kawano et al., 2011). In this study we are characterizing this cell line and evaluate its suitability as a model of polarized intestinal epithelia. When grown on transwells, RTgutGC cells develop
a transepithelial electrical resistance comparable to in vivo measured values (Sundell et al., 2003), express the tight junction protein (ZO-1) and show clear formation of desmosomes as shown by transmission electron microscopy. Moreover, confocal images show evidence of polarization such as distinct apical/basolateral actin staining. Other important features of RTgutGC cells include active enzymes involved in ionic osmoregulation and xenobiotic detoxification such as Na/K-ATPase and CYP1A, respectively. Our current research focuses on the development of transport assays using fluorescent molecules and nanoparticles.

References


II-17-700

The fish embryo test beyond validation – improvements of the fish embryo acute toxicity test and potential applications for reduction of chronic fish toxicity tests

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Despite successful validation there was concern, that for certain compounds the fish embryo acute toxicity test (FET, OECD TG 236) may not predict acute fish toxicity. A retrospective analysis of fish embryo and acute fish toxicity data retrieved a number of compounds with low toxicity in fish embryos. The majority of these compounds were identified as neurotoxic and/or highly hydrophobic. Experimental analysis of selected compounds suggests that including behaviour analysis (embryonic movement) as additional endpoint could quantitatively predict acute toxicity of neurotoxic compounds.

Fish embryos have also been envisaged as an alternative to the fish early life stage test (FELST). The FELST is used for chronic toxicity assessment and is based on exposure of fish from the embryonic to the early juvenile stage. A retrospective analysis of FELST data shows a clear correlation with acute fish toxicity. Outliers from this correlation included, for instance, compounds with a neurotoxic mode of action. Hence, we suggest developing additional endpoints for the fish embryo test for specific modes of action, such as neurotoxicity. Such assays could be used within the concept of Adverse Outcome Pathways in order to replace the FELST or limit the number of compounds, for which FELSTs would be needed.

II-17-704

It is time to develop the ecological Threshold of Toxicological Concern (Eco-TTC)

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The Threshold for Toxicological Concern (TTC) concept is well established for assessing human safety of indirect food additives and has been reapplied for a variety of endpoints including carcinogenicity, teratogenicity, and reproductive toxicity. TTC approaches have benefits for screening-level risk assessments, including the potential for rapid decision-making, fully utilizing existing knowledge, reasonable conservativeness for chemicals used in lower volumes, and reduction or elimination of unnecessary animal tests. TTC has found particular favor in the assessment of chemicals used in cosmetics and personal care products as well as other chemicals traditionally used in low volumes. Use of the TTC in environmental safety is just beginning and initial attempts are being published. Key questions focus on hazard extrapolation of diverse taxa across trophic levels, importance of mode of action, and whether safe concentrations for ecosystems estimated from acute or chronic toxicity data are equally useful and in what contexts. This paper will provide an overview of the theoretical basis for developing an eco-TTC with an initial exploration for chemical assessment and boundary conditions for use. An international collaboration under the ILSI Health and Environmental Sciences Institute has been established to address challenges related to developing and applying useful eco-TTC concepts.

II-17-765

Alternatives to animal testing for the environmental assessment of cosmetic and personal care products / a review of achievements, challenges, vulnerabilities and future needs

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Fish are key aquatic organisms, but fall into the scope of several international regulations for the protection of animals used for scientific purposes. As a consequence, replacing animal testing for the safety assessment of cosmetic and personal care products faces significant
challenges when addressing environmental issues such as fish long term toxicity, environmental endocrine modulation and bioaccumulation, where fish BCF data are still required for regulatory PBT/vPvB classification.

This review will present the progresses made in these domains over the past decade. It will also highlight promising methodologies to face today’s challenges at anticipating potential environmental long term adverse effects.

Complementary efforts at optimizing QSARs, cell-based assays, invertebrates and fish-embryo tests provide promising tools to assess bioaccumulation and to potentially replace the standard fish acute toxicity assay. Additional endpoints to the Fish Embryo Test as AOPs and functional assessments (cardiovascular or behavior analysis) are gaining serious consideration to better predict fish chronic toxicity. In addition, benefits are expected from the field of human toxicology screening, where developing methodologies with aquatic models are gaining much interest.

II-17-827

Development of an alternative testing strategy for the fish early life-stage test for predicting chronic toxicity

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Testing for chronic fish toxicity is one of the most animal demanding areas in environmental risk assessment. The Fish Early Life Stage (FELS) test (OECD TG 210) is the primary guideline used to estimate chronic toxicity of regulated chemicals to fish. Industry and regulatory bodies have expressed the need for developing alternative testing strategies focusing on non-animal alternatives and mechanistic information. The development of alternative testing approaches requires however a detailed understanding of the mechanisms leading to chronic toxicity. In 2013 we started a project funded by CEFIC (LRI-ECO20-UA) to develop an alternative testing strategy to reduce the need for FELS tests. The project uses four putative AOPs linking molecular initiating events to relevant adverse outcomes at higher levels of biological organization: 1) narcosis leading to respiratory failure, 2) thyroperoxidase inhibition leading to impaired vision, 3) thyroperoxidase inhibition leading to impaired swim bladder inflation, and 4) acetylcholinesterase inhibition leading to motor activity impairment. We will use a combination of modified ZebraFish Embryo Toxicity (ZFET) and in vitro tests to study (molecular initiating) events at lower levels of biological organization along the selected AOPs and investigate the predictivity of these events for FELS toxicity.

Session II-17: Poster presentations

II-17-052

Using Chlorella kessleri for monitoring of water quality

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Pollution of surface waters is an increasing problem in the modern world. Causes are various like over-fertilization, insufficient capabilities or lack of wastewater treatment plants, littering, ecological disaster or even chemical and biological warfare. In the presented work the algae chlorella kessleri was used as a unspecific signal transducer for water quality. The algae was immobilized on a BioChip with a cellulose membrane, supplied with algae culture broth and monitored using the IMOLA-IVD technology (Wiest et al., 2006; Staudacher et al., 2011) which was developed in cooperation with the Hein; Nixdorf Lehrstuhl für Medizinische Elektronik at Technische Universität München. Here, the oxygen production due to photosynthetic stimulation of the algae with light emitting diodes and the extracellular acidification is monitored. If the water quality changes, the photosynthetic activity of the algae is also altered and that can be detected with the set-up. In a proof of principle study, probes from Indonesian palm plants were investigated. The technology showed that it can be used as an unspecific early warning system for water quality monitoring. The technology and first results using probes from Indonesian palm plants are presented. Future work will be on the transition from the laboratory to a real world scenario.

II-17-093

Strategies to reduce the number of fish used in ecotoxicity tests

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In 2011, nearly 180,000 fish were used for toxicological and other safety assessments in Europe, and this number is likely to rise in advance of the 2018 REACH deadline. As few nonanimal methods are available to assess the ecotoxicity of chemicals, strategies that reduce the number of animals required are urgently needed.

In ecotoxicity tests, a test substance is usually added to the tank water. To overcome practical issues associated with testing poorly soluble substances, a small volume of solvent is often used. As the solvent can influence the outcome of the study, two controls – one in the presence and one in the absence of solvent – are currently required. Using two controls doubles the number of control animals and has ani-

References


Novel in vitro tests for analysis of indoor environment toxicity correlated to human health

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New tests have been developed for analysis of the ecotoxicity for indoor environments (Finnish Ministry of Health and Social Welfare, 2013). Swab samples from different houses have been processed with 1) porcine sperm cytotoxic test (Andersson et al., 2010) and 2) E. coli loss of luminescence test (Atosuo et al., 2013). Human inhabitants health parameters have been simultaneously collected and grouped into systemic, respiratory, skin, eye and myalgia categories. In parallel, the health status of the building has been assessed by an expert survey. The meta-analysis (Gasik et al., 2012) of the data has revealed a high correlation between the sum of symptoms and especially respiratory ones with porcine sperm cytotoxicity.

Bayesian analysis allowed selection for the threshold values for ill-health symptoms allowing high sensitivity (>75%) and selectivity (nearly 100%) values to classify the toxic status of the indoor space. The “in vitro-in vivo” correlation shown also high relevancy of new toxicity tests capable of prediction of correct positive and negative results with 70-75% tolerance. New in vitro tests confirm their relevancy and high throughput analysis of samples vs. traditional tests with cultured mammalian cells (hours vs. days).

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Revisiting Hydra in the wake of vision Tox21

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Toxicity testing has been redefined with the landmark publication of the National Academy of Sciences and National Research Council report: Toxicity Testing in the 21st Century A Vision and a Strategy which proposes the use of emerging technologies based on non-animal methods but also adoption of animals belonging to the lower ranks of the phylogenetic tree, with lower sentence levels, as complement to in vitro and in vivo toxicity testing. Besides animals such as Danio rerio, Drosophila melanogaster and Caenorhabditis elegans that are discussed in the scientific community about their potential use in risk assessment, Hydra, a well-studied organism for over 200 years, offers a unique alternative for testing toxicity. Indeed this small cnidian polyp, which is easy to culture and produce in large numbers, exhibits a simple anatomy organized along the gut, and complex behaviors due to its sophisticated nervous system. Thanks to its pool of stem cells Hydra shows a low senescence. However, Hydra is highly sensitive to environmental insults. In this study we monitored the acute toxicity of copper and cobalt in Hydra magnipapillata. Morphological analysis of isolated cells together with comet assays suggests genotoxicity of these compounds and activation of the caspase cascade leading to apoptosis.

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Theme III – 3Rs in Academia and Education

Coordinators
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Monika Schäfer-Korting, FU Berlin, Germany

Session III-1: 3Rs in academic education, training programs and anticipated needs

Co-chairs
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Session III-1: Oral presentations

III-1-190
Assessing current practice on the Three Rs literature search: analysis and key findings from the national survey
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Despite the tremendous amount of information on the Three Rs available online, it can be very difficult to obtain the specific information desired to replace, reduce and refine the use of animals for teaching and research, as required by legislation in Korea and elsewhere. The KNIC3Rs was established on August 2011 in collaboration with government, academia and national and international experts from animal welfare organizations, with the goal of exchanging knowledge and resources. One of its functions is to better prepare Korean scientists to identify and use relevant Three Rs techniques and strategies. In 2012, the KNIC3Rs conducted a survey of the Three Rs literature searching practices of Korean IACUCs and investigators. This paper presents key findings from the survey results and the progress made in Korea through practical workshops under the title of “Reducing the use of animals in research and education through better experimental design” and “The 3Rs Good Practice: Effective Search Strategies to comply with the 3Rs” conducted in 2012 and 2013. Educating scientists and IACUCs is key to giving them the skills to find useful information and is a good place to start in helping them embrace the use of alternatives.

III-1-396
Integrated 3Rs education
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Rather than the 3Rs being taught as an abstract concept, with information on each provided separately in different courses or defined subsections, we have incorporated all elements under an overarching heading of experimental design. Replacement is considered through literature searching and setting the experimental aims, questioning not only whether non-animal alternatives might be used but also whether an alteration of the experimental aim might avoid animal use. Refinement is discussed with the decisions on what types of data to gather, the effect of animal discomfort or distress on the reliability and variability of the data gathered, the choice of procedures to be used, and the application of humane stopping points. Reduction is taken as minimising numbers overall by using an efficient and appropriate design and including in it proper controls, avoidance of bias, and sufficient

III-1-315
Doerenkamp-Zbinden Foundation’s vision to reduce animal use in education and research in India through MGDC adequately realized
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MGDC was established in 2009 by Doerenkamp-Zbinden Foundation to promote adoption of 3Rs in India. MGDC adopted a pragmatic approach in which the academic fraternity was enlightened about and trained in ICT tools in education as alternatives to animal dissections and experiments in 40 seminar-cum-workshops across the country, in parallel with training to more than 200 researchers in in vitro and in silico toxicology in 15 workshops conducted at MGDC’s HQ. Sensitisation was also achieved through popular lectures, talks in conferences, including Indian Science Congress, and meetings with regulatory authorities. High profile workshops were conducted in collaboration with The Hanner Institutes, USA; In Vitro AdMet Labs, USA; SkinEthic Academy, France; and ExCel Matrix, India. Original articles and reports were published in peer reviewed journals. A 4 credits elective course entitled “Alternative Methods to Animal Experimentation” is offered. MGDC’s relentless effort, supported by PFA, PeTA, and I-CARE, was rewarded with UGC, and Medical and Pharmacy Councils bringing up guidelines limiting use of animals in education to a bare minimum. Also, MGDC partnered with organizations in working out reforms in animal use in risk assessment. Highly satisfactory outcome indeed but achieved through persistence and perseverance.
numbers to detect worthwhile effects. Along with a varied delivery which recognises both the range of ways people learn and the difference between adult and schoolchild learning; this provides a package that participants rate highly and for which responses to problem solving and pre and post testing indicate good acquisition of knowledge, understanding, and skill.

III-1-454

An integrated practical course on cell biology on in vitro cytotoxicity assays for Brazilian health/biology undergraduate students

D. Silva, J. Côrtes, R. Bachinski, C. Spiegel and G. Alves

Cell Biology (CB) is present on curricula of Health/Biology undergraduate courses. While practical CB classes are usually employed to review theoretical concepts discussed on expository classes, sometimes undergraduate students miss the relevance of cell culture on in vitro assays, and, moreover, as alternative methods for animal use on their future career. This work presents the development of a practical CB 10-lessons course, applied to students from the Fluminense Federal University (Brazil), proposing that students participate in the whole process of drafting and performing a cytocompatibility assay, with production of a scientific report by each group of 5 students (n=80 groups). Students selected reference papers and presented a seminar on cytotoxicity tests and each class proposed a protocol, feasible with the available infrastructure. Students were trained in laboratory and cell handling. Students from all classes agreed upon the use of a colorimetric assay with murine pre-osteoblasts, using many replicates (3-5) and including adequate controls. Groups were able to produce sound, scientifically adequate reports, and students recognized, as stated on a qualitative assay, the relevance of practical lessons and CB on their professional development, as well as cell culture as alternative to animal testing, indicating the adequacy of this practical approach.

III-1-554

Use of animals for the purposes of education and training

M. Jennings and E. Lilley

The use of living animals for education and training purposes which may cause the animals pain, suffering, distress or lasting harm will require project authorisation under Directive 2010/63/EU. Until recently, the extent to which such animal use is permitted has varied considerably among Member States – from routine use in acquisition of skills to use only under very specific circumstances.

There is still considerable debate regarding if and when animal use for education and training is justified. However, there is agreement that a considered and structured approach is needed. The learning outcomes need to be well defined and use of alternative strategies fully explored before any request is made to use living animals. Ideally, within the EU, there should be a common understanding of the specific circumstances under which animal use is likely to be authorised, and agreed constraints, for example limiting the severity to mild and reducing numbers of animals to a minimum.

This presentation will discuss the ethical and practical factors to be taken into account when developing a harmonised approach.

III-1-624

The Berlin-Brandenburg research platform BB3R and integrated graduate school


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Fundied by the German Government the Berlin-Brandenburg Research Platform BB3R with integrated graduate education has started in April 2014. Joint research of scientists from FU Berlin, Potsdam University, Charité Berlin, TU Berlin, BfR, and Zuse-Institute Berlin will focus in gaining substantial progress in the fields of alternative and humane testing and in strengthening the national 3R expertise. BB3R aims to accomplish the following goals:

- Establishment of alternative methods for preclinical drug development and basic research; facilitation of research collaborations and sustainable research activities in the region Berlin-Brandenburg
- Expansion of regional research activities by establishment of three junior research groups; successful candidates will be qualified for management positions in professional areas related to the 3Rs
- Sustainable establishment of the BB3R graduate school for structured training of graduate students who complete a specific mandatory course program on alternative test methods to animal experimentation and related fields in addition to the research project
- Creation of a pool of 3R experts for advice and assistance
- Increasing the awareness of the society for 3R-related issues

The research platform BB3R along with the associated graduate school will close substantial knowledge gaps in the fields of 3Rs and alternatives to animal experimentation in the years to come.

III-1-642

Training for the Three Rs – the CCAC research fellowships in animal policy training

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Since 2003, the Canadian Council on Animal Care has offered 6 research fellowships in animal policy development. These fellow-
Session III-1: Poster presentations

III-1-072
Survey of Canadian research and teaching institutions on strategies for implementation of the Three Rs

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The Canadian Council on Animal Care (CCAC) guidelines provide assistance in the implementation of best practices and achievement of Russell and Burch’s Three Rs for animal use in science. Two CCAC guidance documents (Canadian Council on Animal Care, 1997, 2006) suggest animal use protocols include details on replacement, reduction and refinement, as one strategy for implementation of the Three Rs. To identify, more comprehensively, current and proposed strategies for Three Rs implementation, we conducted a survey of Canadian institutions to query how they encourage investigators to incorporate Three Rs principles in their research or teaching programs. The survey gathered information on the size and type of animal care program, challenges and the various strategies employed with respect to implementing the Three Rs. The survey also asked institutions to provide specific examples to illustrate how investigators purposefully address the Three Rs within their research or teaching programs. The data is collated and presented descriptively but clearly identifies the survey as a useful tool to collect information from the various institutions. We intend to share the information with Canadian institutions so that they may institute new ideas or procedures for Three Rs implementation into their institution’s animal care and use program.

References

III-1-165 *
Experience in the use of alternatives to animal experimentation in the learning capability of undergraduate students of human physiology at the Universidad de Guadalajara, Centro Universitario de la Costa 2008 to 2014

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The complexity of teaching is increasing with the advancement of scientific knowledge. The level of detail at which science needs to be explained in any field of knowledge makes observation and macroscopic experiments insufficient.

Health education is a challenge: imparting knowledge to explain life using all the resources available and implementing new strategies to facilitate this transfer of knowledge.

Animals have been used in scientific research in many ways, allowing the advancement of scientific knowledge in human and veterinary medicine as well as in the chemical industry (Nuffield Council on Bioethics, 2005).

New alternatives to the use of animal models can replace old models that injure and destroy lives of animals.

The methodology used in the world include the following: Models, mannequins and mechanical simulators; Simulation and virtual reality in computers (Dewhurst, 2006); Experimentation in humans and plants; Use of biological material from slaughterhouses; In vitro cell lines studies; Reuse of dead animals from donations (Vinardell, 2012).
Multiple studies have attempted to measure the level of effectiveness based on the use of these alternative learning methods based on computers (Dewhurst, 2004; Clarke, 1987).

Results: 65% of surveyed agreed that animals are not necessary in health education, 56% indicated learning better with autoexperimentation and 76% concluded that alternatives are better.

References

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III-1-222

An Indian educational initiative to promote the use of alternatives to animal testing with special reference to 3D reconstructed human tissues

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In 2013, the Mahatma Gandhi Doerenkamp Center and SkinEthic Academy signed a partnership to organize a yearly national workshop on alternative methods to animal testing. The aim of this workshop is to increase awareness to 3R by illustrating how in vitro alternative methods have revolutionized modern toxicology and to train people to handle human reconstructed epidermis in the context of validated in vitro method for skin corrosion/irritation.

The workshop is an outcome of the effort of MGDC in propagating modern tools for risk assessment to the Indian community and the commitment of SkinEthic Academy to promote 3R and the use of alternative methods based on 3D reconstructed human tissues. The workshop is organized in a phased manner. The first leg of workshop is held for faculties and scientists from universities and research institutes. The second leg is for scientists from cosmetic, pharma and CROs. The workshop schedule is aptly distributed between lectures, hands-on and interactive sessions.

The decision in 2013 to ban animal testing for cosmetics in India reinforces the importance of such workshop to participate to the constitution of a community of scientists and toxicologist able to implement current alternative to animal tests and to develop new alternative strategies.

III-1-324

Student perspectives on harmful animal use as a teaching method in the life sciences

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Practices involving harmful animal use are still employed as a teaching method in the life sciences at the Autonomous University of Nuevo Leon in Mexico. Several students have expressed their unwillingness to participate in these labs, which are an obligatory requirement. To address this problem, surveys were designed to assess the students’ perception and attitude towards these practices. They were conducted upon 576 students from the following majors: Biology, Parasitology and Biotechnology. A Likert Scale was used, in which students were asked to express their degree of approval towards specific statements or questions. Simple yes or no questions were also included. The results overwhelmingly favored the use of alternatives. Fifty two percent expressed having felt an ethical conflict when performing these practices, yet only twenty percent voiced their concern to their teachers. Eighty five percent declared that they would prefer to use an alternative, and ninety four percent stated that they support the implementation of a conscientious objection policy. These positive results prompted the director of the Biology School to take an active interest in the issue and the School has now begun to take steps towards replacing harmful animal use with humane alternatives.

III-1-263

Continuous education and trainings promotes the 3Rs alternative methods development in China

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Since 1990s, the 3Rs principles have begun to be known in China. Compared to Europe with a long tradition of animal warfare, 3Rs alternative technology in China is progressing slowly. In recent decade, China is undergoing significant changes. Several workshops and trainings hosted by GDCIQ have played a positive role in outspread of 3Rs, in which nearly 200 people from different industries attended. An alternative organisation, the Chinese Centre for Alternatives Research and Evaluation (CCARE), was established for sharing information and resources in 2007. The first Chinese alternative textbook, Alternative Laboratory Animal Methods Principles and Applications, was published in 2010 with the support from international contributors; another book, Alternative Animal Testing Guideline, will be published soon. With the support of EPAA, the Chinese version of the three Rs and Humane Criterion has been issued for the 55th anniversary of Russell and Burch’s marvellous book The Principles of Humane Experimental Technique. Thousands of copies have been provided free of charge to Chinese public libraries and universities. With the cooperation of ECVAM, IIVS and other organizations, we are constantly making progress in this field. It is believed that continuous education and trainings will promote 3Rs alternative forward in China.

References
Conscientious objection to harmful animal use within veterinary and other biomedical education

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Laboratory classes in which animals are seriously harmed or killed, or which use cadavers or body parts from ethically debatable sources, are controversial within veterinary and other biomedical curricula. Along with the development of more humane teaching methods, this has increasingly led to objections to participation in harmful animal use. Such cases raise a host of issues of importance to universities, including those pertaining to curricular design and course accreditation, and compliance with applicable animal welfare and antidiscrimination legislation. Accordingly, after detailed investigation, some universities have implemented formal policies to guide faculty responses to such cases, and to ensure that decisions are consistent and defensible from legal and other policy perspectives. However, many other institutions have not yet done so, instead dealing with such cases on an ad hoc basis as they arise. Among other undesirable outcomes this can lead to insufficient student and faculty preparation, suboptimal and inconsistent responses, and greater likelihood of legal challenge. Accordingly, this paper provides pertinent information about the evolution of conscientious objection policies within Australian veterinary schools, and about the jurisprudential bases for conscientious objection within Australia and the USA. It concludes with recommendations for the development and implementation of policy within this arena.

An inspiring book telling the personal stories on the replacement, reduction and refinement of laboratory animal use

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In 2012 and 2013, Inge Toussaint from the Netherlands Knowledge Centre on Alternatives to animal use (NKCA) interviewed 56 experts involved in the Replacement, Reduction and Refinement (3R’s) of laboratory animal use, including Coenraad Hendriksen, Bert van Zutphen, Vera Rogiers, Herman Koetter, Marie-Jeanne Schifflers and Erwin L. Roggen. The interviews nicely illustrate the dilemma’s, opportunities and barriers that appear on the long road from development to implementation of 3R-alternatives. Their stories show that there is not a straightforward, easy way to implement 3R-methods. Some of these experts even claim that the 3R-principle has become outdated and that there is a need for a completely new approach. Initially these interviews were only published online. In December 2013 “De V van Verhalen” was published, in which all interviews were brought together in a beautifully designed hardcover book. Illustrations were done by NKCA employee Marjolein Schilders-van Boxel. The book was made available to schools, animal laboratories, researchers, policymakers, animal protection organizations, science journalists and individual persons, all free of charge, and it has been highly valued. Currently, the book is only available in Dutch, but the NKCA would be interested in publishing it in English as well.

EPA’s toxicity forecaster research effort communications and outreach overview

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Recent scientific advances provide innovative solutions to persistent and pervasive issues facing risk assessments and policy decisions made about the safety of chemicals. USEPA has been using in vitro testing methods to accelerate the pace of chemical evaluations, reduce reliance on animals, and address the lack of data on the thousands of chemicals. In 2013, EPA’s Toxicity Forecaster effort released high-throughput screening data on 1,800 chemicals. These chemicals were screened for potential health effects in ~800 HTS assays. All data is publicly available for stakeholders to analyze and use to help inform chemical safety decisions. Using ToxCast requires changing a regulatory paradigm that has been used for decades. EPA recognized early that an outreach strategy with the goal of helping increase usage and analysis of the data was needed. This presentation will describe EPA’s strategy including an overview of:
- Communication and outreach goals and approach and how EPA is measuring effectiveness.
- Summaries of research collaborations and stakeholder groups.
- Strategies implemented (Communities of Practice, websites, videos, scientific media outreach, educational workshops, research collaborations worldwide, requesting stakeholder feedback) and information about which strategies were the most successful.
- Summary of stakeholder feedback.
- Future plans for outreach efforts.

For more information: http://www.epa.gov/comptox

4-year experience teaching alternative methods to animal use in toxicology

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Since AY 2010/2011, a course for students with bachelor’s degree in sciences has been offered at the University of Milan, Master of Veterinary Biotechnology Sciences titled “Alternative Methods to Animal Use in Toxicology” (Caloni et al., 2011). Several topics include contextual laboratory activities featuring the illustration and protocol of the main in vitro toxicological tests. An ad hoc lesson is reserved for the illustration of in silico models, provided by experts. An interactive approach adopted during the course, through video conferences and workshops with national and international speakers proved to be very effective for students. At the end of each lesson, the feedback of the students is requested through a questionnaire with 4/5 questions about the specific topic, in order to assess learning capacity. Finally the students are also asked to investigate an ongoing issue with a critical approach by providing ideas and possible improvements through presentations or display of posters, followed by a discussion. The result after 4 years is definitely positive, with high appreciation by students. For the next AY 2014/2015, a mandatory course in the first
Graduate training program BB3R

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In April 2014, Berlin-Brandenburg research platform BB3R with integrated graduate program “Innovations in the 3R Research – Genetic engineering, tissue engineering and bioinformatics” has been established. The BB3R graduate program aims to prepare doctoral students and junior professors for later career in the field of life science or science administration.

The post-graduate qualification is based on the own research project of the PhD student and is supplemented by a broad, clearly structured training program in the wide field of 3R. Every PhD student is supervised by a team of three professors (research survey and personal mentoring).

The graduate program includes compulsory courses offered in spring schools (seminars and practical training) of alternative test methods and laboratory animal science and a forum for ethics and laws. The professional development program includes targeted training and workshops for transferable skills and provides mentoring, coaching and individual support in the career planning. Training of related methods in a lab of a research partner is recognized as elective course. In addition, the PhD students can attend seminars of the Dahlem Research School (DRS), established under the Excellence Competition of German universities, covering the fields of academic performance, managerial skills, information technology/languages and career development.

Experiments on animals: ideals of humane education & real practice

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In the frame of the UNESCO project 3-level system of bioethics education, including ethical issues of animal experimentation in life science, has been formed in Belarus in the last decade. Training courses, curricula, textbooks and guidance manuals have been developed. Owing to co-operation with InterNICHE and Russian Animal Center Rights educational animal-free courses have been introduced in some Belarusian universities. The workshops on actual ethical issues of humane education and experimentation take place annually at International Scientific Conference “Sakharov Readings”.

Nevertheless, the practical application of 3Rs concept has local and sporadic character due to experience shortage of institutionalization process and ethics management by modern managerial techniques. So in Belarus there is no law on the protection of animals, alternatives are not included in the biomedical and veterinary educational standards. Obligatory ethical expertise of biomedical research on animals and GLP standards are not completely and widely applicable. Dissemination of alternatives to everyday practice is limited by high cost of modern models and small number of teaching materials in Russian.

Humane alternatives application on biology school lessons for the purposes of eco-ethical thinking formation among youth

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Formation of eco-ethical thinking among youth is a complex, multistage process. A school educational system plays a significant role in creating the preconditions for this type of thinking. Training and education of adolescents during biology school lessons provide large opportunities in this regard.

For many years in Belarus adolescents during of the course “Zoology” were forced to get acquainted with the animal world by using sacrificed and canned or dried animals. This led to the development of the younger generation utilitarian way of thinking.

Nowadays, thanks largely to the development of computer technologies, the application of humane alternatives (such as video and audio materials, multi-media presentations, etc.) during biology lessons has become possible.

Modern approach with application of humane alternatives has been introduced in course “Zoology” in secondary school 98, Minsk, Belarus. Furthermore, during studying the course “Human Anatomy” students become acquainted with the program “Virtual Physiology” and were informed about the existence of humane alternatives to experiments on animals, which they could use in the future in higher education.

Humanising and modernising medical education: change in the Ukraine

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An important element of enhancing the teaching process for future medical doctors is to make it humane. In 2012, following national and international media exposure of very poor conditions and severe animal experiments at Donetsk National Medical University, the De-
Department of Physiology signed an agreement with InterNICHe and Doctors Against Animal Experiments (DAAe) (Germany) to develop collaboration and to fully replace animal use in practical classes. In 2013, the Department of Pathological Physiology signed a similar agreement. InterNICHe and DAAe donated laptop computers, multimedia projectors, models, trainers, interactive multimedia software, and video films on DVD. Approximately 90% of the animal use was replaced over a period of several months, and the final 10% is being replaced over one year. The annual use of over 700 animals in physiology and over 5000 animals in pathological physiology has been ended. The departments value the concept of humane education and the possibility to implement replacement alternatives. In addition, the impact of interactive learning approaches and computer literacy in helping to modernise medical education in the Ukraine is being recognised.

Session III-2: Funding agencies and funding programs

Co-chairs
Takashio Omori, Doshisha University, Japan
Vicky Robinson, NC3Rs, UK

Session III-2: Oral presentations

III-2-024
The Danish 3R-Centre
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Background: The new Danish 3R-Centre was established in 2013, as a unique collaboration between government, the pharmaceutical industry and animal welfare organizations. The centre works to promote alternatives to animal testing (replacement), increase awareness of methods that use fewer animals (reduction) and endorse improvements to scientific procedures and husbandry which minimise pain and suffering and improve animal welfare in situations where the use of animals is unavoidable (refinement).

Materials and methods / Who and how: The centre has a Board consisting of seven recognized experts within the field. They are actively involved in the development of alternatives and animal testing in practice. A Secretariat assists the board in their daily work. The role of the centre is to collect and disseminate knowledge as well as initiate and support research within the 3R’s.

Mission: The 3R-Centre will:
– work to initiate useful activities that may lead to the immediate implementation of the 3Rs.
– provide a forum for collaboration, discussion, exchange and dissemination of information on the 3Rs.
– initiate research projects and recommend funds allocation of resources within the area.

Vision: It is the goal of the Danish 3R-Centre to generate a leading environment within the implementation and dissemination of the 3R’s.

III-2-074
More than 20 “years” research funding by ZEBET – measures and impact
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Research funding is a general instrument trying to promote the advancement of science by providing financial and organizational resources. Particularly, research funding is an appropriate action for those research areas, which are currently underestimated, where data gaps exist, and where a vision needs to be pursued. In 1959 William Russell and Rex Burch published already the 3R principle. At a time, as science on reduction, refinement, and replacement was not the highest priority. ZEBET (The Centre for the Documentation and Evalua-
Experience from a UK 3Rs research funder

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It is now ten years since the NC3Rs was launched. During this time we have introduced a number of funding schemes to support research, and early career training and development. This presentation will cover our experience as a 3Rs research funder including lessons learnt. It will focus on the impacts of the science we have supported.

Motivating synthesis of evidence part of more knowledge with fewer animals programme

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The More Knowledge with Fewer Animals programme, is developed by the Netherlands Organisation for Health Research and Development (ZonMw). The programme aims to reduce the use of animal testing, without impairing the quality of scientific research and the safety of developed products. The programme is subdivided into modules with varying focus, aiming at developing 3R methods across a broad spectrum. Three modules are commissioned, one module promotes and develops 3R knowledge infrastructure. The focus of this module is divided into two parts: First part, by stimulating the performance of a synthesis of evidence by biomedical researchers. Second part, by stimulating the publication of solid negative results of animal experiments. Hands-on workshops “From information to knowledge through systematic reviews” are organised by SYRCle in collaboration with ZonMw. Junior and senior researchers, involved in animal experiments, are invited to join the workshop. To stimulate publication of solid negative experimental results, researchers can apply for compensation of a one month salary in order to publish their negative results. Publications must be in peer reviewed, open access journals and researchers are required to use the “GSP Checklist” or “ARRIVE” in order to improve both the accessibility and the quality of the published results.
Session III-3: Innovative teaching and training tools

Co-chairs
Hans A. Braun, Philipps University, Marburg, Germany
Nick Jukes, InterNICHE, UK

Session III-3: Oral presentations

III-3-247

Evaluation of the multimediaroom/training clinic at the Norwegian University of Life Sciences

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At WC8 in 2011 we presented the Multimedia Room/Training Clinic at the Norwegian School of Veterinary Science (now part of the Norwegian University of Life Sciences). One of the conclusions at WC8 was the need for more research into the use of such facilities. In 2012 a simple questionnaire was used to assess the students’ self-confidence in anatomy, physiology and basic clinical skills such as blood sampling from dogs. There was a significant increase in the students’ self-confidence in performing basic clinical skills after the Multimedia Room/Training Clinic was opened in 2009. While we cannot conclude categorically that this improvement was caused solely by use of the new facilities, the results agree closely with our subjective impressions, following discussions with the students. The results were published in the Norwegian Veterinary Journal in 2014 and will be presented here. Other benefits include easier access to multimedia and training products for the students, and lower running costs after the initial investment. This is likely to become even more cost-efficient as the number of students enrolled on these courses increases, compared to traditional teaching methods.

Reference

III-3-497

The potential of Drosophila in neurosciences – education of undergraduates and junior researchers

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Many practical and ethical obstacles severely limit the scope for experiments using mammals in fundamental biology and biomedical science. Invertebrate models are an increasingly appealing alternative. Simplicity and economy of breeding and housing in combination with extremely powerful genetics have made the fruit fly Drosophila a predominant model to understand how genes direct the development of an embryo from a single cell to a mature multicellular organism. Many of the genes that they defined as being important for fly development have since been shown to be critical for all animal development, including humans. Particularly, many of the underlying building blocks and engineering processes have been conserved through evolution and are strikingly similar. Over the last three decades, the use of Drosophila has been extended into areas as learning, behavior, and aging. I will present exemplary studies demonstrating the versatility of Drosophila for studying elementary mechanisms of cognitive aging (Gupta et al., 2013), and ways of protecting from it. Moreover, I will discuss and illustrate of how we use Drosophila in the education of undergraduates and junior researchers.

Reference

III-3-516

The birth of “SimDonkey”; the develop of a high fidelity donkey patient simulator

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High fidelity human patient simulators (HPSs) provide medical students with opportunities to develop important clinical competencies, including technical skills, clinical reasoning, teamwork and communication, within environments which are reasonably realistic, yet comparatively stress free. However, very few high fidelity animal patient simulators have been developed for use by veterinary students. Accordingly, we transferred working parts from “SimMan” – a high fidelity HPS – into an animal mannequin. We chose a donkey – subsequently “SimDonkey” – for several reasons. The size of the HPS circuitry and equipment precluded the use of a smaller mannequin. Additionally, we make significant use of donkeys in our clinical training program, and there remains a dearth of simulators for teaching equine clinical skills. Our SimDonkey has a range of cardiovascular and respiratory features derived from the HPS, including bilateral palpable pulses in the regions of the carotid arteries and front legs, an airway that can be intubated, with or without a range of intubation problems, spontaneous chest excursions to simulate breathing, auscultable heart and breath sounds, with a range of pathologies available, and ECG and defibrillation connection points – although the internal structure of this mannequin, including its potentially inflammable components, currently preclude defibrillation.
Successful replacement of animals with a CAL software in pharmacology education

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Our department has been striving hard to replace animals with alternatives in education (Raveendran and Gitanjali, 2011) and was able to completely replace the animal experiments by incorporating the “ExPharm Pro” software (written by the last author) in undergraduate syllabus. ExPharm Pro (http://www.animalsimulator.com) is a new and online version of ExPharm and includes many new features such as pre-test, post-test, full experimental set up, data entry page, logs and admin features. It can simulate 5 animal experiments each of which includes a learning module and 2-3 examination modules. The software was introduced in the syllabus in 2012 for medical undergraduates doing a course in pharmacology in our institution after removing all the animal experiments. After demonstration of each simulated animal experiment in a regular practical class, the students were asked to perform the experiment individually in the department computer laboratory. One batch of students completed the course in December 2013 and took the final examination using the software. The new method worked well as indicated by the successful and smooth completion of the course and the examination. The general feedback was good and our experience demonstrates the successful replacement of animals using a CAL software without compromising the quality of education.

Reference

The real and virtual laboratory: computer simulations in life science education and research

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The Marburg Neurodynamics group combines the experience from experimental studies with profound knowledge of mathematical simulations to develop realistically appearing computer models for education and mechanism-based models for research.

The educational tools, the “Virtual Physiology” series (SimNerv, SimMuscle, SimHeart, etc.) are already used since many years at universities and schools all over the world in refinement and/or replacement of animal experiments (Bahar, 2001; Braun, 2003). They are currently reprogrammed as platform independent versions with new features (demoversions at http://www.virtual-physiology.com).

Likewise, advanced mathematical simulations can significantly reduce the number of animal experiments and clinical studies. According to the goals of a huge EU Network of Excellence (BioSim, http://en.wikipedia.org/wiki/BioSim), the idea is to make use of computer simulations for the design of more goal directed experimental and clinical studies at universities as well as pharmaceutical companies thereby avoiding unnecessary suffering of animals as well as of patients – at lower costs.

Examples from own research (Postnova et al., 2010; Tchaptchet et al., 2013) shall illustrate how simplified, nevertheless realistic models of neurons and synapses can be designed that allow to consider all major drug effects and that can be connected to models of higher autonomic and mental functions for the examination of their disturbances and the evaluation of more effective treatment.

References
Session III-3: Poster presentations

III-3-119  
NORINA: information on animal alternatives in a changing world  
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The introduction of the personal computer in the 1980’s made it possible to produce simple computer simulations to replace the classical animal experiments performed in physiology and pharmacology classes. Increasing resistance to the use of animals in schools led to the production of dissection alternatives. The NORINA database (http://oslovet.norecopa.no/NORINA) was launched in 1991 to provide a source of global information, since many items were produced by small companies or university departments with little advertising. A supplementary database, TextBase, was produced to provide information on written material (e.g., anatomical illustrations) and textbooks within laboratory animal science.  
Many of the technologies in use 20 years ago are, however, irrelevant to course providers today, and students expect to find material for the latest platforms. In addition, many suppliers have ceased to operate, changed address or placed their products on the web. Similar developments are occurring within human medicine, and some products are almost directly transferable for the replacement or reduction of animal use.  
This presentation will describe the developments within the area of animal alternatives, highlight the ways in which educators can contribute to the process and describe how NORINA has evolved to meet today’s needs.

III-3-188  
Development of the new Korean teaching aids on the Three Rs principles: translation of “the Three Rs and the humanity criterion”  
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The principles of the Three Rs of Russell and Burch (Replacement, Reduction and Refinement) have now been guiding animal use for more than 50 years. In 2008, this came into effect to Korean law and a number of activities toward promoting the Three Rs have been undertaken ever since. Despite such a ground-breaking publication of The Principles of Humane Experimental Technique over half a century ago, and an abridged version The Three Rs and the Humanity Criterion, which is written more simply and accessibly for non-English readers, it is still necessary to read certain sentences over and over again to access the meaning. We have finally been able to address the ongoing absence of Korean teaching aids relating to the general basic theory and concepts of the Three Rs. This paper details the development of the new Korean teaching aids on the Three Rs based on The Three Rs and the Humanity Criterion. This book is designed for academic textbook and teaching aids in the field of bioscience. It provides user-friendly instructions and informative graphics including Korean legal requirements. It is designed as a loose-leaf publication which is suitable for regular updating.

III-3-194  
Animal alternatives in veterinary teaching-status and scope in an Indian veterinary university  
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In India, animal experimentation is restricted in veterinary curriculum due to intervention of Animal Welfare Board. Various alternative tools, i.e., histological/ histopathological slides, video demonstrations, preserved specimens, phantom box technique have been used in veterinary teaching since long back to address this issue. A survey with various faculty members revealed that many alternatives which are used in other countries have application in Indian veterinary curriculum. Ethically sourced cadavers, which provide real tissue feeling, can be a good educational aid in veterinary anatomy, pathology and surgery. Rumen Simulation technique and Hohenheim gas production Techniques are some in vitro tools to analyze feed degradability, volatile fatty acids and rumen microbes, which replace painful rumen fistulation and relevant in nutrition and physiology. Videos of well performed dissection, surgery and autopsy, animal models, surgical training models and mannequins (animal handling, blood collection and intubation) have good application in the field of veterinary surgery, anatomy, pathology and medicine. Mannequins can be well utilized in veterinary gynaecology to study foetal presentation. However, many of the faculty members remain unaware of the tremendous scope of the alternatives. Therefore, wide awareness on the use of animal alternatives should be given before its popularization.

III-3-409  
Peer mentoring and alternatives to using animals as a strategy for education in the life sciences  
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The Centro Universitario de la Costa de la Universidad de Guadalajara, implemented since 2008 a program of alternatives to using animals in teaching physiology based on self-experimentation, a mixed
system of hardware and software, and a group of outstanding students known as physiology lab instructors, which support learning of physiology students from lower semesters studying medicine and nursing. Mentoring relationship is important in career advancement (Ragins and Scandura, 1997; Sands et al., 1991; Aagaard and Hauer, 2003; Barczyk, 2011; Buckenmeyer et al., 2011).

The training of the instructors includes the review of current scientific articles in the field, the introductory course management of the Biopac©, and a course of human development.

In this paper we try to detect the level of satisfaction among the instructors.

The results are: from 35 answered surveys the 52% responded that their expectations of the group were completely fulfilled, 48% said their expectations were partly met, 97% felt identified with the program practices without the use of animals, 3% not identified himself, 97% felt satisfied serving as peer tutor, only 3% reported feeling unsatisfied in this area, 94% agreed that belonging to the group improved their academic training and finally the 6% was partially agree.

References

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III-3-433

Overview of the free virtual experiments – computer simulations for teaching pharmacology
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Objectives: The scope of this investigation was to determine and promote the available free educational tools for teaching pharmacology, according to the 3R principle.

Methods: Using the databases for humane teaching alternatives, such as InterNICHE and NORINA and the Internet search engine Google, we have searched for appropriate educational tools for teaching experimental pharmacology that are: 1) stand-alone programs capable for off-line teaching; 2) interactive alternatives to animal experiments – capable of simulating real experiments and producing realistic data; and 3) completely free of charge. Therefore, the following programs were omitted from this overview: 1) web-based, internet-dependent; 2) available primarily from a commercial source, or 3) non-experimental-based forms of computer assisted learning (CAL) such as online textbooks or multiple-choice questions.

Results: We have identified only two, rather old programs that meet all the above-mentioned criteria: Microlabs for Pharmacologists and Strathclyde Pharmacology Simulations (Jukes and Chiuia, 2003). Microlabs for Pharmacologists represents a series of computer simulation; we have recently written a free-of-charge E-handbook to accompany this program (Modun and Bach-Rojecky, 2013). Strathclyde Pharmacology Simulations is a suite of programs simulating physiological experiments on isolated tissues or whole animals.

Conclusion: There is a need for new, free, interactive and stand-alone programs capable of simulating experiments for teaching pharmacology.

References

III-3-487

Emphasis on zoology curriculum based on non-invasive teaching methods of physiology and anatomy has significantly reduced the animals use in practical classrooms in India
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The efforts and pursuance of some progressive academicians and organizations of India made possible that University Grants Commission issued circular to universities to discontinue dissection in a phased manner and use appropriate alternatives. The Save Environment and Welfare of Animals Society, Rajasthan, and Mahatma Gandhi – Doerenkamp Center, Tiruchirapalli, organized several training programmes all over the country for effective implementation of alternatives. Anatomy and physiology of vertebrates are taught using non-invasive software-based methods. Digital Frog 2.5, Pro-Dissector Frog, Froguts, PhysioEx 9.2, Dogfish, etc., are the common digital tools. Feedback response were obtained from the participants, which indicated more than 95% acceptance of digital alternatives over the age-old obsolete wet lab practices involving extensive animal killing. Institutions that practice digital methods of anatomy and physiology have discontinued animal dissection with improvement in understanding and learning skills. Survey carried out in some parts of Western and Southern India has shown positive sign of improvement in the biodiversity status of many animal species which were over-exploited in dissection and vivisection. Although, many digital alternatives for anatomy and physiology have been introduced in the recent past, there is need to develop 3D virtual dissection and physiology experiments using the modern digital tools.

III-3-489

Digitization of zoology museums can reduce the number of wild animals sacrificed for teaching of biosystematics: another dimension to implementation of 3Rs principle in teaching
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In conventional protocols formalin – or alcohol-preserved or stuffed museum specimens are used for teaching biosystematics and identification of animals. For this purpose thousands of animals are collected from the wild and sacrificed for making museum specimens. This method of teaching biosystematics not only causes loss to biodiversity but also poorly-effective because badly maintained specimens do not provide proper information for identification. Protected animals also not spared because often these specimens are supplied by illegal poachers and traders. Hoplobatrachus tigerinus, Euphlyctis hexadactyla, Uperodon systoma, Saara hardwickii, Chemeleo zeylanicas, Spalerosophis diadema atriceps, Naja naja, Geochelone elegans, etc., are some species which are facing serious threats in Western India because poachers are taking them from wild for supply of museum specimens. In order to circumvent this problem we have digitized the museum and histological slides using high resolution imaging systems to make 2D and 3D images using image processing and analysis software. These digital images are hyperlinked to live videos. Teaching of biosystematics using digitized specimens significantly improves the knowledge and skill. The students also like the digitized museums rather than look at the mutilated specimens. This concept of virtual museum can save millions of animals, particularly those that face threats.

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iii-3-490

Prokaryotes can be used as an effective animal alternative in genotoxicity testing of antifertility and xenobiotic compounds in routine lab courses

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Large numbers of animals are sacrificed to learn toxicology, teratology, and pharmacology. To conduct the very preliminary / pilot experiments large numbers of animals are sacrificed. In the recent past while studying genotoxicity of the male antifertility drug RISUG on Salmonella typhimurium TA 97A, TA 100 and TA 1537, it was found that these prokaryotes can be conveniently used as alternatives for animals in genotoxicity testing in preliminary screening. Vibrio fischi, E. coli tester strain K12 and E. coli BMX 100, can be used in the environmental toxicity assay of xenobiotics. With these prokaryotes the results can be brought out within 24 hours with an efficacy ratio of 9.4% where as animal models require 5 weeks, with an efficacy ratio of only 7.2%. Another advantage is that F1 hybrid is obtained within one day as compared to mouse model which takes 35 days. Designing prokaryotic strain for testing molecules is more convenient than the animal model. The analysis of byproduct of breakdown pathway is also user-friendly. When prokaryotes were used as alternatives in routine laboratory demonstration of genotoxicity of antifertility molecules and xenobiotic compounds, experience revealed to be highly efficacious, fast, cost-effective and subscribed to the principles of 3 R.

iii-3-515

The development of a clinical skills laboratory at Ross University School of Veterinary Medicine

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Several trends have altered the teaching of clinical skills to veterinary and medical students, including increasing recognition of clinical errors as a cause of adverse patient outcomes, the evolution of clinical skills educational research and theory, increasing class sizes, budgetary constraints, and increased focus on alternatives to animal use, for humane and ethical reasons. Accordingly, medical and veterinary schools have established dedicated laboratories for teaching clinical skills, using models, mannequins and simulators. Although these laboratories are being established in medical schools for more than two decades, their incorporation within veterinary curricula has occurred more recently.

In 2007 we decided to establish a clinical skills laboratory (CSL) at Ross University School of Veterinary Medicine. We visited two established, successful CSLs elsewhere. We then considered the range of skills we wished to teach, the physical space, equipment and infrastructure required, including facilities to deliver PowerPoint presentations and case simulations, and others to handle cadaver specimens. We converted an appropriate campus building, hired teaching staff, and sourced models and mannequins for teaching veterinary clinical skills.

Our CSL currently offers instruction in a diverse array of surgical, medical and other clinical skills. We hope this description of our experiences may assist others establishing CSLs elsewhere.

iii-3-564

“Live zoology” an effective, non-invasive replacement alternative to animal dissections in zoology curriculum

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The pedagogy in zoology laboratory in India involves dissections where in animals are purpose killed. The zoology teachers teach the importance of biodiversity/wildlife conservation in the theory courses, and it is paradoxical that the same teachers kill animals for purpose of dissections. Animal welfare groups, educationists and students protest animal kill for dissections. The University Grants Commission, has brought up a specific Guidelines, that animal dissections should be phased out. The Guidelines suggests that during field visits the students shall observe the animals and make record of the observations; the animals shall not be removed from the natural habitat. Conscientious zoology teachers consider learning through dissections as “live zoology”. The various factors such as pedagogical concern, ethical issues, environmental problems, biodiversity conservation, societal
accountability and legal issues are pressures on the educators to replace the present practice of animal kill for dissections to learning of live zoology.

III-3-601

Virtual physiology: computer laboratories for life science education

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Computer laboratories of the Virtual Physiology series (SimNerv, SimMuscle, SimHeart, etc.) are used in lectures, seminars and practical courses at university institutes and schools all over the world and, in many cases, have replaced previous experiments with animal preparations, e.g., with the frog nerve and muscle or the rat heart.

The Virtual Physiology programs are particularly well accepted because of their realistic lab design. They offer completely equipped laboratories on the computer screen with all necessary stimulation and recording devices. All settings of the devices are freely adjustable to perform physiological and pharmacological experiments almost as in the real world. Mathematical algorithms guarantee for physiologically appropriate reactions of the virtual preparations.

Experimentation in the virtual laboratories has particular didactic advantages (Bahar, 2001; Braun, 2003). The students are doing the experiments without negative emotions due to the killing of animals and without the fear that another animal will be killed when they are making a mistake that destroys the preparation. The virtual labs allow free experimentation thereby promoting the most effective type of learning, namely “learning by doing”.

The Virtual Physiology tools are currently reprogrammed with several new features as platform and resolution independent versions. Fully functioning demo-versions can be downloaded from http://www.virtual-physiology.com.

References

III-3-626

3Ts teaching touching toxicology in veterinary medicine degree: a project

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The use of the touch screen technology has grown more and more popular with a new generation of students, leading to the need for an up-dated way of teaching, through a software easily accessible always and everywhere, with an interactive and stimulating approach, designed for the students. A collaboration has started between the University of Milan and the University of Budapest, in order to create such educational tools in the Veterinary Medicine Degree, in the discipline of Veterinary Toxicology, following the 3Rs approach. The educational software, structured in accordance with the requirements of each institution, will contemplate 3 main areas: The General Principles of Toxicology, Systems Toxicology and Clinical Toxicology.

Each main folder will be set up in order to contain specific topics, opened by the user. The clinical toxicology section will also be supported by virtual case-based learning (Balogh, 2014), giving students the possibility to have a tool in a practical discipline, avoiding the use of animals. Implementing technology in didactic program gives advantages for educators in relation to the dissemination of concepts and for the students increasing the interest through new manners.

Reference
Balogh, M. (2014). Veterinary Record 174, 63-64

III-3-631

Synthesis of evidence and systematic reviews of animal studies: urgently needed innovations in laboratory animal science education

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According to Lancet series 2014: “Methodology and reporting of animal studies is currently inadequate and improvements are urgently needed”. Education is a first step to make scientists aware of this situation and to provide guidance on improvements. In health research, transparency on quality of methodology of clinical studies has shown to be an effective method to raise awareness and as a result major improvements have been made. Transparency has been achieved by analyzing clinical trials in a transparent, structured and thorough way using systematic reviews. Recently, systematic reviews of animal studies, as a methodological approach of synthesis of evidence, were introduced within laboratory animal science (Leenaars et al., 2012). Besides stimulating better science, the potential benefits of systematic reviews encompass: (1) leading to better informed ethical review, (2) helping to implement the Three Rs, and (3) improving translational transparency to inform clinical trials (Ritskes-Hoitinga et al., 2014). This innovative topic in education was introduced by SYRCLE in recent years and has been funded by the Dutch Ministry and Health Funding Organization. Content, progress, availability and future needs of education and training programs for systematic reviews of animal studies will be presented. Moreover, effects and results of the training programs will be discussed.

References
Humane education strategies for veterinary education and training in Colombia

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Making the right decisions from an ethical and legal point of view within veterinary science is a complex issue, and learning how to do it should be one of the fundamental pillars of education in our work as teachers. One way to achieve this goal is to integrate into the learning process a theoretical approach to animal welfare and ethics. Another is to develop an assessment of the needs and concerns of students during the learning process. In the last years, research has been conducted in the Faculty of Veterinary Medicine and Zootechnics at the Universidad Cooperativa de Colombia on the need for adopting effective and humane strategies which contribute to the development of students’ skills. The use of non-animal alternative models for anatomy, clinical skills and surgery has become an efficient resource for this purpose. Students have been trained using non-animal models for large animals, small animals and wildlife. Students and teachers are very open to the use of alternatives. It is recommended that curricular committees of each institution acknowledge and approve non-animal models with the aim of establishing the academic guidelines required for their implementation.

Three databases for alternatives in education and training

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InterNICHe provides three online databases to support the implementation of replacement alternatives in education and training. Available at the multi-language website http://www.interniche.org, the free-access resources are continually updated with new information and functionality. The Alternatives Database provides information on over 1000 alternative tools, with descriptions, specifications, images and links to producers. It can help teachers and others identify products to enhance specific practical classes and replace harmful animal use. The resource can be linked into alternative search strategies for universities and training centres. The Studies Database is an academic database, providing references, abstracts and in some cases full papers of over 1000 published studies. Entries are included for their relevance to the pedagogical, ethical and economic issues presented by the use of animals, alternatives and technology. The Downloads Database is a new resource with a range of software alternatives, images, video clips, presentations, posters and banners being added for download. As the resources evolve, a degree of integration of the databases will be developed. Users will be able to search for a training tool, watch a demonstration clip, check availability for borrowing the item, read a related academic paper and contribute a review.

Session III-4: Implementing the “Montreal Declaration on the Synthesis of Evidence”

Co-chairs
Gilly Griffin, CCAC, Canada
Merel Ritskes-Hoitinga, Radboud University, The Netherlands

Session III-4: Oral presentations

Systematic reviews of animal studies; missing link in translational research?

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The methodological quality of animal studies is an important factor hampering the translation of results from animal studies to men. Systematic reviews of animal studies may provide a suitable method to assess and thereby improve their methodological quality.

In our research we investigated: 1) risk of bias assessment in animal-based systematic reviews, and 2) internal validity of the primary animal studies included in systematic reviews of animal studies.

We systematically searched Pubmed and Embase for SRs of pre-clinical animal studies published between 2005 and 2012. A total of 91 systematic reviews met our inclusion criteria. The risk of bias was assessed in 48 (52.7%) of these 91 systematic reviews. Thirty-three (36.3%) SRs provided sufficient information to evaluate the internal validity of the included studies. Of the evaluated primary studies, 24.6% was randomized, 14.6% reported blinding of the investigator/caretaker, 23.9% blinded the outcome assessment, and 23.1% reported drop-outs.

To improve the translation of animal data to clinical practice, systematic reviews of animal studies are worthwhile, but the internal validity of primary animal studies needs to be improved. Furthermore, risk of bias should be assessed by systematic reviews of animal studies to provide insight into the reliability of the available evidence.
III-4-541

Implementation of the Montreal Declaration on the synthesis of evidence

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The Montreal Declaration at WC8 resulted from discussions and research about the insufficient progress of 3R implementation (Leenaars, 2012). Systematic reviews (SRs) are used as a method for the synthesis of evidence, which should lead to better scientific quality, implementation of the 3Rs and better patient safety simultaneously (Hooijmans 2013). Since WC8, international symposia on SRs of animal studies have been held in Nijmegen (SYRCLE, 2012) and Edinburgh (CAMARADES, 2013); and SRs of animal studies were discussed at the Cochrane Collaboration meeting in Quebec in 2013 (Ritskes-Hoitinga, 2014). SRs have demonstrated insufficiencies in reporting of animal studies, and several reporting guidelines and their harmonization will be presented in this session. Developments in toxicology supported by SRs will also be discussed. Finally, stimulation of SRs by the Dutch Parliament and by funding education and development of tools by government agencies will be presented. This session will ask what critical factors are needed to achieve further worldwide progress in synthesis of evidence for stimulating quality of animal studies and creating more transparency on translational evidence.

References


Session III-5: Sharing best practices in LAS education and training

Co-chairs
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Jan van der Valk, Utrecht University, The Netherlands

Session III-5: Oral presentations

III-5-081

The European Platform for LAS Education & Training

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The EU Directive 2010/63/EU requires Members States (MS) to publish minimum requirements with regard to education and training (E&T) of persons involved in laboratory animal science (LAS). In order to facilitate harmonization and subsequent free movement of personnel, an EU Platform & Information Portal for LAS Education & Training (Platform) has been proposed to enable information sharing and communication between approval/accrediting bodies, course providers and MS authorities. One of the Platform’s key activities will be to establish a website to serve as an information portal and database to facilitate information exchange between stakeholders.

The Platform goals involve establishing criteria for mutual recognition of E&T in LAS within Europe and to identify and maintain lists of approval/accrediting bodies and courses. It will help facilitating the establishment of new courses if required, by exchange of information and experiences. Furthermore, the Platform aims to facilitate sharing of information on standards for supervision and assessment of competence. All (non-personal) information will be freely available to trainers, accrediting bodies, potential trainees and other interested parties.

The activities of the Platform are coordinated by a Steering Committee with representatives from course providers, MS and accreditation bodies.

III-5-485

Training in the Three Rs under Directive 2010/63/EU

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The adoption of the principles of the Three Rs within the text of Directive 2010/63/EU requires that animals may only be used in procedures where there is no alternative and that the minimum numbers are used, and that all refinements are used to minimise pain suffering and distress in animals used or bred for use on procedures.
Education, training and, supervision (as appropriate) and competence assessment are essential requirements for all persons carrying out procedures, designing procedures, caring for and killing animals. Knowledge and implementation of Three Rs is an essential element in the training for such personnel.

In February 2014, a common education and training framework was agreed among Member States. Within this framework are a series of Modules, each of which contain a number of Learning Outcomes (LOs) which have to be achieved. There are a series of LOs relating to the Three Rs which all relevant staff have to achieve, and a separate series, requiring a more comprehensive understanding, which have to be attained by those involved in the design of procedures and projects. Working towards the agreed standards throughout the EU will also facilitate free movement of staff and scientists.

Animal user training in Canada

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In 1999, the CCAC published guidelines on: institutional animal user training. This guidelines document defined the need for training all those who were going to be carrying out scientific studies with animals ie, principal investigators, research technicians, post-doctoral fellows, graduate students. The guidelines document provided a recommended syllabus for an institutional animal user program, but it was not prescriptive, recognizing that many institutions already had good training programs in place. For some institutions, however, implementing the recommended syllabus was a challenge, due to lack of resources. The CCAC responded by developing a series of training modules which are available on the CCAC website. The training modules support both the core and non-core topics included in the recommended syllabus. In general, they are developed by experts in the area, and are based on CCAC guidelines. The guidelines document on: institutional animal user training is currently undergoing revision to place more emphasis on the attainment of competency, and to make CCAC’s requirements clear for all categories of personnel (including animal care staff and undergraduate students).

Harmonization of education and training in laboratory animal science

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The International Council for Laboratory Animal Science is a scientific organization dedicated to advancing human and animal health by promoting the ethical care and use of laboratory animals in research worldwide. ICLAS fosters education and training in laboratory animal science, particularly in regions of the world where such opportunities are lacking or few, for veterinarians, animal health technicians, and researchers who are responsible for carrying out animal-based studies. In 2008, the ICLAS governing board published a document on The International Harmonization of Guidance on the Ethical Review of Proposals for the Use of Animals, and on the Education and Training of Animal Users in Science. This document described guiding principles for the education and training for researchers, and pointed to suitable international references. The goal of this endeavor remains to encourage high quality science and animal welfare globally.

Practical training of basic laboratory animal handling technique

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Since 2010, international and national regulations, standards and guidelines were newly implemented or revised. OIE sets international standard of animal welfare including “Use of animals in research and education” in 2010. EU Directive of protection of animals used for scientific purpose was adopted in 2010. ILAR in USA revised its “Guide for the care and use of laboratory animals in 2011. CIOMS revised “The International Guiding Principles For Biomedical Research Involving Animals” in 2012. ISO10993-2 Animal Welfare Requirement for biological evaluation of medical devices will be revised soon. These standards and regulations emphasized 3Rs and training and education of any people involved in animal experimentation for refinement. These should be reflected all over the world including Asian region where practical implementation of laboratory animal welfare used to be less stringent. The author developed “Koken Rat” for a training tool in this field and then remodeled this mannequin as NATSUME RAT which is now available in the market. The practical training of basic laboratory animal handling technique can be achieved using this model. Using NATSUME RAT, the instructors can demonstrate students and researchers not only appropriate handling techniques but also inappropriate mishandlings which cause pain and distress on laboratory animals.

References


With an increasing need of training in Laboratory Animal Science, e-learning appears to be a promising solution to issues of limited time and resources. As e-learning is increasingly used, it is important to understand how students perceive this approach to learning. At IBMC we have integrated e-learning in our advanced (FELASA Category C) training, as a complement to classroom lectures (blended learning) and in our introductory (FELASA Cat B) course (theoretical part exclusively delivered by e-learning). We assessed participants’ acceptance of the e-learning platform and level of satisfaction of its use during the last 3 years with E-learning Acceptance (QeLA), a concordance likert-type scale. This study included 127 participants (60% from the C course), 21 to 50 years old (M=28.42; SD=6.22), of which 76% were women, mainly PhD students and postdocs.

Results revealed that participants from advanced and basic courses show, in general, a positive acceptance to this approach (94.4%), and a very positive perception of the platform usability (70.9%). In both courses participants strongly agreed that e-learning was useful for time management (71.4%). Moreover the majority of the participants recognized that e-learning had a positive influence in practical classes with animals (66.1%).

### Session III-6: Discussion: The role of journals in implementing the 3Rs

**Moderator**

Iratxe Puebla, PloS One, UK

### Session III-6: Oral presentation

**III-6-941**

**Journal publishers: who exactly do we serve?**

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For 41 years, the journal, *Alternatives to Laboratory Animals* (ATLA), has played a significant role in the dissemination of Three Rs-related information, including research papers on the development of alternative methods, reports on validation studies, project findings and conference proceedings, and news and comments and opinions on relevant issues. The journal’s editors have always been mindful of the need to help authors in any country in the world, regardless of their economic circumstances, to promote and further the implementation of alternatives.

Recently, two issues have been pushed to the forefront of the minds of journal publishers, both of which threaten to skew the emphasis away from the needs of authors and toward the interests of the journal itself. These issues are the popular concepts of “open access” and the oft-misused journal Impact Factor. Unfortunately, these two issues have the potential to impact on the already inherently slow progress of Three Rs implementation. With real-life examples of the issues involved, it is hoped that ways in which this impact can be mitigated can be positively discussed in the session.

**III-6-952**

**ALTEX – documenting and disseminating visions and progress on alternatives to animal experiments**

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The topic of alternatives to animal experiments is unique in that it combines basic and applied research, legal regulations, regulatory control mechanisms, activist groups, political decisions and ethical discussions. ALTEX – Alternatives to Animal Experimentation (www.altex-edition.org) provides a scientific platform for constructive communication for all interest groups in this area by publishing full articles and reviews, short communications, letters, comments, workshop reports, corners, news and a calendar of events. The Food for Thought column allows the development of visions for future strategies in the area of alternatives to animal experiments. Any party may submit contributions to the news or the calendar.

ALTEX, like ALTEX Proceedings, a journal for abstracts and proceedings of scientific conferences on alternatives to animal testing and TIERethik (www.tierethik.net), a German-language journal on the bioethics of the relationship between humans and animals, is issued by the Society ALTEX Edition, which is a non-profit organization financed by subscriptions, author publishing fees, member contributions and sponsorship.

The main interest of the Society ALTEX Edition is to promote the 3Rs, thus ALTEX is devoted to the publication of research on the de-
development and promotion of alternatives to animal experiments according to the 3R concept of Russell and Burch: Replace, Reduce, and Refine. Articles having no 3R relevance are rejected before entering the evaluation procedure and ALTEx requires that authors reporting on animal experiments adhere to the ARRIVE guidelines. Consequently, ALTEx chooses to rank the potential direct and indirect impact of an article on implementing the 3Rs over its citability.

ALTEx is an open access journal, which means that all content is freely available without charge and unedited versions of each accepted manuscript are published in the ALTEx Online first section to allow interested parties rapid access to new information. ALTEx articles were accessed on average 1400 times per month from PubMed in the last year and the website was visited by 3800 different users per month. Alerts on new issues of ALTEx are sent out via the Altweb Newsletter.

Taken together, ALTEx strives to promote the implementation of alternatives to animal experiments by informing all types of stakeholders, documenting progress and encouraging strategy-building and networking.

PloS ONE consideration of ethical aspects in publication of animal-based research

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PLOS ONE receives a wide range of manuscripts reporting research from all biomedical fields and many of these involve animal experiments, which can range from experiments on primate models to research on amphibians and cephalopods. PLOS ONE considers that the ethical aspects of experimentation are a critical part of the design and completion of research and we apply strict scrutiny to the aspects of the research related to the handling and care of animals.

We consider it our responsibility as editors to maintain internationally acceptable standards for research involving animals. We see the editors’ duty as going beyond the application of specific journal policies, and consider that as editors we can and should facilitate progress in standards and the implementation of the 3Rs in research by raising awareness in different settings, PLOS ONE does this regularly by following up with institutions and ethics committees when we encounter situations that present ethical challenges.

Open Access publications allow the re-use of published research, increasing its use and reproducibility. These goals are in line with those behind the 3Rs and the PLOS journals support a broader implementation of these principles. Journals play an important role in maintaining standards of research involving animals and we would welcome a discussion on how journals and publishers can raise awareness about best practice in animal research and facilitate progress in this area.

Supporting RRRs through “Special Issues” and the role of journals in “big data”

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Testing for embryo-fetal developmental toxicity and fertility measures is among the largest consumer of animal resources. Obvious concern exists for the appropriateness of animal models for predictivity of teratogenicity in embryo-fetal developmental testing and the RRRs especially in light of the increased requirements from REACH legislation in the EU. The current status of in vitro alternatives is directed at integrative testing strategies focused on promising results from stem cell-based assays, free-living zebrafish embryos, and high-throughput screening (HTS) methods. The integration of in vitro data with in silico models has not, however, been accepted for regulatory purposes.

This is an area of active investigation and scientific publications and reviews. Questions remain as to how to best define and assemble test batteries into predictive models that cover essential steps of fertility and prenatal/postnatal development across different lifestages of the reproductive cycle, including maternal-filial interactions during pregnancy and lactation and the impact on children’s health and well-being to puberty and beyond. One way a scientific journal can support the RRRs is ‘Special Issues’. Since 2009, RTX has published 11 special issues (http://www.journals.elsevier.com/reproductive-toxicology/special-issues). Several have focused explicitly on the 21st century toxicity testing paradigm: the EU’s ReProTect project (vol 30:1, 2010); one devoted to zebrafish embryogenesis (vol 33:2, 2012); and one devoted to the EU ChemScreen project (2014, in progress). Other special issues centered on the annual meeting of the affiliated society (European Teratology Society) have included novel solicitations for manuscripts and review articles addressing symposium themes for the annual meeting. In general, these special issues have been well-cited, generally above the normal for regular submissions to the journal, and have also stimulated subsequent regular submissions in those areas. Journals can adopt guidance statements for validation of in vitro manuscript submissions, targeted testing strategies, and inanimate (virtual) models for embryo-fetal developmental testing strategies. Approaches that use fewer animals but deliver scientifically valid information are highly desired but constrained by the lack of understanding of mechanisms by which drugs and chemicals interact with biological systems and the relevant pathways of developmental toxicity. Journals can encourage harmonization of discovery-based or hypothesis-driven approaches to toward standard Adverse Outcome Pathway (AOP) databases for which quantitative mechanistic relationships can be made, as well as building novel resources for Virtual Tissue Models (VTMs) for spatiotemporal predictive modeling across lifestages. Finally, publishers can play a role in the article of the future that posits large and complex ‘big-data’ (from terabytes and exabytes to zettabytes and yottabytes) difficult to process using conventional data management tools or traditional data processing applications. The trend to big-data enables mining for hidden correlations that are difficult to extract from smaller individual studies, and can address the interplay of complex biological pathways, exposure considerations, and lifestyle considerations (nonchemical stressors) for predictive toxicology. As such, the publishers need to address the issue of data format and availability and novel search strategies to make this happen as big-data unfolds.
Session IV-1: Information requirements on project proposals

Co-chairs
David Anderson, Pentlands Management Systems, UK
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Animal ethics approval and monitoring process in an Australian institution

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Animal based research in Australia requires approval by an institutional Animal Ethics Committee (AEC) and must demonstrate adherence to the principles of the Australian Code (2013) and relevant Commonwealth, State and Territory legislation, policies and guidelines.

The Code details the ethical principles and decision-making framework to guide all involved with the care and use of animals for scientific purposes; an obligation to respect animals throughout their involvement in any project underpins the Code. To ensure appropriate evaluation of project proposals, the Code specifies criteria for categories of AEC membership including scientists, veterinarians, community and animal welfare members.

A duty of care and reciprocal responsibilities of investigators and AECs to ensure ethical acceptability throughout the lifetime of the project encompass all aspects of the planning, review approval and conduct of a project (Rose and Grant, 2013). Applications to the AEC must set out the case that a proposed project is ethically acceptable not only justifying the proposed use of animals but also weighing evidence of benefits and potential impact on animal wellbeing. Alternatives must be applied at all steps of animal care and use.

This presentation will outline the approval process and continuing assessments of an animal ethics application by an Australian institution.

References

Information requirements for project proposals under EU Directive 2010/63/EU

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In September 2013, the National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes endorsed recommendations prepared by an Expert Working Group convened by the European Commission to develop guidance on Project Evaluation. This document contains advice and guidance on the information which should be included in a project proposal to facilitate subsequent evaluation by competent authorities. Applications which contain all the necessary and appropriate information are likely to be processed more quickly. The provision of correct, complete, current and relevant information is a prerequisite.

The guidance provides details of the issues which need to be addressed, offers suggestions on how these can be addressed and gives examples of the common faults in provision of information.

The key requirements are information on the scientific objectives of the procedures they are exposed to; however, this lack of openness may further undermine the public’s trust.

Public trust in animal research practices

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Research institutions often respond to criticisms of animal use by increasing the level of secrecy around how animals are used, including access to information about the types of animals used and the specifics of the procedures they are exposed to; however, this lack of openness may further undermine the public’s trust. The objective of this study was to assess whether people’s willingness to support the use of animals in research varies depending on the openness of the university governance system in place. Participants (n=279) took part in an online survey where they were presented with four different options for the governance of animal research at universities: a) status quo (no information shared), b) some information made publicly available, c) detailed information made available for public comment, and d) detailed information made available for both public comment, plus animal facility inspections. Results indicate that participants were progressively more willing to support animal research under systems that have higher levels of openness, and that have opportunities for public feedback. These results suggest that research institutions may benefit...
from developing mechanisms for better sharing of information and for constructive dialogue regarding animal-based research.

* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

Requirements on project proposals in Singapore

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In Singapore, all research institutions using animals must be licensed under the Agri-Food and Veterinary Authority (AVA) and comply to the “Animals and Birds Act (Chapter 7) Animals and Birds (Care and Use of Animals for Scientific Purposes) Rules 2004”. The project proposals for animal studies must be reviewed by the local IACUC in accordance to the requirements stated in “Guidelines on the Care and Use of Animals for Scientific Purposes” developed by the National Advisory Committee for Laboratory Animal Research (NACLAR) in 2004. In general, written project proposals must be submitted on proposed projects or significant changes in ongoing projects. All proposals must contain sufficient information to justify the proposed use of animals and compliance with the 3Rs Principles including the rationale for involving animals, the species and numbers of animals to be used, and that the activities do not unnecessarily duplicate previous experiments. The project proposal must give a complete description of procedures designed to assure that discomfort, distress or pain to the animals will be limited to that which is unavoidable for the conduct of scientifically valuable research. Further, humane end-points, including a description of any euthanasia method to be used, must be described and justified.

References

Session IV-2: Scientific reporting standards (in vivo and in vitro)

Co-chairs
Mardas Daneshian, University of Konstanz, Germany
Merel Ritskes-Hoitinga, Radboud University, The Netherlands

Session IV-2: Oral presentations

Classifying editorial policies on animal use in science: the EXEMPLAR scale

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Along with external regulation of animal use (e.g., legislation), self-regulation by the scientific community also impacts researchers’ attitudes and practices regarding animal experimentation. One example is journals’ editorial policies (EP) on animal welfare. Researchers’ drive to publish can motivate compliance with EP, particularly for journals of high impact factor (IF). The latter, however, often demand less details on how studies are carried out (Hooijmans et al., 2010). To overcome the shortcomings found on a previously available scoring system (Osborne et al., 2009), the EXEMPLAR (Excellence in Editorial Mandatory Policies on Animal Research) scale was developed. Scoring is divided into four categories (4 points each, maximum 16 points): A) Regulatory Compliance; B) Quality of research and reporting of results; C) Animal Welfare and Ethics and D) Criteria for the exclusion of papers. An analysis of journals publishing papers on murine tuberculosis (n=49) and diabetes (n=29), revealed remarkably low scores (mean=3.56, Median=3). Typically, category A scored higher than all other categories, which were overwhelmingly neglected. This suggests journals’ main concern is regulatory compliance, and displacement of their responsibilities for animal welfare/ethics to institutions. No linear relationship between IF and EXEMPLAR score was found. Higher scoring journals (>8 points) and the highest mean were found in journals with average IF (2,000<IF<4,000).

References

Animal studies: more than a modern sacrifice ritual?

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Currently, one of the main factors for evaluating the performance of scientists is the number of publications in high impact factor journals. This has led to a situation in which editorial boards of journals have gained a decisive power on how science is reported. It appears that many essential details are not reported, not even the two basic starting points of good scientific practice, namely randomisation and blinding, as is demonstrated again and again when performing systematic reviews. Moreover, a lack of correlation between the impact factor of a journal, the number of citations and the quality score of the publication has been reported. SYRCLE (http://www.SYRCLE.nl) is dedicated towards education and performing systematic reviews of animal studies to create more awareness on scientific quality when planning,
executing and reporting animal studies. Besides insufficient reporting, also the quality of planning and execution is in great need of improvement. In addition, it is critical that negative results and other raw data are made available always. The current situation is unacceptable for scientific, ethical and societal reasons. It also raises the question whether animal studies in science are regarded as more than a modern sacrifice ritual?

The ARRIVE guidelines: improving the reporting of in vivo research

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The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is leading an initiative to improve the design and reporting of animal research. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al., 2010; http://www.nc3rs.org.uk/ARRIVE) were developed in consultation with scientists, statisticians, journal editors and research funders with the aim to maximise information published and minimise unnecessary animal use. The guidelines consist of a checklist of 20 items, which researchers can use when designing and reporting scientific experiments to ensure animal studies are robust, transparent and reproducible. Over 350 journals, major research funders, research intensive universities and learned societies endorse the guidelines; the recent translation of the guidelines into popular languages has contributed to their widespread international adoption. The guidelines are now available as a pocket-sized reference – last year, over 3,500 copies were distributed to researchers in 24 countries.

Following on from the ARRIVE guidelines, the NC3Rs is developing an online tool – the Experimental Design Assistant (EDA) – to guide researchers through the design of their experiments and improve the internal validity of animal studies. Through a graphical representation of the experiment, the EDA also provides a tool to improve transparency of design and analysis.

Reference

IV-2-750

Research reporting standards, scientific validity, animal welfare and the 3Rs

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Scientific journals are instrumental in the communication of knowledge around the world and play a key role in disseminating information on animal welfare, the three Rs (replacement, reduction and refinement) and good scientific practices. When reporting research, critical information includes experimental design and analysis, housing and care arrangements, experimental procedures, pain management, humane end-points and euthanasia methods. These details are crucial to the development of more humane science, but are also vital to enable an assessment of whether results are reliable, reproducible and scientifically valid.

This presentation will provide an overview of the analysis and output of a recent ICLAS working group on harmonisation of animal research reporting standards. This will include discussion of data from published studies of journal publication policies (Osborne et al., 2009, 2010) and existing efforts to raise research reporting standards (Hooijmans et al., 2010; Kilkenny et al., 2010; ILAR, 2011). By improving the reporting of animal research, the validity of the research will become easier to assess, whilst also promoting animal welfare and implementation of the 3Rs. This in turn can help facilitate an informed discussion of the ethical issues that are integral to the use of animals in research.

References

IV-2-904

Quality standards for publications dealing with in vitro test systems

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Every study is different and needs to be presented in a different way. But, there is a broad interest in more transparency and clarity of published data. Moreover, publications should provide sufficient information to repeat experiments described in the literature. However, each and every issue of toxicological journals still contains publications dealing with in vitro methods, but that lack essential information. These publications are slowing the progress of in vitro toxicology, not necessarily because there is a problem with their quality and the relevance of their science as such, but simply because it is difficult, and sometimes even impossible, to understand what was really done in the experiments and what was the outcome. Therefore, the Center for Alternatives to Animal Testing – Europe (CAAT-Europe) started a project to define a set of criteria, which should be met as quality standards for publications dealing with in vitro test systems in order to ensure sufficient and thus responsible information. This project involves over 70 co-authors working on different aspects of in vitro data, such as minimum requirements on descriptive information on biological and non-biological tools, methods and techniques, data processing approaches, statistics and data presentation as well as on the wealth and richness of information regarding the message of the publication. The list of criteria compiled here is thought to provide some support and guidance and poses as a plea for a code of good practice, guided by common sense.

References
Kilkenny, C., Browne, W. J., Cuthill, I. C. et al., 2010; http://www.nc3rs.org.uk/ARRIVE

IV-2-677

IV-2-750

IV-2-677
Session IV-2: Poster presentation

IV-2-111
Study on the scientific validity of animal experiments in Switzerland
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Accumulating evidence indicates that poor experimental design and conduct may be widespread in animal experiments (Kilkenny et al., 2009), thereby contributing to poor reproducibility and translational failure (Van der Worp et al., 2010). Journal editors, researchers, and research funders therefore call for improvements, with more rigorous reporting guidelines (e.g., ARRIVE guidelines) being a first step (Howells et al., 2014; MacCallum, 2010; Kilkenny et al., 2010). An important question, however, is whether funders and licensing authorities have sufficient information on experimental design and conduct to decide whether a planned study is worthwhile both economically and ethically. In an attempt to assess this systematically we are currently screening all application forms submitted for approval of animal experiments in Switzerland in 2008, 2010, and 2012 (n=1600). The forms are screened for key indicators of internal validity (e.g., randomization, blinding, sample size calculation) and external validity (systematic variation of study population), and the data will be compared with data reported in publications resulting from these experiments to determine how well the application forms predict the scientific validity of the published reports. The expected results should allow us to identify problems underlying poor experimental design and conduct, and facilitate the implementation of new strategies to effectively prevent them.

References

Session IV-3: Retrospective analysis / non-technical summaries (2010/63)

Co-chairs
Barbara Grune, ZEBET BfR, Germany
Stephen Ryder, Home Office, UK
Moderator
Ursula G. Sauer, EUSAAT, Germany

Session IV-3: Oral presentations

IV-3-283
Establishing a web-based IT system for publishing non-technical project summaries in Germany
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Based on the EU Laboratory Animal Directive2010/63/EU the public has the right of access to information concerning projects using live animals in the European Union (EU, 2010). In order to fulfil this legal mandate the so-called non-technical project summaries are published by the European Member States. Non-technical project summaries provide information on the objectives of the projects, predicted harm and benefits, the number and types of animals to be used, a demonstration of compliance with the requirement of replacement, reduction and refinement. The German Animal Welfare Law and the new German Regulation on the implementation of Directive 2010/63/EU give the German Federal Institute for Risk Assessment the responsibility for publishing non-technical summaries on the Internet (Federal government of Germany, 2013a,b). The Federal Institute for Risk Assessment is currently establishing a web-based IT system for publishing non-technical project summaries in closed cooperation with the competent authorities of the German federal states. The IT solution should cover the key workflows between applicants (scientists), competent authorities at the state level and the Federal Institute for Risk Assessment as well as it should allow user-friendly information retrievals.

References
Requirements for non-technical summaries and retrospective assessment under Directive 2010/63/EU

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Non-technical summaries: To improve transparency and availability of information on the use of animals in scientific procedures, all Member States shall publish non-technical summaries (NTS) of authorised projects as described in Article 43 of the Directive. A consensus on the approach to publication has been agreed by Member States with a significant number already publishing in the agreed common framework. The NTS should include information on the objectives and expected benefits, the numbers and species projected, the expected harms and fate of the animals, and how the Three Rs have been addressed. The information should be presented in a format understandable by the general public – and an example was prepared to help scientists meet this need.

Retrospective assessment: Article 39 of the Directive describes the requirements for Retrospective Assessment (RA). Retrospective assessment is considered a powerful tool to facilitate critical review of the use of animals in scientific procedures, to identify future Three R improvements and, where published, to inform future studies and to enhance transparency to public. In September 2013, the Member States endorsed recommendations prepared by an Expert Working Group convened by the European Commission to develop guidance on Retrospective Assessment.

Annual statistical reports and actual severity

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Directive 2010/63/EU changes how statistics on animal use will be reported, including, for the first time at EU level, information on the impact of scientific procedures on animals. This is in addition to information on the purposes for which animals are used in science within the European Community.

While providing greater transparency and opportunities for targeting of 3Rs initiatives, the assessment of actual severity creates difficulties; it is not a wholly objective process. Without accurate and consistent classification of actual severity across the EU there is a risk that the quality of the data presented will be compromised. The intended improvements in communication and transparency may not be fully achieved.

The criteria set out in Annex 8 of the Directive relate predominantly to prospective classification of techniques and provide little guidance on how actual severity of entire procedures should be assessed.

Additional guidance on severity assessment has been provided for UK users (http://www.gov.uk/research-and-testing-using-animals#annual-returns), but areas of difficulty remain, in particular in the severity of breeding of genetically altered animals; the assessment of the severity of animals found dead; and in the differences between procedural and non-procedural harms.

Some examples of these challenging areas will be presented and discussed.

Experience with publication of non-technical summaries

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The Directive 2010/63/EU calls for publication of the non-technical project summaries of authorised projects and any updates thereto. This regulation opens for questions of, i.e., practicality, safety, academic ownership, intellectual property and economical matters.

The Danish authorities have on its website for ten years published both non technical and the technical parts of all applications for a license to perform animal experimentation.

Experience and problems with this procedure will be presented and discussed.

Making numbers count: 3R gains from animal statistics

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Statistics can be a powerful tool in gaining 3R benefits for animals used for scientific purposes. To make this happen you need to collect correct and relevant data as well as successfully disseminate the results.

Statistics in this field has been collected for over two decades in the EU. Since research areas are continuously developing and the understanding of the 3Rs has grown, the demands for the statistics need to follow this evolution.

Directive 2010/63/EU on the protection of animals used for scientific purposes states more detailed demands for the statistics than before.

Actual severity, origin and generation of non-human primates, and an increased spectrum of diseases and disorders are among the new data to be collected.

With actual severity for each individual noted we will know what severities the animals have been exposed to in which areas and be able to direct 3R efforts to the most urgent ones.

Reporting will be done yearly on a newly designed Microsoft Excel-sheet. It has built-in validating systems enhancing the correctness of the submitted data. The data will be stored and analyzed in an EU database in which the public will have access to reports facilitating the dissemination of data.
Session IV-3: Poster presentation

**Public openness in laboratory research: a survey study**

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The objectives of this study were to model a system that makes animal protocols available for public comment, and identify key factors that affect public acceptance of animal research. Participants (n=247) completed an online survey where five different research scenarios were presented: a) Parkinson’s Disease with chimpanzees, b) organ transplant research with pigs, c) smoking research with mice, d) cancer research with zebrafish, and e) chronic pain research with mice. Participants were asked “Are you willing to support this use of animals in research?” They could choose “yes,” “neutral,” or “no.” Participants were also asked to provide a reason for their choice. Willingness to support the proposed use of animals varied with scenario. The proposal to use mice for smoking research received the lowest level of support (26% of participants voted “yes”). Reasons provided for not supporting this research were framed around a belief that science is well informed on the negative effects of smoking, and that the research is therefore unnecessary. This study illustrates one way in which research protocols could be open to public scrutiny and comment, providing institutions a better sense of how their practices meet public expectations, and which practices are the most contentious.

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Session IV-4: Information systems and databases

**Development of the new Korean guidance on the Three Rs search: adaptations and use of the European Commission’s search guide**

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In Korea, investigators planning to use animals for scientific purposes must complete an animal use protocol and submit it to an Institutional Animal Care and Use Committee (IACUC) for approval prior to commencement of the study. The animal use protocol outlines how the Three Rs (Replacement, Reduction and Refinement) will be implemented in the proposed animal-based procedures after searching for the most up-to-date information on the Three Rs. Based on our national survey of the Korean IACUCs and investigators in 2012, 25% of the respondents were not aware of how or where to find the Three Rs alternatives. We consider that the promotion and protection of animal welfare is one of the core competencies of well educated investigators and other personnel involved in uses of animals. This paper details the development of the new Korean guidance on efficiently and effectively finding information on the Three Rs based on the EURL ECVAM Search Guide of the European Commission. This guide sets out a step-by-step search strategy which provides detailed instructions based on their roles, i.e., investigator or IACUC, and a loose-leaf publication which is suitable for regular updating.

**Considering alternatives: searching for the 3Rs**

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Types and sources of information continue to multiply at a phenomenal rate. While daunting and some times exhausting, mastering the unregulated disordered world of information, and managing the diverse results and outcomes, is achievable. Successful navigation requires a basic understanding of scholarly communication applied to a well-planned and thoughtfully considered search inquiry. Additionally, a clear understanding of the 3Rs and how they are practically reflected in a search is required in order to also address alternatives and produc-
Effectively searching for 3Rs information, specifically, has been researched, results have been published, and guides have been created; notable examples are the NCRBs ARRIVE Guidelines, the systematic review work of SYRCLIE at Radboud University Medical Center in the Netherlands, and the EURL ECVAM Search Guide recently published by Roi and Grune (2013). The question is how laboratory scientists and principal investigators make use of the research in order to comply with regulations and to find useful 3Rs information that may affect their research design. As significant obstacles to scientific inquiry and information acquisition, time, access and expertise are the primary concerns to be addressed.

Reference

IV-4-524
Promotion of the use and development of alternatives by the European Commission: the EURL ECVAM databases
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Ready access to comprehensive information on (non-animal) methods is a prerequisite for their use within decision making processes by regulators for safety assessments or any end-users. To satisfy this need, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) of the European Commission’s Joint Research Centre (EC-JRC) is managing public submission platforms (EC, 1991) providing peer-reviewed information on suitable and adequately described experimental and in silico methods in the field of biomedical sciences and toxicology, such as, the:

i. DB-ALM (Database service on Alternatives Methods) providing information on well over 250 mainly experimental in vitro methods, at all stages of development, validation and regulatory acceptance used for research or regulatory purposes (http://ecvam-dbalm.jrc.ec.europa.eu/)

ii. QSAR model database providing structured information on 70 in silico models for physicochemical, environmental and human health effects (http://qsardb.jrc.it/qmrf/)

iii. TSAR (Tracking System on Alternative testing methods towards Regulatory acceptance) monitoring the method’s progress from proposal for validation until its adoption into the regulatory frameworks (e.g., EU, OECD) (http://tsar.jrc.ec.europa.eu/).

Moreover, additional ongoing projects refer to the EURL ECVAM Search Guide (http://bookshop.europa.eu/en/the-eurl-ecvam-search-guide-plb124391/) and improvements to the DB-ALM and QSAR models databases for easier access. “Non-stop” online demonstrations will be given at the JRC booth during the congress.

Reference

IV-4-689
Sources of information needed to comply with requirements of Directive 2010/63/EU on animal welfare in the field of biomedical sciences in Lithuania – a researcher’s point of view
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Planning investigations in life sciences, researchers have the main task to find the best experimental model which can enhance the experimental design and diminish data variance. Directive 2010/63/EU and best scientific practices, moreover, require consideration of the Three Rs when experiments involve animal use. Having a precise knowledge and, furthermore, access to relevant information resources is a prerequisite to achieve this as a basis for the permission to be granted by Lithuanian Ethics Committee on the Use of Laboratory Animals at State Food and Veterinary Service. However, the only information source suggested by the authorities is the link to EURL ECVAM webpage on their local internet site. The approach generally used by scientists was mainly the search of PubMed® database for available bibliographical data on similar investigations. Recent publication of EUR’L ECVAM search guide was a long expected breakthrough in information retrieval. Further expectations of researchers would be the availability of thematic reviews including basic research topics in reviewed databases. Problems and needs for accessing relevant information to comply with sound scientific practice and demands for animal welfare will be presented from the perspective of a researcher active in biomedical sciences in Lithuania.

IV-4-831
Single database versus multi-database searches for alternatives: a comparative review
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The US Animal Welfare Act requires that investigators consider 3Rs alternatives when proposing to use painful or distressful procedures involving animals. One method of examining potential alternatives is a comprehensive literature search. Often, only one database is consulted despite recommendations that variation in subject coverage and sources indexed requires searching more than one. For this project, we asked whether a single database might perform reasonably close to multiple databases at returning seminal articles for a particular 3Rs question, and if so, which singular database(s) would perform best. We conducted a comparative evaluation of common citation databases including PubMed, Scopus, Google Scholar, and a proprietary multi-database system. A “gold standard” collection of pertinent articles was
established for each question by identifying references used in recently published review articles and guidance documents. After identifying a relevant 3Rs question (for example, eye irritation testing), we conducted simple keyword searches in each database and evaluated the search results against references identified in the “gold standard” collection. Each database was analyzed for sensitivity and specificity to test whether it and the gold standard collection were correlated. Preliminary results show little agreement between references in gold standard collections and those from single database searches.

Session IV-4: Poster presentations

IV-4-035
The status of a new database construction for the safety results of Korean alternative researches
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During the cosmetic development, animal experiments have been used to confirm safety. However, recently the banning of animal test in cosmetics is on progress, and thus various alternative methods to replace animal tests are currently under development and/or being validated in the world.

In Korea, in Nov 2009, the KFDA established the Korean Center for the Validation of Alternative Methods (KoCVAM) that can undertake the developing and validation of alternative methods to animal testing. KoCVAM enables the institutionalization of animal alternative tests by building cooperative system that verifies and reviews alternative methods, and develops internationally certified standards for domestic animal alternative tests.

In developing new animal alternative tests, the research data related to the test substance are important. Specifically, in case of the safety management of cosmetic materials in Korea, the sharing of the safety information is currently missing. It is thus imperative to build a database of safety data, and toxicity mechanism and pathways as in silico method, so we would like to introduce the status of a new database construction for the safety results during Korean alternative researches. This DB will give more information to establish new alternative researches, and to conduct safety evaluation of cosmetic materials.

IV-4-459
Monitoring and evaluation of developments in animal use and 3R-alternatives
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Over the last years the annual numbers of laboratory animal use in the Netherlands remained stable. Annually approximately 600,000 animals are used for animal experiments and testing. Does this mean that not much was done to reduce these numbers or to develop 3R-methods? No. These numbers do not say much about the development and application of 3R-alternatives, or about the value of animal experiments.

There is a political and societal need for more insight into 3R developments and the effectiveness of policy measures. In 2013, the Netherlands Knowledge Centre on Alternatives to animal use (NKCA) published a report that proposes an integrated approach for data storage, monitoring and evaluation of laboratory animal use and 3R-alternatives in the Netherlands.

Developments in the field of the 3Rs could be monitored by using a set of indicators that give information on specific subjects within the broad 3R spectrum, such as the implemented 3R-methods in research institutes; animal use per procedure/test substance; welfare (objectively measured); substitution by evolutionary less complex organisms; publications about the use of 3R-methods; effects of Codes of Practice; adaptations of guidelines; increased awareness/understanding. Experts will have an important role in explaining the stories behind the numbers and figures.

IV-4-476
Use of ASPCA Animal Poison Control Center’s toxicology database for characterizing toxicity of human medications in pets
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The ASPCA Animal Poison Control Center (APCC) maintains a state of the art, fully relational and searchable database containing information on more than two million animal exposure cases. In 2013, approximately 20% of the 180,000 cases received by the APCC involved pets exposed to human medications. The APCC database has been used for characterizing minimum and lethal toxic doses of several human medications, onset time, duration of clinical effects, species sensitivity, new syndromes, trends, better treatment options, and other useful clinical information by using retrospective or prospective approaches. For example, APCC data has shown that pamidronate, a bisphosphonate, is a useful adjunct treatment for treating hypercalcemia from ingestion of calcitriene in dogs. Similarly, high mortality rate associated with acute 5-flourouracil ingestion, isoniazid-induced seizures at low doses, serotonin syndrome from 5-hydroxytryptophan ingestion and its treatment with cyproheptadine in dogs also came from this database. These aforementioned syndromes and treatment options are now well recognized and are great examples of the benefits of leveraging clinical data, in place of laboratory animal studies, to gain critical and valid medical knowledge. These examples also show the significance of finding alternatives to animals in research highlighting the principles of the 3Rs (reduction, replacement, refinement).
There is a bewilderingly large number of websites listing databases, information centres, guidelines and regulatory policies which may be of relevance to researchers attempting to implement the 3Rs of Russell & Burch. The majority of these lists merely give links to the resource’s website, leaving it up to the reader to try and assess the relative importance of the individual items. We are very aware of this problem, having spent nearly 20 years building such a website (http://oslovet.norecopa.no), which now consists of approx. 7,000 pages. Many of the resources on this site were identified by a collaborative effort between the Norwegian Reference Centre for Alternatives and AWIC to locate global resources.

The information gathered is being converted on the oslovet website into a “database of databases”, known as 3R Guide. The resource will function as a fully searchable, English-language tool for researchers, project leaders, animal welfare bodies and others who need to identify relevant resources as quickly as possible.

The oslovet website aims to be a “one-stop-shop” for those needing help to find direct links to global 3R-resources.

**The EURL ECVAM Search Guide – promoting global standards for searching 3Rs information**

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- its promotion by EU Member States implementing provisions of the EU Directive 2010/63/EU
- inclusion of the Guide in the 2012 June/July Top 10 (4th position) of the most downloaded publications by the European Union’s Bookshop
- use of the Guide within laboratory animal science training courses in Europe, Asia and South America
- use of the Guide to produce a Korean Guidance on three Rs searches

The Guide is aimed at untrained database users and provides examples of search procedures, suggested search terms and user guidance. It includes descriptions of relevant sources of information and thesauri.

A check list is moreover offered (the seven golden steps) to allow for searches in a structured way and to document them – a fundamental step in preparing a research project in biomedical sciences and toxicology. In this way, the Guide contributes to a standardised approach for information retrieval that ensures systematic, unbiased and comprehensive searches, as well as it improves transparency.

**An optimized hypothermic storage medium capable to maintain histology and functions of skin tissue and allow epidermal stem cell isolation**

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The human skin models have become indispensable for the cosmetic industry. The full thickness skin as an organotypic in vitro model represents a valuable tool: 1) in toxicology for assaying the effects of dermatological and cosmetic products, 2) in the study of skin inflammatory process.

In our last study we saw that after hypothermic storage during 5 days at +4°C, we found some resistant cells after this extreme storage and we hypothesised that they are epidermal stem cells (eSC).

For characterized ESC, we used following methods: HES staining to check the integrity of junction line at epidermal-dermal interface, specific staining with p63, ki67 and keratin 19 to study the presence of ESC and keratinocyte proliferation and clonogenicity assay to check the ability of a single cell to grow into a colony.

After long storage of skin in our new storage medium, we observed the presence of proliferative cells in epidermis and the high clonogenicity potential of cells.

We demonstrated that our new cold storage medium allows stored skin to maintain self-renewal, clonal growth and cell differentiation of cells.
Data sharing and joint submission as tools for avoiding unnecessary testing for registration under the REACH Regulation

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The obligation to share data applies to any registrant under REACH Regulation irrespective of the phase-in or non-phase-in status of their substance. The analysis of the registration dossiers provides statistical insights as to how registrants have met their obligations to share data. The first ECHA Article 117(3) report from 2011 concluded that the joint submission of information worked well in general as shown by the proportion of total registrations submitted jointly. Further insights will be gained from the second Article 117(3) report due in June 2014.

References
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Data sharing of alternative evidence in replacement research and safety assessment supported by OpenTox and ToxBank

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Data sharing is a key ingredient for advancing alternatives development and application, but its successful implementation involves a complex mixture of scientific, technical, social, legal, quality and business issues. We discuss here the potential of OpenTox in providing open standards and services for data sharing for both federated and
local solutions. Within OpenTox security approaches for authorisation and authentication for component resources were implemented and the use case for the creation of validated predictive models based on a combination of public and private data was demonstrated.

ToxBank created an infrastructure supporting SEURAT-1 at different levels of data sharing: local, partnership, consortium, cluster, and international collaborations and resources (e.g., Tox21, TG-GATEs). Particular attention was given to the processes of data sharing to support in a quality-based controlled and evolving manner different contexts. Protocols linked to datasets emerging from laboratories are provided an accelerated pathway to validation situations. Careful consideration is given to both IP protection and Open Access goals for datasets.

We will discuss a security, privacy and IP review of our resources and others providing some recommendations for the healthy development of secure open, mixed and private big data toxicology clouds for the scientific community.
Theme V – Efficacy and Safety Testing of Drugs and Biologicals

Coordinators
Tobias Schnitzer, Roche Diagnostics GmbH; Penzberg, Germany – IQ consortium
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Session V-1: Pathways based assays in drug development

Co-chairs
Humberto Pinheiro de Araujo, INCQS/FIOCRUZ, Brazil
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Session V-1: Oral presentations

V-1-066

Development of functional human cell based cardiovascular construct for cardiotoxicity assessment

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Functional human cell based in vitro cardiac construct would benefit the present need for reliable in vitro model to replace animal biology based test systems in cardiotoxicity assessment (Guo et al.). Here the aim was to develop an in vitro cardiovascular construct modeling human heart tissue.

In this study, a vascular-like network formed by human adipose stromal cells/fibroblasts and human umbilical vein endothelial cells is used as a natural scaffold. Human embryonic/induced pluripotent stem cell-derived cardiomyocytes (CM) are seeded on top of the vascular-like network to form a cardiovascular construct. Vascular-like network formation, CM orientation and functionality as well as drug responses of the cardiovascular construct were characterized.

Results showed that the vascular-like network increases the viability and functionality of the cardiomyocytes. CMs were elongated and aligned with the vascular-like network and formed a synchronously beating cardiovascular construct. The electrical activity, calcium metabolism as well as response to adrenalin were shown to be normal in the construct.

In conclusion, the vascular-like network supports the orientation and contractile properties of CMs. Our results suggest that the developed cardiovascular construct has the potential to serve as a model suitable for toxicological and safety pharmacological testing of compounds targeting human cardiovascular system.

Reference

V-1-229

Bioengineered organs-on-chips for modeling disease, drug safety and efficacy screening

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More than 30% of promising medications have failed in human clinical trials because they are determined to be toxic despite promising pre-clinical studies in animal models, and another 60% fail due to lack of efficacy. The challenge of accurately predicting drug toxicities and efficacies is in part due to inherent species differences in drug metabolizing enzyme activities and cell-type specific sensitivities to toxicants.

To address this challenge in drug development and regulatory science, the NIH launched the Organs-on-Chips Program to develop alternative approaches that would enable early indications and potentially more reliable readouts of toxicity or efficacy, and provide suitable alternatives for animal testing. The goal of the program is to develop bio-engineered microdevices that represent functional units of the 10 major human organ systems: circulatory, respiratory, integumentary, reproductive, endocrine, gastrointestinal, nervous, urinary, musculoskeletal, and immune. The opportunities for significant advancements in the prediction of human drug toxicities require a multi-disciplinary approach that relies on an understanding of human physiology, stem cell biology, material sciences and bioengineering. This unique and novel in vitro platform could help ensure that safe and effective therapeutics are identified sooner, and ineffective or toxic ones are rejected early in the drug development process.

V-1-295

Fishing for teratogens: a consortium effort for a harmonized zebrafish developmental toxicology assay

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Reference
A consortium of biopharmaceutical companies was formed to develop a harmonized zebrafish developmental toxicity assay (ZDTA). In the first phase of this project, an optimized ZDTA method was developed to evaluate 10 known teratogens and 10 non-teratogens, which resulted in an overall predictive value of 88%. In this project, 38 proprietary pharmaceutical compounds from four companies were evaluated in two laboratories using the optimized ZDTA method in pond-derived or cultivated-strain wild type zebrafish embryos. Compound uptake analysis was also performed with all compounds. Compounds with confirmed embryo uptake of >5% achieved an overall predictive value of 82% & 75% at the 2 respective laboratories. Overall predictivity of the entire test set was 73% for one laboratory and 57% for the other when compared to their respective in vivo mammalian data. Low uptake (<5%) compounds classified as non teratogenic were re-tested up to 1000 µM, which improved ZDTA predictivity to 75% & 60%. When only logarithmic concentrations were considered (0.1, 1.0, 10.0 & 1000 µM), ZDTA predictivity improved to 79% & 62%. Subsequent data analyses showed that technical differences rather than strain differences were the primary contributor to inter-laboratory differences in predictivity. Based on these results, the ZDTA is viewed as a harmonized zebrafish developmental toxicity assay (ZDTA) with an overall predictive value of >80% in a multi-center project by two laboratories using the optimized ZDTA method in pond-derived or cultivated-strain wild type zebrafish embryos.
Session V-1: Poster presentations

V-1-011
Cardiotoxicity of captopril in chick embryos
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Captopril is one of angiotensin-converting enzyme (ACE) inhibitors and is indicated for initial therapy in patients with hypertension. ACE inhibitors are known to alter myocardial function manifested by electrocardiogram changes. With the recent concern for animal rights, experimental studies using mammals have been limited in number and methods. In order to develop alternative methods, we have studied the biological effects of drugs on the cardiovascular system of chick embryos using physiological techniques. The present study evaluated the effect of captopril on the heart in chick embryos.

Fertilized eggs of White Leghorns were incubated and investigated. Captopril was injected into the air sac of a fertilized egg. After injection with drug, the values of heart rate were measured. Electrocardiograms (ECGs) were recorded after the drug injection, and heart rate was determined from ECG wave cycles (Hiroyuki et al., 2009).

After the administration of captopril 0.5 mg/egg, the heart rate was not different compared with control. However, the heart rate was significantly decreased by the administration of 5 mg/egg, 10 mg/egg and 20 mg/egg captopril and arrhythmia was produced.

We have demonstrated that our recording system for ECG of chick embryos is useful for investigating the cardiotoxicity of captopril.

Reference

V-1-018
A zebrafish bioassay to evaluate gastrointestinal function
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Zebrafish are an important vertebrate model system with established and emerging applications for toxicology, drug discovery, and target optimization. Zebrafish fecundity, small size, and transparency through organogenesis, make them suitable for evaluating developmental toxicity of drug candidates, as well as safety assessment of cardiac, visual, and gastrointestinal (GI) functions. GI intolerance is a common preclinical finding and can be a serious safety concern in the clinic. Establishing a rapid, inexpensive, and predictive assay for GI transit would improve flexibility in drug screening and could have significant 3Rs impact as an alternative to conventional GI evaluation. Effects of test compounds on GI transit of fluorescent food were evaluated using fluorescence microscopy and spectrophotometry techniques. Results suggest strong agreement between the two measures and observed effects on GI function match known drug effects in humans. Results indicate significant inhibition of GI transit for atropine (p<0.001) and stimulation of transit for tegaserod and metoclopramide (p<0.001). We conclude that the measurement of GI transit time in zebrafish using a medium-throughput fluorescence assay can be useful for early drug candidate triage and may contribute to the reduction of GI safety testing required in more sentient mammalian species.

V-1-019
Inhibitory effects of ambroxol on type A seasonal influenza virus infection in vitro
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Objectives: Mucolytic agent including, ambroxol and l-carbocisteine is known to have antioxidant and inflammatory properties with reduction of the release of inflammatory cytokines. L-carbocisteine also inhibits influenza A virus (IAV) infection by the reduced expression of the receptor on the human tracheal epithelial cells. Previous studies revealed that the protective effective of ambroxol, a mucolytic agent which has antioxidant properties and stimulates the release of pulmonary surfactant, against influenza-virus proliferation in the airway in vivo was investigated (1). To study whether ambroxol exerted anti-influenza A virus activity on MDCK cells in vitro.

Methods: To determine the inhibitory effect of ambroxol on influenza A virus infection, MDCK cells were treated with ambroxol and l-carbocisteine after/before influenza A virus infection.

Results: Ambroxol and l-carbocisteine treatment after influenza A virus infection had no effect on influenza virus replication, whereas, treatment with ambroxol and l-carbocisteine before influenza A virus infection induced distinct reduction in IVA replication.

Conclusion: These finding suggest that ambroxol may inhibit IAV infection via reduced expression of the receptor for influenza virus in the MDCK.

Reference

V-1-051
Electron paramagnetic resonance (EPR) spin labeling study of HT-29 colon adenocarcinoma cells after hypericin-mediated photodynamic therapy
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Aims: One of the alternative treatment methods for colon cancer is photodynamic therapy (PDT). Hypericin (HYP) derived from Hypericum perforatum is a strong candidate photosensitizer for PDT (1). Membrane fluidity of the cancer cell is related to cancer metastases (2). Presence of cancer cells increase the heterogeneity and also fluidity of plasma membranes (3). Our interest was focused on the biophysical status of plasma membranes in relation to HYP-mediated PDT.

Methods: HT-29 colon cancer cells were treated with 0.04, 0.08 or 0.15 μM HYP concentrations irradiated and examined (24 h). Cells incubated with 10^{-8} M 16-doxyl-stearic acid (16-DSA) spin label suspension for 60 min at 37°C. After centrifugation the pellet was transferred to a capillary. EPR measurements were performed on a Bruker EMX-131 spectrometer at 23 and 37°C and repeated three times.

Results: The obtained spectra were evaluated by EPRSIMC program which provides the calculation of heterogeneous structures up to four spectral components with different fluidity characteristics. Generally, three spectral components were obtained. As the order parameters of the most populated components compared, an increase was observed with increased HYP concentration.

Conclusions: HYP-mediated PDT was observed to be effective on the fluidity of the HT-29 cells.

V-1-083

Nerve-muscle-cultures as an in vitro tool for pharmacologic testing

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Background: The in vivo mouse LD50 assay is routinely used for potency testing of botulinum toxins (Adler et al., 2010). However, this test is associated with severe pain and distress in animals and requires large quantities of mice (Bitz, 2010). Here we established co-cultures of spinal cord and muscle tissue as an alternative in vitro system for pharmacologic testing (Braschler et al., 1989).

Methods: Nerve-muscle-cultures were prepared from mouse embryonic tissue (C57/BL6J) and cultured for 21-24 days (Gähwiler, 1981). In these cultures spontaneous muscle activity was quantified in sham and botulinum toxin-treated cultures for up to 5 days by video microscopy.

Results: Exposing the cultures to different concentrations of botulinum toxin A (1, 2, 5, 50 mouse units) reduced concentration-dependently the frequency of spontaneous muscle contractions.

Conclusions: The strength of the described in vitro assay is that spontaneous muscle contractions are monitored as read-out (Drexler et al., 2011). Thus, the pharmacological endpoint of botulinum toxin, which means a muscle relaxing effect, can be directly tested. This in vitro nerve-muscle-culture system might be a valuable tool for drug testing in the future.

References


V-1-161

Use of upcyte® human hepatocytes to CYP inhibition and induction screening

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upcyte® human hepatocytes are “up-regulated” primary hepatocytes (PHHs) which have been driven into proliferation, whilst retaining important adult cell markers (Burkard et al., 2012). Phase 2 activities are similar to those in freshly isolated PHHs, but CYP activities are equivalent to 5 day cultures. We have improved the upcyte® technology which resulted in higher basal CYP activities and greater responsiveness to inducers. For inhibition studies, upcyte® Hepatocytes were pre-cultured and CYP1A2 wells were induced using omeprazole. All other CYPs did not require pre-induction. The inhibitors were pre-incubated prior to the addition of substrates. For induction studies, upcyte® Hepatocytes were pre-cultured prior to the addition of inducers. The “Relative Induction Score” (RIS) prediction model was used to predict the in vivo induction potential. The RIS prediction model correctly identified the CYP3A4 potency of all compounds analyzed. These results support the use of upcyte® Hepatocytes for CYP inhibition and induction screening. upcyte® Hepatocytes are standardized such that the results are reproducible across experiments; this combined with the sheer quantities that can be generated from one vial of PHHs makes them a promising and unique alternative to PHHs for drug interaction screening.

Reference


V-1-268

Using mode of action framework in prediction of liver toxicity

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The aim of the study was to develop and benchmark different liver toxicity Modes of Action (MOA). MOA, as inner layer of AOP concept, describes key events and processes starting with interaction of a compound with the cell towards biological response. MOA, as a representation of existing knowledge, concerns the linkage(s) between initial chemical binding, defined as the molecular initiating event (MIE), subsequent events on cell, tissue and organ level and biological outcome. In order to identify key events for which non-animal tests can be developed, MOA can be used, thereby facilitating mechanism-based, predictive toxicological assessments. Our focus was on 3 well studied liver toxicants and based on the existing knowledge, we created a computational model of biological pathways by manually annotating and processing molecular data from the literature from the
Trypanosoma cruzi: in silico and in vitro alternatives for identifying new antiparasites

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Identification of new antiparasites involve in vivo assays in the early phases. This includes Chagas disease, an endemic parasitosis caused by Trypanosoma cruzi (Chagas, 1909). Approximately 18 million people are currently infected, and 50,000 individuals die yearly (WHO, 2008). Only two drugs, nifurtimox and benznidazole, are available since 1970s, mostly due to toxicity issues. The objective of this work was to identify new antiparasites by using only in silico and in vitro alternatives. Therefore, we explored lapachone derivatives using cell culture and in vitro assays with T. cruzi infective forms, which revealed a potent biological activity for some of them (Ferreira et al., 2006). Molecules that are toxic for parasites and non-toxic for mammalian cells are then evaluated in vitro and obtained data support the synthesis of candidate drugs (Da Rocha, 2013). This approach showed a structure-based relationship for safety and efficacy of some molecules. The in vitro method was developed by our group (Ferreira, 2006) using CBBR250 dye, whereas for the in silico approach we used free online programs. Using this strategy, we could select the best and most promising molecules hugely decreasing the use of animals on the pre-clinical steps. This process led to two patent submissions that are under evaluation.

References


Antithrombotic potential of synthetic 1,2,3-triazoles evaluated through in vitro and in silico approaches

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Cardiovascular diseases, in which platelet aggregation and blood coagulation are involved, represent a major cause of disability and death worldwide. The current antithrombotic therapies have unsatisfactory results and may produce side effects. Therefore, alternative therapies have been extensively investigated. In this work, we evaluated, through in vitro experiments, the antithrombotic potential of a series of synthetic 1,2,3-triazole derivatives. Platelet aggregation was monitored on a Whole Blood Aggregometer (Chrono log 490 2D) using human platelet-rich-plasma (PRP). Coagulation assays were performed by Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) tests (Wierner Lab), on a Digital Coagulometer (Amelung KC4A). Results showed that some of the derivatives inhibited plasma coagulation on PT and aPTT assays as well as inhibited platelet aggregation on PRP induced by collagen and ADP. Theoretical toxicity studies using the software Osiris Property Explorer also revealed that these derivatives have low toxicity and a drug-score similar to commercial anticoagulant (warfarin) and antiplatelet (aspirin) drugs. In conclusion, triazoles are promising candidates for molecular modeling of new antithrombotic drugs. Moreover, in vitro and in silico studies should be firstly carried out as a trial, before the use of animal models, in the early studies of drug development.

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The effect of additional rodent enrichment on local and systemic bacterial infection models, hematology, clinical chemistry, and serum cortisol

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Objectives: To determine the effects of foraging enrichment added to standard enrichment (SE) in a mouse skin (MS) infection or rat endocarditis (RE) model. Effects on hematology (H), clinical chemistry (CC) and serum cortisol (SC) were examined.

Methods: Animals were group housed into SE (mice, Nestlets™; rats, acrylic tubes) and additional enrichment (AE)(SE with autoclaved hamster food (mice) or sunflower seeds (rats)) groups. After 28 days, a group of mice or rats were euthanized and blood was collected for H, CC, and SC (rats). The remaining animals participated in a S. aureus MS or RE model. In both infection models, untreated animals were compared to vancomycin-treated animals.

Results: Additional foraging material had no apparent effect in either infection model with respect to bacterial load at the infection site or the efficacy of vancomycin. Additionally, H and CC values were similar for each group. SC in rats was lower in the AE group (p<0.03), suggesting that the animals did not experience additional stress from the added enrichment, and may have benefited.

Conclusion: Animals could benefit from the additional foraging enrichment to enable natural behavior. Clinical chemistry and hematology should be evaluated prior to implementing in a research program.

References

Characterizing the gut microbiota as a way to reduce the group size in rodent studies

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The gut microbiota of animal models has a substantial impact on the expression of and the variation in the models (Bleich and Hansen, 2012). E.g., in models of type 2 diabetes the correlation between essential parameters and the gut microbiota composition is 30-40% (Ellekilde et al., 2014), while it in the oxazolone model of atopic dermatitis is more than 80% (Lundberg et al., 2012). Today, high throughput sequencing enables a full characterization of the microbiota based upon a non-invasive fecal sample. In animal experiments group size is calculated as 2 x Z-values (Significance + power)/(Average effect level/Uncontrolled variation) (Ellekilde et al., 2014). Therefore, it is possible to characterize the microbiota composition of individual animals in sensitive studies and thereafter turn the “uncontrolled” variation into “controlled variation” by incorporating the characterization in the data evaluation model. Alternatively, only mice with a gut microbiota coding for a strong expression of the disease could be used. We have previously shown that mice might be inoculated with tailor-made microbiota around weaning to achieve the immunological phenotype induced by the early life colonization (Hansen et al., 2012). A third approach might be to feed the mothers a microbiota-modulating diet that will induce a specific phenotype in their offspring (Hansen et al., 2014).

References
Ischemic preconditioning against myocardial infarction: a systematic review of animal models

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Systematic review of available animal data has shortcomings in the design and quality of animal studies which may play major role in the poor translation of animal data to clinical benefit (Kilkenny et al., 2009; Van der Worp et al., 2010). For cardiac ischemic preconditioning (IPC), a protective strategy in which brief bursts of ischemia induce protection against myocardial infarction, several clinical trials in humans have not replicated the promising results reported in over 500 preceding animal studies (Hausenloy and Yellon, 2011; Ludman et al., 2012).

To identify factors hampering the translation of results from animal studies on IPC into clinical practice, we are performing a systematic review of all animal studies investigating the effect of IPC on myocardial infarction. We performed a systematic literature search in Pubmed and EMBASE. After study selection, study characteristics were extracted from the 556 studies which presented data on myocardial infarct size in an in vivo model. We performed meta-analysis of the impact of study design characteristics (e.g., species, IPC protocol used, co-morbidity and gender) and study quality indicators on IPC efficacy. Results of these analyses will be presented here for the first time.

The results of this systematic review will be used to optimize future, evidence-based, experimental animal studies and may inform the design of future clinical trials.

References


From scientific failures to policy changes: moving away from animal use in research

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The ethics of using animals for research purposes has historically been a focus of attention, but there is an increasing body of literature calling the validity of animal models into question—historically considered the “gold standard.” As a result, there is a growing community of scientists, legislators, regulators, policy makers and members of the industry that support strides toward replacing the use of animals in research and toxicity testing with more human relevant alternatives.

Critical examinations of animal use are also leading to policy changes. As one example, the Institute of Medicine, commissioned by the National Institutes of Health (NIH) in the United States, concluded that the use of chimpanzees in research is largely unnecessary (Altevogt et al., 2011). Following this report, NIH announced that they will retire nearly 90% of their chimpanzees into sanctuaries.

Furthermore, several papers investigating disease models, including Alzheimer’s (Langley, 2014), asthma (Buckland, 2011), shock trauma and sepsis (Seok et al., 2013) have recently been published and have been instrumental in highlighting the lack of reproducibility and validity of animal models in the development of cures.

This growing body of knowledge and how it can ultimately lead to policy and regulatory changes, as well as how science is studied will be discussed.

References


Murine models of human disease: why we must think outside the cage

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In the life sciences, *Mus musculus* is considered to be the quintessential human mimetic species suitable for studying human disease mechanisms and drug responses. Despite extensive murine-based research efforts, mechanisms of disease pathogenesis remain unclear and effective pharmacotherapeutics without adverse side effects remain elusive for many human diseases. Comparative data show major limitations of mouse models for many human diseases, notably type 1 and type 2 diabetes (Roep, 2007; Chandrasekera and Pippin, 2014) heart disease, inflammatory disorders (Seok et al., 2012), ageing (Demetrius, 2005), cancer, stroke, and neurological disorders (Cavanaugh et al., 2014). Immutable species differences have been identified at every level of biological action—from gene expression to whole organism—severely restricting the ability to reliably replicate human molecular mechanisms, pathophysiology, and treatment responses in mice. Despite evidence questioning the purported merit, murine models continue to form the cornerstone of biomedical research today. We review here the most noteworthy murine-human species differences that significantly impair translation and propose a step-by-step strategy by which researchers and funding agencies across the globe can begin to “de-murinize” and, conversely, humanize the study of disease mechanisms and therapeutics. Given the extent of the intractable translational barrier, research and clinical advances require a paradigm shift, systematically incorporating human-relevant technologies that can improve bench-to-bedside success.

References

retained their similarity of the origin donor characteristics of histology and biologic stability better than those established by HCC cancer cell lines. Our results demonstrated that YM155 combination with sorafenib inhibited the PDX tumor growth than sorafenib only-treatment. By Immunohistochemistry assay, the expressions of cleaved caspase-3 (apoptosis marker), Ki-67 (cell proliferation marker), and CD31 (angiogenesis marker) all correlated to the antitumor activity of YM155 combination with sorafenib treatment. In comparison, the drugs treatment reduced serum levels of h-AFP. In summary, we established four HCC PDX mouse models that can serve as a useful platform for evaluating the antitumor activity of drug candidates. This result will provide the future clinical trial of the combination of YM155 with sorafenib in HCC therapy.

V2-746

Refined models for GLP-conform translational studies in the preclinical safety assessment of cell, tissue or organ transplantation with human therapeutic biologicals

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Recent advances in regenerative medicine are based on the understanding that for the majority of pathological conditions there are three therapeutic solutions: replacement strategies using artificial materials and devices; the use of cells, tissues or organs for repair and substitution; the stimulation of the self-healing potential. Cell therapies and cell-scaffold combination products play a major role to deliver an effective therapeutic platform able to heal a variety of chronic, acute, and age-related diseases.

However, one major bottleneck to translate stem cell based science into therapies is the potential risk of some cellular products to induce malignancies in vivo. Regulatory agencies have responded to this risk and have introduced several preclinical safety standards. Nevertheless, the greatest hurdle to test the safety of cell therapies, cell-scaffold combination products and endogenous factors resulting from cell therapies in animal or alternative models is the lack of suitable models able to detect single cells that may develop into a tumour within a cell population of millions of “healthy” cells.

Here, we present an experimental and refined in vivo model that uses immune tolerance induction strategies to provide preclinical, GLP-conform safety studies for human cell therapies able to complement and validate ex vivo test systems.
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Introduction: Animal research is controversial but vital to understand disease mechanisms and to establish new drugs. These studies are burdening, labour intensive and their numbers are rising. We developed a system monitoring animals automatically, free of stress and manipulation.

Methods: The system with artificial intelligence and web access for remote control, recording temperature and activity profiles of multiple animals simultaneously by thermography was evaluated in models of infection/inflammation.

Results: In (semi-)lethal experiments, the combination of temperature and activity allowed prediction of death at least 7.7 h before this event.

A clear discrimination between survivors and non-survivors was achieved. Antibiotic rescue was able to change expected outcome until a distinct degree of prediction. All animals undergoing infection demonstrated a characteristic temperature trajectory, indicating disease progression and severity, but also definition of disease phenotypes. The system allowed non-subjective decision making for euthanasia in survival analyses and pre-mortal tissue sampling from animals predicted going to death. It reduces harm and numbers of animals and enables humane endpoints.

Conclusions: Automated, infrared monitoring offers the opportunity to fulfill 3R-standards by reducing numbers and suffering of animals, improves quality of data by stratification and allows definition of a uniform state of a progressive disease.

Session V-2: Poster presentations

V2-064
Comparative assessment of cytotoxic effects of garlic oil and 2,2′-dithio-bis(N,N-diethyl)ethanamine on human umbilical vein endothelial cells in culture
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It is known that garlic oil (GO) and its sulfur-containing components such as diallyl sulfide, diallyl disulfide and diallyl trisulfide exhibit a broad spectrum of anticancer properties, both in vitro and in vivo (Shukla and Kalra, 2007). However, the exact mechanism of effects of these substances is not known. Earlier, it was suggested that they can provide inhibiting effects on carcinogenesis by acting on endothelial cells and inhibiting neoangiogenesis (Xiao et al., 2006; Thejass and Kuttan, 2007a,b).

We have conducted a comparative assessment of GO and abioticogenic disulfide 2,2′-dithio-bis(N,N-diethyl)ethanamine (DS) on viability of human umbilical vein endothelial cells in culture. Three methods were applied: the neutral red uptake assay, quantification of intracellular ATP and modifications of Mosmann method. We revealed that DS and GO have a similar cytotoxic effect upon the endothelial cells (EC50 ~ 0.6 mM). In addition, it was demonstrated for the first time that DS and GO at concentrations of 0.2 mM and more can serve as mediators of plasma membrane oxidoreductases activity, tetrazolium salts (MTS and MTT) being as the substrate. The mediator effect of DS developed more intensively, so we suggest it could possess the antiangiogenic and anticancer properties, not less or even more than those of GO.

References

V2-386
Trends in the use of genetically modified mice and their efficacy as a model for human disease
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There has been an overall decrease in the total number of animals used for scientific procedures in the past 40 years. However, there has been a consistent increase in the breeding and use of genetically modified animals, mostly mice, over the same period. Genetically modified mice (GMM) have become a routine model for researchers in a number of fields, yet many uncertainties and inconsistencies remain. Specifically, there is concern over the relative efficacy of GMM as an in vivo model for human disease and drug development. This work outlines the relative trajectory of GMM in research and questions their validity. Investigation into the scope of the areas of research, the factors related to the rise in numbers, and the varying mechanisms for genetic modification are made, as well as projections for where GMM research may be heading. The advantages and limitations of GMM are contrasted to determine the relevance and applicability of GMM. It is concluded that, although some GMM models do provide insights into areas of human disease research, the reliance and assumed ability to correlate results with humans may be overstated and requires significant further analysis.

V2-903
Diet-induced iron deficiency anemia in rats – in vivo model
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Objective: The aim of the study was to induce iron deficiency and assess its bioavailability in rats.

Material and methods: During 28 days of the experiment 12 male Wistar rats received the deficit in iron AIN-93M diet and 12 male rats received a complete AIN-93M diet. After experiment blood was collected from the tail and haemoglobin, haematocrit and Fe concentra-
tion in serum were determined. After the Fe deficiency was detected the animals were killed and blood, heart, liver, spleen and kidneys were collected for analyses.

Results: It was found that level of haemoglobin, hematocrit, Fe in serum, Fe in the liver and Fe in kidneys significant decrease in rats fed with the iron-deficient diet. Significant reductions were also recorded for MCV, MCH and MCHC values. No changes were observed in other analysed parameters.

Conclusions: Such an animal model was successfully established within a relatively short time. The applied model can be used for nutritional experiments for the evaluation of the bioavailability of iron.

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V2-931
Role of drug transporters in pancreatic adenocarcinoma therapy
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References

We investigated mechanisms of action of paclitaxel and second generation taxane SBt-1216 in vitro and in vivo models.

Effects of taxane drugs were followed by cytototoxicity test and flow cytometry in model cell lines in vitro and by real-time PCR with relative quantification of gene expression of 15 drug efflux ATP-binding cassette (ABC) and 13 drug uptake solute carrier (SLC) transporters in mouse xenografts in vivo.

IC50 for paclitaxel and SBT-1216 ranged from 15 to 65nM in BxPc-3, MiaPaCa-2, and PaCa-44 cell lines. Cell lines differed in induction of apoptosis and G2/M cell cycle block after exposure to both taxanes. Administration of SBT-1216 caused increase of gene expression of ABCB1, ABCB2, and SLC22A1 and concomitant downregulation of ABCC1, SLC29A1, and SLC29A2 in PaCa-44 tumor xenografts.

Our data shows that both taxanes are highly cytotoxic in pancreatic cancer cells but mechanisms of their action substantially differ. Considering recent data demonstrating activity of nab-Paclitaxel in PDAC patients (von Hoff et al., 2011; Awasthi et al., 2013), underlying mechanisms of taxane action should be further studied.

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V-2-931

Session V-3a: Potency testing of human and veterinary vaccines

Co-chairs
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Session V-3a: Oral presentations

V-3a-108
Replacing the NIH test for rabies vaccine potency testing: a synopsis of drivers and barriers
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Approximately 70% of animal use is utilized to demonstrate quality control of vaccines. Especially rabies vaccine potency testing, using the NIH challenge test, involves objections in terms of scientific relevance, animal welfare concern and costs. Several 3R models have been proposed to refine, reduce or replace this test. Some are formally incorporated into regulatory requirements, but actual regulatory acceptance and use by industry lags behind, raising the question concerning which factors influence this process. This question is answered by a combination of literature review, interviews and a survey among 50 rabies vaccine experts. The findings are analyzed using the multilevel perspective on technology transition, which distinguishes 3 levels of factors influencing innovation acceptance. At the micro level (where 3R models are developed and validated) the dis-advantages of, and fractional experience with, 3R models, scarce data sharing and demanding validation processes exist. The meso level (existing regulatory regime) encloses the barriers of the “gold standard”, the lack of harmonization and the driving force of legislation stimulating 3Rs use. The macro level (the societal context) combines risk aversion an increased concern for animal welfare. Regulatory acceptance and use of 3R models requires dedicated stakeholder communication, cooperation and coordination at all three levels.
V-3a-147

The deletion of the target animal batch safety test from monographs in the European Pharmacopoeia and waiving possibilities at VICH level

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In the past, a target animal safety test (TABST) was indispensable for the release of batches of veterinary vaccines. A double dose of vaccine was injected into each of two animals. The batch passed the test when no systemic or local reaction occurred within 14 days after injection.

The need for release of each batch was questioned. The vague pass criteria and poor statistical relevance of the outcome called the test into question. Moreover it could not be excluded that the test produced wrong negative results.

As a consequence the TABST was deleted from the European Pharmacopoeia in 2013. Nevertheless, the TABST might still be performed due to the lack of international harmonisation. Lead by Europe, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) worked on the “Harmonization of criteria to waive the target animal batch safety testing for inactivated vaccines for veterinary use” and the relevant GL50 is in force since 1st April 2014. European manufacturers can now apply for a waiver of the TABST when exporting to the other VICH regions (Japan, North America) or countries following VICH. VICH is currently working on a comparable guideline for live veterinary vaccines.

V-3a-340

The consistency approach in lot release testing of vaccines

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Vaccine lot release testing is characterised by its extensive use of laboratory animals. Successes have been achieved in implementing Three R methods, but progress is tedious and time consuming.

A new paradigm in lot release testing of established vaccines is the consistency approach. This approach starts from the idea that subsequent lots of vaccine produced can be compared to a clinical/historical lot, which is thoroughly tested and has a well defined profile. The consistency approach has come into reach by improvements in production and control: optimised production processes, a tight protocol for in-process testing using innovative physico- and immunochemical techniques and a state-of-the-art quality monitoring system (GMP, QA).

Consistency testing may lead to a significant reduction in animal use since a narrow set of animal tests performed on each final lot, sometimes with questionable relevance, may be replaced by a battery of meaningful physicochemical-, immunochemical- and in vitro functional tests with enhanced capacity to measure equivalence with batches of proven safety and efficacy.

The paradigm of consistency is an interesting strategy for vaccine manufacturers as it might allow for a reduction in costs and shortening of testing period. This presentation will introduce the consistency approach and discuss advantages and limitations.

V-3a-462

Toward the replacement of the Rabies NIH potency test: International Working Group for Alternatives to NIH test

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Numerous studies have shown that immunization with the native immunogenic form of the rabies glycoprotein G results in the production of neutralizing antibodies and protection against lethal challenge. In human rabies vaccines, antigen quantification is used at final bulk stage, allowing definition of the vaccine final antigen content. Following a NICEATM, ICCVAM meeting, an EPAA project meeting in 2012 focused on gaps in technical knowledge of in vitro G antigen quantification methods and proposed solutions for the replacement of the NIH test. Regulators and manufacturers stressed that the NIH test should be replaced and emphasized that the current in vivo assay should not be used for correlation, since it is highly variable and therefore a concordance strategy should be followed. The ELISA methods under development should be able to discriminate between potent and sub-potent batches: Concordance study. An International Working Group was formed to coordinate a more harmonized approach of the alternative assay developments through the acquisition and distribution of a common set of rabies vaccines. A protocol was established to allow comparison of different ELISA methods. Results from these studies will be presented end 2014 at a Workshop on Alternatives to NIH to form the basis for an EDQM collaborative study leading to the replacement of the NIH test.
Session V-3b: Potency testing of human and veterinary vaccines

Co-chairs
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Session V-3b: Oral presentations

V-3b-120
Residual live virus detection test for rabies vaccine for human use: a 3Rs proposal for the Brazilian pharmacopoeia test method
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Currently, the majority of rabies vaccines for human use are inactivated by betapropriolactone, an efficient and safe agent. However, it is necessary to demonstrate that inactivation was efficient, what can be tested in both animals and cell cultures.

WHO, used to recommend the mouse inoculation test, in at least 20 mice (WHO, 1980, 1987), but, since 2007, this test was deleted (WHO, 2007). However, testing in mice is still recommended in Brazilian Pharmacopoeia (2010) that preconizes for this purpose: A) the inoculation of 20 sucking mice and 20 adult mice; B) the test of viral amplification in cell culture alternatively.

This study, approved by FIOCRUZ ethical committee, evaluates: A) a reduction and refinement of the Brazilian Pharmacopoeia test for detection of residual infectious rabies virus by testing only ten sucking mice euthanized five days after inoculation and tested by the rabies immunofluorescence test; and B) a replacement by a cell culture test.

Results confirm the higher sensitivity of baby mice to rabies virus, that group provided equivalent titers to those obtained with adult mice observed for 21 days. The Cell Culture test also showed satisfactory sensitivity to detect the rabies virus when compared to WHO mouse test.

References

V-3b-125
A cell line assay for in-process toxicity and antigenicity testing of clostridium septicum vaccine antigens
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Safety and potency are basic elements of the quality of vaccines. Clostridium vaccines must be free of residual toxicity and confer a protective immunity. Classically toxicity and potency are evaluated in an animal test; pass/fail criterion is death of the animal. The animals, usually mice, are basically used as the detector system.

Alpha toxin is the major potent toxin produced by the bacterium Cl. septicum. This toxin is toxic for VERO cells as well as for mice. This characteristic should allow the replacement of the detector system mouse by VERO cells. Cell line assays to replace mouse based assays for control testing of various clostridial toxoid vaccine antigens have been developed supported by an NC3Rs. In order to introduce the cell based assay in the regulatory framework a collaborative study under the aegis of the EDQM Biological Standardisation Programme, with the full support of ePAA has been initiated. Batches of toxin and toxoid from different manufacturers are tested by several laboratories from industry and authorities in parallel in mice and in VERO cells.

Successful completion of the collaborative assay should allow the replacement of the animal test in pharmacopoeias.

V-3b-197 *
Functional in vitro testing of the consistency of Bordetella pertussis vaccine production
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Batch release testing of vaccines is primarily based on animal models that are costly, time-consuming and sometimes of questionable relevance, while accounting for approximately 10% of the animal use. A new paradigm in batch release testing is the consistency approach, which is based on the integrated strategy of thorough process- and product-characterization and does not require animal testing. A panel
of physico-chemical assays has been developed, but these assays don’t assess the capacity of the vaccines to induce immune responses. Therefore, we used several functional in vitro assays to study various aspects of innate immune activation by Bordetella pertussis (Bp) vaccines. To mimic inconsistent production, the production process was manipulated resulting in altered expression of proteins that are regarded as important for inducing protective immunity. Using ELISA and mass spectrometry, we demonstrated that the “inconsistent” vaccine contained significantly less of these proteins and a different LPS form than the “consistent” vaccine. In addition, the inconsistent vaccine induced lower innate immune activation compared to the consistent vaccine by different cell types: HEK293-hTLR4, MM6 and dendritic cells. The results of our study show that a combination of functional immunological assays can be used to assess Bp batch consistency in vitro.

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**V-3b-707**

**Consistency approaches for the replacement of Diphtheria Tetanus Pertussis potency assays**

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For Tetanus and Diphtheria potency tests, the replacement of the challenge procedure by a single immunogenicity assay involving antibodies titration using non-animal methods, e.g., ELISA has been described in European Pharmacopeia for several years as an alternative for the release of combination vaccines. Pertussis activity is already assessed using a consistency test with an immunogenicity assay where the lot is compared to a reference vaccine.

The implementation of such immunogenicity assays for the routine control of vaccines is difficult and not always successful mainly due to the variability of these in vivo tests and their current design.

The limitations of current in vivo immunogenicity assays involving a reference vaccine and the alternative approaches based on unidose assays (Geometric mean titer – GMT – of antibody responses) as consistency tools will be presented. For Pertussis vaccines, the unidose assay with GMT read-out is already accepted in North America and described in WHO recommendations.

Reduction and refinement of the in vivo methods currently used for vaccines quality control are important improvements and their complete replacement with in vitro consistency assays will be the ultimate target for the future.

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**Session V-3: Poster presentations**

**V-3-057**

**Thermal imaging – alternative concepts for refinement and reduction of animal use for testing of biologicals**

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Latest technical developments in high resolution Infrared Thermography (IR) provide further prospective opportunities for its use in biomedical research (InfraTec, VarioCam®, high resolution, technical specification). Here, we evaluated five potential refinement and reduction alternatives to long-established animal experiments. Experimental settings comprise (1) safety testing of veterinary vaccines, (2) the rabbit pyrogen test, (3) thermometry in guinea pigs, (4) investigation of oral ovalbumin allergy in mice and (5) tumor modeling.

Results: (1) IR-Thermography is well suited to assess local reactivity of veterinary vaccines causing significant increase of the surface temperature at the injection site. (2) Only pronounced pyrogenic reactivity can be detected by IR. (3) Thermography appears to be a suitable non-invasive tool to monitor body temperature in guinea pigs. Thermometry analysis of the tuberculin skin test did not yield promising results. (4) Thermal monitoring of mice to study clinical aspects of food allergy seems to be a promising application of thermal imaging. (5) Evaluating the tumor status by IR-thermometry promotes timely assessment of efficacious therapeutic intervention and provides the opportunity for early humane endpoints.

Conclusions: IR-thermography holds the potential to reduce pain and discomfort of animals involved in biomedical studies. Methodological limits are related to peculiarities of animal physiology like fur, skin thickness, and metabolic properties in small laboratory animals (Pascoe et al., 2007; Eckert, 2002).

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References


V-3-170
An animal-free batch testing method for alum-adjuvanted veterinary rabies vaccines
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Since many years efforts are ongoing at the Paul-Ehrlich-Institute to develop modern batch testing methods for vaccines and immunologicals according to the 3R concept. Here, we aim to establish an animal-free test method for the antigen quantification of alum-adjuvanted rabies vaccines. The challenge with alum-adjuvanted vaccines is to detach the adsorbed immunogenic components from the adjuvant and make them available to immunological quantification. To this end an electrochemical method was developed by which the antigens are detached from the aluminium salt matrix and simultaneously immobilized on a nitrocellulose membrane. In a second step the amount of desorbed antigen is determined using a quantitative immunoblot.

We can demonstrate that the method is highly specific. The antigens are near quantitatively desorbed and detected. Additional antigens present in the vaccine do not influence the results. The method is reproducible and suitable for all veterinary rabies vaccines currently available on the German market.

Together with additional tests assessing the quality of the adjuvant matrix this versatile and robust antigen quantification method holds promise to form an important component for a new animal-free test strategy for alum adjuvanted vaccines based on the consistency approach.

V-3-201
Notes on the validation of Vero cell test for the absence of residual diphtheria toxin
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Toxicity tests are mandatory during diphtheria toxoid production to verify the presence of residual toxoid after formaldehyde treatment (Specific Toxicity Test – STT) and after six weeks at 37°C (Reversion to Toxicity Test – RTT). Once Brazilian Pharmacopoeia (2010) does not provide for the use of alternative methods, every toxoid batch must be tested in guinea-pigs (500/year). The use of Vero cells as alternative has been described (Hoy and Sesardic, 1994) and according to the institutional QC activities modernization plan, observing the 3R’s concept, we started the methods validation.

Based on the national rules (ANVISA, 2003) this work aimed to validate the Vero cell based RTT and STT in animal proteins-free medium. All the experiments were performed as previously described (WHO, 2013) with modifications: use of serum-free medium and synthetic trypsin; and identification of cytopathic effect by crystal violet staining.

Vero cells cultivated in SFM are 7.5 times more sensitive to diphtheria than in 2% FCS supplemented MEM (Minimum Cytopathic Dose: 1.17x109 Lf/ml versus 1.5x1011 Lf/ml). Interfering components in the toxoid (thiomersal and formaldehyde) had their concentration lower than the citotoxic concentration simplifying the test. Reproducibility and robustness were satisfactory. Therefore, the modified Vero cell test for residual diphtheria toxin can be considered valid according to Brazilian requirements.

References

V-3-203
A serological assay to determine the potency of inactivated rabies vaccines for human use
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For potency determination of inactivated rabies vaccines for human use a classical mouse challenge test is prescribed. Groups of mice are injected intracerebrally with live rabies virus following immunization with serial dilutions of a test vaccine or a standard preparation. The potency of a vaccine is calculated on the basis of the survival rates. This test is imprecise, time-consuming and causes severe distress as animals develop clinical signs of rabies and finally die.

Based on the serological assay mandatory for veterinary rabies batch potency testing (Kraemer et al., 2009, 2013) we have developed a multi-dilution serological assay for human rabies vaccines. This test is performed by immunizing mice with different dilutions of either the test vaccine or the standard vaccine preparation. After two weeks blood samples are taken and tested individually for rabies virus neutralizing antibodies using a modified version of the rapid fluorescent focus inhibition test. The determination of the relative potency of a vaccine batch is based on the serum activities. This assay has several advantages compared to the mouse challenge test: The animals do not suffer from rabies as no challenge infection is necessary, the animal number is reduced, the test is more precise, less expensive, easier and faster to perform.

References

V-3-249
In vitro characterization of adjuvant biological activity and safety
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The use of recombinant proteins as vaccine antigens requires the combination with adjuvants capable to overcome the low immunogenicity
of such antigens. Sanofi Pasteur R&D evaluates new adjuvants combining either emulsion or aluminum particles with or without TLR agonists. Such adjuvants are used to stimulate the innate immune system by triggering the secretion of pro-inflammatory cytokines, however the level of pro-inflammatory response induced by these adjuvants must be moderate to be well tolerated. To document and compare different adjuvant formulations, an in vitro assay based on the measurement of human pro-inflammatory cytokines secreted by a human monocytic cell line, was developed and used as alternative to animal model to evaluate the adjuvant biological activity and safety. Thanks to this monocyte activation test, we documented the benefit of TLR agonist incorporation into emulsion.

Development of relevant serological methods for replacing in vivo seroneutralization tests used for determining potency of diphtheria, tetanus and whole-cell pertussis vaccines

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Multi-dilution challenge tests have been traditionally used for determining Potency of Diphtheria, Tetanus and whole-cell pertussis antigens in vaccines. In Latin-America, we use the classical WHO challenge method (Kendrick test) for pertussis antigen, while a variant developed at NIH and adopted by FDA, USA, based on in vitro toxin neutralization, has been used for Diphtheria and Tetanus toxoids for decades. However, both types of assays have been criticized in terms of variability, ethical and technical issues. As serological tests are more suitable to monitor the quality and manufacturing consistency than challenge assays, the present paper aims to show the progresses we have had in the development and implementation of serology for Tetanus, Diphtheria and Pertussis as alternatives to the seroneutralization assays. In all cases, validation processes were successfully performed for serology, including the demonstration of significant correlation against the routine in vivo seroneutralization assays. Vaccines with proven clinical efficacy were used for validating the relevance of antibodies in mice-serology for Diphtheria and Tetanus. Likewise, immunological functional tests were evaluated for sustaining the role for protection of the total antibodies in pertussis serology. Thus, we demonstrated the significance of using relevant serological methods as Potency testing instead of the challenge assays.

Study on the establishment of standard material for pertussis toxin and standard pertussis strain

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As the National Standard for the residual pertussis toxin (PT) testing of acellular pertussis vaccines in our current national lot release has characteristics of whole cell pertussis, PT having characteristics of acellular pertussis vaccines should be manufactured. And, as B. pertussis strain for use of modified mouse intracerebral challenge assay (MICA) is subcultured by every potency test, results of potency shows high variances. Therefore, establishment of Standards pertussis strain requires to improve quality control level of pertussis vaccines. To manufacture PT, the pertussis Tohama strain was cultured by optimized culture condition. PT was purified from the culture supernatant using various column chromatography. The final PT was prepared by optimization of large scale pertussis culture condition and lyophilization condition. Our study demonstrated that manufactured PT has specific characteristics of PT using in vitro both carbohydrate binding assay and enzymatic HPLC assay. Additionally, the potency of PT was obtained by histamine sensitization test (HIST) from co-work of laboratories and was determined into 27.3 ng/dose (LD50). Strain 18323 of B. pertussis was used for MICA. The final Standard pertussis strain (200 vial) was manufactured and its viable bacteria count was 4x108 cfu/ml. The potency of final Standard pertussis strain was determined into 93 viable bacteria/0.025 ml (LD50).

Determination of effectiveness of immunized chickens’s Immunoglobuline Y against Salmonella infection in mice

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This study was aimed to determination of effectiveness of immunized chickens’s Immunoglobuline Y (IgY) by inactive monovalan (Salmonella Dublin, S. Typhimurium, S. Kentucky or S. Anatum), bivalent (Salmonella Dublin, S. Typhimurium) and combined (Salmonella Dublin, S. Typhimurium, S. Kentucky and S. Anatum) salmonella vaccines. Chickens were immunized with inactive monovalan, bivalent and combined salmonella vaccines, three times at 21 days intervals. Immunized chickens’s eggs were collected after last vaccination and IgY were purified. A total of 100 mice were challenged with salmonella strains by oral route. Then, IgY to mice were administrated by oral route after challenge. Mice were observed for occurrence of morbidity and mortality. Also, feecal samples were analyzed to determination of IgY on spread of salmonella species by feacal. The antibodies titre in blood and eggs of immunized chickens were found to be high by ELISA than before vaccination and controls. Any cases of morbidity and mortality were observed in mice of groups. The numbers of re-isolation of salmonella strains were lower from internal organs and feecal samples of mice in all groups. In conclusion, IgY of immunized chickens were useful for protection against salmonella infections.
Currently, the rabbit test is practiced as end-product pyrogen test for national lot release of blood derived products. However, the replacement of rabbit test needs to be considered due to the high consumption of animals and ethical issues. It does not coincide with international efforts for minimization of animal using test, also represented as 3R.

The aim of our study was to develop an alternative pyrogen test for final products of blood derived products. We established conditions and test method of bacterial endotoxin test (LAL test) for seven blood products. In addition, we conducted monocyte activation test (MAT) based on human fever reaction to study on the possibility of its application to live blood products.

Three laboratories collaborated to examine the validity of established LAL test method for 20 lots of each final products. As a result, we confirmed that endotoxin contents were less than the detection limits of each product respectively, and that recovery rate of the spiked endotoxin met acceptable standard 50~200%. Furthermore, data showing that MAT is capable of covering the total pyrogens and applicable to blood derived products.

However, further studies are needed to verify the propriety of these alternative pyrogen tests for other blood products.

V-3-458

Potency evaluation of Bothrops snake venom: a cytotoxicity assay as an alternative to the murine model

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Snakebites affect about 30,000 persons yearly in Brazil, and 90% (27,000 cases) are due to Bothrops genus. LD50 and ED50 mice assays are the official methods for potency evaluation of venom and antivenom. This work proposes an alternative Vero cell cytotoxicity assay for potency evaluation of Bothrops venom and antivenom. Moreover, this assay can evaluate the cytotoxic effect of B. jararaca venom and its main toxic components (metalloprotease, phospholipase A2) by using enzymatic inhibitors. When tested in the presence of Orthophenantroline (metalloprotease inhibitor) inhibition of 50% Cytotoxic Dose (Cd50) was achieved (from 4.07 µg/ml to 15.62 µg/ml) and with bromophenacyl bromide, (phospholipases A2 inhibitor), values of Cd50 increases from 4.09 µg/ml to 6.92 µg/ml. Therefore, we can conclude that this in vitro potency assay is sensitive to metalloproteinases and phospholipases. These results qualify in vitro Vero cell methodology, as candidate to an alternative method to the murine assay for potency evaluation of Bothrops venom and antivenom.

References

V-3-512

Optimisation of an in vitro assay system as an alternative to current murine histamine sensitization test for acellular pertussis vaccines

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The histamine sensitization test (HIST) is a safety test for batch release of acellular pertussis vaccines (ACV) which monitors residual toxic pertussis toxin (PTx) or reversion to toxicity of the toxoid. HIST is a lethal test requiring large numbers of animals and it is difficult to standardize. Therefore, there is an urgent need to develop an in vitro alternative to this test. An in vitro test system has been developed as a potential alternative to HIST. This test system examines both functional domains of PTx (enzymatic and carbohydrate binding domains) using a combination of enzyme coupled-HPLC and carbohydrate-binding ELISA. A previous international collaborative study demonstrated that the test system is transferable between laboratories and is suitable for differentiating three types of ACV products which sup-
Development of a common immunogenicity assay on guinea pigs for the potency testing of multicomponent vaccines

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A serological potency assay on guinea pigs is now referenced in the European Pharmacopoeia for DTacP vaccine. This alternative assay will reduce the number of animals due to the ability to use the same animals for multiple antigens. We developed and validated according to Ph. Eur. requirements a common immunogenicity assay on guinea pigs with a multiplex antibody detection method. Concordance studies have been conducted on several lots using a multi-dilution assay design. A one-dilution assay design is preferable for routine testing in order to diminish the number of animals. Managing homologous reference on the long run is challenging, especially qualifying a new reference that is similar to the previous one for the response to several antigens. Therefore, we consider an alternative one-dilution assay design that would assess lot conformance based on the geometric mean antibody response to each antigen.

Avian encephalomyelitis live vaccines – alternative methods for batch testing

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Avian encephalomyelitis virus (AEV) causes severe neurological symptoms in poultry worldwide (Calnek, 2008; Tannock and Shafren, 1994).

Control of AEV can be achieved by vaccination of breeder flocks during the growing period (Calnek, 2008). Most available vaccines contain a live, enterotropic, virulent virus strain and are administered via drinking water. The guidelines of the Pharmacopoeia require a titer determination for the final product - a titration in eggs with hatching of chicks or virus titration in cells (European Pharmacopoeia, 2012). The virus titration in cells is not established yet. Therefore, egg titration and the counting of chicks, that show clinical signs (tremor, paralyisis), are the only titer determination methods currently used.

The objective of the project is to refine or replace animal experiments – according to the 3R principles – with a specific, sensitive method. Project one aims at refining the egg titration, by replacing the hatchout approach with antigen detection in the embryonic brain using an AEV-Antigen-ELISA. In project two, cellular aspects of AEV infection will be investigated in order to facilitate the development of a cell culture infectivity assay for vaccines.

References


Refinement and reduction of 3R; in vivo imaging method can refine experimental procedures with less number of animals

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Among 3R, reduction of animal number is the shortest way to improve the animal experiment procedure for the aspect of animal welfare. Usually IACUC of each institute controls the number of animals for in vivo experiments to avoid excessive or unnecessary animal death.

In vivo imaging using molecular signals such as fluorescence or luminescence are already utilized as a routine procedure to diagnose many symptoms for clinics and to analyze cellular phenomena for in vivo imaging methods without

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In vivo imaging using molecular signals such as fluorescence or luminescence are already utilized as a routine procedure to diagnose many symptoms for clinics and to analyze cellular phenomena for in vivo imaging methods without
**The BINACLE (binding and cleavage) assay allows in vitro detection of active tetanus neurotoxin in vaccines**

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Tetanus toxoid (inactivated tetanus neurotoxin) is an essential component of tetanus vaccines. In order to exclude a residual toxicity, safety tests in guinea pigs are prescribed for each toxoid bulk (Council of Europe, 2014a,b). Our aim is to replace these tests, which require thousands of animals annually, by an *in vitro* method.

Tetanus neurotoxin consists of two protein subunits: The heavy chain mediates the toxin binding and uptake by neurons, and the light chain cleaves the neuronal protein synaptobrevin, thus causing a spastic paralysis. We have developed an *in vitro* method for the detection of active tetanus toxin which takes into account the toxin’s receptor binding capability as well as its proteolytic activity (Behrensford-Nicol et al., 2010). By employing this “BINACLE” (binding and cleavage) approach, false-positive results induced by partly inactivated or fragmented toxin molecules can be avoided. The applicability of this assay for the safety testing of toxoids has been examined in an in-house validation study and an international transferability study (Behrensford-Nicol et al., 2013, 2014). The results demonstrate that the method could allow at least a partial replacement of the prescribed animal safety tests. An overview of the current validation status will be given.

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**References**


we attempted to establish an alternative in vitro method. To this end we sensitized guinea pigs with inactivated mycobacterial wet mass. Four weeks later peripheral blood mononuclear cells (PBMCs) were isolated, stained with the green fluorescent dye CFSE and stimulated with serial dilutions of WHO standard and test tuberculin. After 5 days loss of CFSE staining was determined by flow cytometry as a measure for antigen-specific cell proliferation. The potency for test tuberculins was calculated from the dose response curves of the standard and the test batches. The method is robust, sensitive and specific. It yielded reproducible results and it has a wider detection range and is more sensitive than the current skin test. In summary, the method has the potential to replace the most stressful part of the current potency test. It refines the read-out and reduces the number of animals required because more batches can be tested in parallel.

V-4-363

Proteomic analysis of tuberculin purified protein derivatives

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Rationale: PPD tuberculins are heat treated products derived from culture material of mycobacteria. Tuberculins reveal a delayed hypersensitivity in individuals sensitized to mycobacteria and are thus diagnostic tools. According to European Pharmacopoeia requirements batch potency needs to be tested in guinea pigs. Because of ethical concerns and poor reproducibility of these tests, there is an urgent need for in vitro alternatives. Therefore, we are evaluating mass spectrometry to characterize tuberculins and to assess batch to batch consistency regarding the presence and abundance of relevant proteins.

Results: So far, we examined PPD tuberculins from three manufacturers and the WHO International Standard. All PPDs passed in vivo potency testing. In total we identified between 19 (WHO standard) and 36 (Sample A2 & C2) proteins. A subset of 12 proteins was present in all PPDs, of which at least 7 appear to be relevant. 5 of these have been explicitly identified to be markers of tuberculin potency (Whelan et al., 2010; Stavri et al., 2012) and/or identity (Whelan et al., 2010; Stavri et al., 2012; Souza et al., 2012).

Conclusions: Mass spectrometry holds potential to assess batch quality and batch to batch consistency of bovine tuberculins and to further characterize the rather heterogeneous product group of tuberculins.

Funded by the German Federal Ministry of Education and Research 3R-methods to replace and refine legally required animal tests for immunologicals (0316009A – C).

References


V-4-593

Towards a full-function molecular-based assay for botulinum neurotoxin

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Thousands of mice are used annually for potency testing of an ever growing number of products that use Botulinum Neurotoxin as the active ingredient. Here we present a first step in the development of a molecular-based assay to detect binding and translocation of Botulinum Neurotoxin type A (BoNT/A). It consists of a liposome (Giant Unilamellar Vesicles, GUVs) with a BoNT/A receptor (GT1b) on the surface. We demonstrate binding of BoNT/A to the GUVs using a fusion protein (eGFP-HcA) consisting of the BoNT/A binding domain (HcA) and Green Fluorescent Protein (GFP). Microscope images of prepared GUVs with HcA-eGFP show binding to vesicles containing GT1b. GUVs prepared without GT1b did not produce the same objects, so GT1b was necessary for binding of the eGFP-HcA. The next step is to demonstrate binding of a construct containing the BoNT/A translocation domain, in addition to the binding domain, to open the way for high-resolution confocal microscopy studies of translocation. When used with an automated sensitive luciferase reporter assay for the detection of BoNT/A proteolytic activity (van Oordt et al., 2013), the developed system has the potential to replace the mouse bioassay, measuring both the translocation and the enzymatic activity of the toxin.

Reference


V-4-705

Incorporation of the 3R’s into preclinical testing programs to support cellular and gene therapy product clinical trials that are regulated by US FDA

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In November 2013 the US FDA finalized the guidance document “Preclinical Assessment of Investigational Cellular and Gene Therapy Products” that included a section providing recommendations on incorporating the 3R’s in a preclinical testing program. Establishing a regulatory framework that is supportive of the 3R’s in preclinical testing of emerging medical product areas such as cellular and gene therapies (CGT), presents a different type of challenge as compared to
the use of accepted alternative methods in established medical product areas, such as vaccines or traditional drugs. There are no “gold standards” to enable validation of alternative preclinical models (both non-animal and animal) for testing the safety of CGT products. Therefore, to enable continued translational development of these promising therapies, the preclinical testing programs rely on the best available understanding of the product characteristics, putative mechanism(s) of action, and proposed clinical use. Thus, components of the 3R’s are integrated in a preclinical testing strategy that is based on continually evolving science and technologies, to help define product benefit/risk elements applicable to human clinical trial design. The 2013 guidance provides examples of opportunities to address the 3R’s in the development of preclinical testing programs for CGT products for regulatory submissions to US FDA.

V-4-885

**The history of the mouse safety test for sera and vaccines**

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At the end of the 19th century the invention of diphtheria antitoxin was an outstanding achievement in medicine. Large-scale production by an increasing number of companies soon required governmental quality control to avoid the release of batches with contaminations or low potency. The first animal safety test which was legally required was used to control the content of preservatives in the final product. A mouse safety test (MST) was suitable to detect phenol in high concentrations to avoid intoxication of patients. The MST became a standard test for all sera and vaccines developed in the following decades and became part of national pharmacopoeia monographs. When the World Health Organization started its work the MST became part of the general safety test. Today this safety test is still required in most national pharmacopoeias and international guidelines where it may be named abnormal toxicity test or innocuity test. However, over the decades the scientific basis to perform the MST has been lost because chemical-analytical tests have been developed to measure the phenol in medicines. Furthermore phenol is hardly used as a preservative nowadays. Therefore, the MST has lost its scientific basis and should be deleted from pharmacopoeias and guidelines.

V-4-070

**Magnetic resonance imaging – a way to reduce animal numbers for safety testing of veterinary medicines**

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Safety-tests are a mandatory part of the licensing procedure for veterinary vaccines. Many experimental animals have to be killed to undergo a pathologic examination (1, 2). In order to reduce the number of animals, this study aimed at testing magnetic resonance imaging (MRI) as an alternative non-invasive method for evaluating local reactions in living pigs. During the study, 96 pigs (6 groups) were vaccinated into the left neck; each group was injected with a different commercial vaccine. At day 1, 3, 8, 15, 22 and 29 after vaccination, the animals were sedated and scanned using MRI. T1- and T2-weighted Spin-Echo sequences were used to examine the extent of local reactions. Imaging software was used to evaluate volumes of local reactions at both neck sides. Half of the animals of each group (n=8/group) were sacrificed for pathologic examination. A paired t-test showed highly significant differences between the left and right volumes of local reaction. Comparable results were found between histopathologic and imaging examination. The results demonstrate that MRI is suitable for safety-testing of veterinary vaccines in order to evaluate and monitor local reactions repetitively and to avoid euthanasia in pigs, which offers the possibility to reduce the number of animals needed.

This research project is funded by the German Federal Ministry of Education and Research (grant number 0316009B).

V-4-135

**Pulmonary artery hemostasis: a model for surgical stapling and sealing**

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Objectives: Pulmonary artery (PA) hemorrhage is a well documented adverse event in thoracic surgery, requiring appropriate animal testing to ensure medical device safety (Yano et al., 2013; Dunning and Walker, 2012; Craig and Walker, 1995). Thoracic anatomical constraints necessitate high animal usage with traditional canine/dog and ovine/sheep PA hemostasis models (Lacin et al., 2007; Izumi et al., 2007). This study aims to develop a novel porcine PA hemostasis model to reduce/refine animal testing of vessel sealing surgical devices.

Methods: Investigative surgery was conducted to improve PA access, integrating a Likert Scale for rating severity of hemorrhage. The model was validated for repeatability and reproducibility and screened for contributing factors. Animal usage requirements were compared to traditional animal models. Appropriate statistical analysis was performed.
Results: Innovative surgical technique greatly increased PA exposure, provided ample device applications, and significantly reduced animal usage. Traditional PA models yielded 5 device firings/animal, while the newly developed model provided 25 firings. The Likert Scale successfully quantified severity of PA bleeding. External contributing factors did not affect hemostasis. Mechanical stapling and Energy sealing devices were successfully evaluated for hemostasis using the novel PA model.

Conclusion: A porcine PA hemostasis model was successfully developed that increased PA access, reducing animal usage by 5-fold. The model greatly increased device firing opportunities and allowed accurate evaluation of PA hemorrhage.

References

In-vitro potency determination of botulinum neurotoxins A and B
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Botulinum neurotoxins (BoNTs) inhibit acetylcholine release from nerve endings, thus causing paralysis. This effect is broadly used for medical applications and in cosmetics. Because of the high toxicity of BoNTs, the potency of every produced batch must be precisely determined (Council of Europe, 2014a,b). This is currently done by a LD₅₀ test in mice.

BoNTs are subdivided into two subunits. After the heavy chain recognizes its receptors on neurons, the light chain is translocated into the neuronal cytosol, where it specifically cleaves neurotransmitter release related proteins.

Botulinum toxins are structurally and functionally similar to tetanus toxin. For the detection of active tetanus toxin a sensitive and robust ELISA-based tool has been developed, relying on the two most relevant characteristics of the toxin: its ability for receptor binding and its proteolytic activity (Behrendorf-Nicol et al., 2010). We are now generating a comparable test for the BoNT subtypes A and B as an alternative to the LD₅₀ test.

Efficient binding of BoNT/B could be obtained using microplates coated with a synthetic receptor peptide in combination with ganglioside GT1b. The proteolytic activity of BoNT/B could be analyzed using recombinant substrate protein and an antibody specifically recognizing the cleaved product. For BoNT/A, assay development is in progress.

References
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Our aim is to develop a tool for the preclinical prediction of immunogenicity for biologics. Therefore, a statistical approach is presented that incorporates various data sources for the prediction of the clinical prevalence of the immunogenicity of biologicals.

The following steps were taken:
- Build a demonstrator database, Pred-Immune with 14 marketed biologics from 2 different categories, antibodies and recombinant proteins, i.e., antibodies blocking TNF-α, recombinant interferon-α (IFN-α) and recombinant IFN-β.
- Incorporated data from preclinical animal models, physical chemical information and human immunogenicity data together with in-house generated in vitro data in a predictive model.
- Describe the relationship between these parameters into a statistical model.
- Using a demonstrator with 14 marketed biologics, we showed that when incorporating biologic class, biological stimulated dendritic cell CD86 expression, and molecular weight in one model, we obtained predictions that follow expected trends, showing promise for our integrated modeling approach.

Using this demonstrator database, we show it is possible to incorporate multiple sources of information, literature data, physical chemical data, as well as in-house generated in vitro analyses. This model combines different assays, parameters and data in one model, generating a first step towards making a well-informed and structured choice of necessary information for immunogenicity prediction possible.

V-4.365

Photographic evaluation of skin lesions in batch potency testing of tuberculins – a reduction approach

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Rationale: Tuberculins are biological products derived from culture material of mycobacteria. When injected intradermally, tuberculins are capable to evoke delayed hypersensitivity in individuals infected by mycobacteria of the same species. Tuberculins are thus used for diagnostic purposes in human and veterinary medicine. To assess batch potency, compendial methods stipulate evaluation of skin lesions in previously sensitized guinea pigs (e.g., Monograph 01/2008 0536. Tuberculin Purified Protein Derivative, Bovine. European Pharmacopoeia, 8th edition). Apart from ethical concerns, the major drawback of this procedure is the poor reproducibility of test results. Here, we evaluated software assisted digital photography and infrared thermography as potential reduction alternatives to on-site manual measurements of lesions.

Results: Software assisted digital photography is suited to assess local reactivity in tuberculin batch potency testing. It allows for repeated measurements and thus helps to meet European Pharmacopoeia requirements for test validity in terms of precision of the potency estimate. It also facilitates documentation of test results in a QM environment. In contrast, infrared thermography did not reflect skin reactivity.

Conclusions: Software assisted digital photography is suited to reduce the number of test repeats and thus the number of animals used for tuberculin batch potency testing. Since covered by the current wording of the relevant monographs, immediate implementation is possible.

Funded by the German Federal Ministry of Education and Research 3R-methods to replace and refine legally required animal tests for immunologicals (0316009A – C).

V-4.461

Local lymph node assay on five medical devices including dental materials for alternative safety evaluation


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ISO 10993 is used for biological safety evaluation on medical devices such as dental materials. Recently 3R’s concept and alternative methods has been adapted to the field of safety evaluation of medical devices with their frequent uses. Non-radioactive LLNA (OECD TG 442), representative alternative method for skin sensitization, have advantages that reduce animal uses and refine their suffer comparing with guinea pig methods (OECD TG 406). In this study, two LLNA (ELISA and noble flowcytometry method, FCM) were conducted to generate background data on medical devices. The noble metal alloys for dental casting (2 items), zirconia block for milling (1 item), biliary stent and vascular stent were selected. All test material was extracted according to “Accelerated Extraction Method” in ISO 10993-12:2012 and all extracts were evaluated in their appearance, pH and concentration of ions using ICP-MS. As results of two LLNA, 5 extracts have all negative responses in both LLNA. Otherwise, HCA (25%) that used as positive control were shown positive responses (SI≥1.6 in ELISA and SI≥3 in FCM method). These results were same with previously guinea pig methods results.

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References

V-4-599

An alternative micromethod to assess the procoagulant activity of Bothrops jararaca venom and the efficacy of antivenom

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The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms relies on the traditional rodent lethality assay (LD50), a time-consuming and expensive test that causes animal suffering and requires a large quantity of venom and animals. Validated in vitro tests are important for assessing the neutralizing capacity of immunized horse plasma and for the quality control of antivenoms. Considering the procoagulant activity of Bothrops jararaca venom in in vitro assays with mammalian plasma and the slow dynamics of fibrin formation in avian plasma, we propose a new coagulant assay. The clotting time was evaluated using the thromboelastometric profile of chicken plasma samples versus standardized doses of venom, either in the absence or presence of anti-bothropic serum (ABS). Ten nanoliters of ABS significantly neutralized the procoagulant effect induced by 0.3 µg of B. jararaca venom. Although it was not possible to obtain a direct correlation between our results and that obtained by the LD50 assay, this micromethod represents a highly sensitive technique for the characterization and quantification of procoagulant activity of small amounts of snake venoms and for the detection of specific antibodies against this activity using a minimal volume of antiserum.

V-4-717

Development of tridimensional murine and primary human osteoblastic cells models for biocompatibility studies

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A considerable number of animals is employed on biocompatibility studies of bone substitute materials. Cell culture models contribute to 3Rs through biocompatibility assessment by cytotoxicity, mineralization and differentiation assays. However, monolaminar bidimensional culture fails to simulate in vivo tridimensional cell-cell and cell-matrix interactions. This work presents trial of protocols of both MC3T3 cell line and primary human bone cells (HBC) to determine the ideal procedure and cell density to obtain spheroidal cultures and access its adequacy to material cytocompatibility tests. The sample included 0.5, 1, 2, 3, 4, 5, 7.5, 9 and 11x10^6 cells (n=15 per group) that were seeded on 24-well plates coated with agar, and incubated in agitation from 1 to 7 days. Cell aggregate morphology and quality was observed by Scanning Electron Microscopy (SEM), Confocal Microscopy and score methodology. Cell cytotoxicity was accessed by an adapted XTT assay. Higher cell numbers formed more stable spheroids. Handling and observation of spheroids was easier starting from 3x10^6 cells. Confocal microscopy and SEM revealed that cells within the core of the cell aggregate are viable. Aggregates were stable and presented good viability when employed on standardized assays, adapted for tridimensional cell culture, testing different ceramic, metallic and polymer-based biomaterials.

V-4-724

The application of rat hepatocyte aggregate for cytotoxicity assay

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It has reported that the cellular functions, especially the metabolic functions, of 3D reorganized tissues convert locally because of their cell-cell communications or the slight difference in the culture environments (Landry et al., 1985; Waentig et al., 2011). Thus, the examination of metabolic functions in 3D tissues should be considered their sizes to obtain physiological response for the application. This study examined the applications of rat hepatocytes aggregates in cytotoxicity assay to demonstrate the size-dependent physiological functions using mycotoxin and anticancer drugs.

The different-sizes rat hepatocyte aggregates were formed on the oxygen-permeable honeycomb microwells (Shinohara et al., 2013). Cytochrome 1A1, 1A2, 3A4 and 2C9 activities of rat hepatocyte aggregates were determined by P450-Glo™ Assay (Promega). Hepatotoxicity of Cisplatin and Aflatoxin B, were examined. The toxicity to human lung adenocarcinoma epithelial cell line (A549) of rat hepatocytes aggregates-mediated metabolites using CPT-11 were also tested.

The cytochrome assays and hepatotoxicity tests of rat hepatocyte aggregates demonstrated that medium size aggregates (52 and 88 mm) had more resistance to hepatotoxic chemical than that of other conditions. The results of cytotoxicity test using A549 cells exposed to rat hepatocyte-mediated metabolite of CPT-11 confirmed that 3D reorganization into certain size was important to obtain physiological response.

References

V-4-727

Development of a mock circulatory system for evaluation of mechanical heart valves

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The simulation of the human circulatory system has been a major focus of cardiovascular research, particularly for the evaluation of mechanical heart valves. This work presents the development of a mock circulatory system, which allows the testing of mechanical heart valves under physiologically relevant conditions. The system consists of a pump that simulates the left ventricle, a reservoir that represents the systemic arterial circulation, and a series of tubing and valves that mimic the arterial and venous structures. The system is designed to allow the control of pressure and flow conditions, enabling the evaluation of valve performance under various physiological scenarios. The mock circulatory system is an essential tool for the preclinical testing of heart valves, ensuring that they can function effectively in the human body before clinical implementation. This approach helps to reduce the risks associated with valve failure and improves patient outcomes. Further research is required to refine the design and functionality of the system, aimed at achieving even closer mimicking of the human circulatory physiology.
Monocyte activation test (MAT): identification of monographs that recommend rabbit pyrogen test (RPT) and bacterial endotoxin test (BET) as a kick-off for showing applicability of MAT

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Purpose: Modelling of mechanical heart valve (MHV) flow dynamics is a challenging biomechanical problem and currently requires extensive in vivo validation. In vitro particle image velocimetry (PIV) techniques can be used to optimize MHV flow dynamics; however accurate pressures and flow rates are required to mimic the native heart. With newer valve technologies under development, PIV must be combined with accurate mock circulatory systems (MCS) to optimise MHV design and, therefore, reduce in vivo experimentation.

Methods: A MCS including the left heart, aorta and systemic circulation was constructed with a clear Perspex chamber to house mitral MHVs. Ventricular contractility, vascular resistance and arterial compliance were controlled respectively via a custom pneumatic controller, gate valve and variable Windkessel chamber. Haemodynamics were recorded and used to validate the MCS against the literature. The MCS was then used in combination with PIV to evaluate MHV flow dynamics.

Results and conclusion: The MCS successfully replicated the haemodynamics typically seen in healthy and failed human hearts. PIV evaluation of MHVs was successful and demonstrated the system’s capacity to optimize MHV design before progressing to in vivo trials. Therefore, the combination of MCS and PIV used in this study should enhance MHV design and reduce the requirement for in vivo experimentation.

Applicability of monocyte activation test (MAT) in the routine of the quality control laboratory

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Some injectables are still tested by Rabbit Pyrogen Test (RPT), mainly biologically. The aim of this study was to use MAT in hyperimmune sera (N=22) previously analyzed by RPT both considered negative and positive. Moreover, Zymosan spiked sample were also studied as non-endotoxin pyrogen. Sample were kept in contact with cryopreserved whole human blood. Saline solution that have passed the RPT were contaminated with different concentrations of Zymosan (from 1,000 to 5,000 ng/ml), kept in contact to cryopreserved whole human blood and in both cases, IL-1β release was quantified. These same concentrations were injected in rabbits in order to verify the fever response. MAT results were compared to RPT (sensitivity: 100%; specificity: 75%). This specificity can be explained due to a higher sensitivity of the MAT, which detected as positive samples that passed the RPT after repetition. It was also established that the 5,000 ng of Zymosan/ml was the threshold limit value for the RPT and MAT. These partial results strongly indicate that MAT may in the future replace RPT.

Assessment of pyrogenic contamination with lipoteichoic acid (LTA) in the monocyte activation test (MAT) and rabbit pyrogen test (RPT)

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LTA is a non-endotoxin pyrogen of great importance in the pathogenesis of sepsis. RPT is able to detect a great number of animals to be used. The Bacterial Endotoxin Test (BET) cannot fully replace RPT since it only detects endotoxins. MAT is sensitive to all types of pyrogens and is based on the same biological characteristics.
mechanism responsible for the fever reaction in humans. ICCVAM has recommended its utilization for other pyrogens than endotoxin since its equivalence to RPT can be demonstrated. This work aims to evaluate the ability of MAT to detect LTA contamination. MAT used cryopreserved human whole blood and IL-1β release was measured. Rabbits were injected with different LTA dosis and the rectal temperature measured for 3 hours. The same curve was tested for both assays. Rabbit fever response was observed from 75,000 ng of LTA/kg and in MAT the limit detection was established in 50,000 ng/ml of LTA, or 5.41 UIE/ml. MAT showed to be more sensitive than RPT. Results suggest that MAT was efficient in detecting LTA and may contribute to the acceptance of this test by the Brazilian regulatory agencies and the replacement of animals.

V-4-946

Isolation of Shiga toxin-producing Escherichia coli (STEC) using magnetic beads

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In order to isolate pathogenic microorganisms from the natural environment, foods, or human-derived specimens, we commonly apply methods that utilize selection media or antimicrobial resistance. In cases where those methods are not applicable, desired microorganism for isolation was selected using laboratory animal passage. This research is an attempt to use magnetic beads for isolating STEC from mixed microorganisms in specimens. Monoclonal antibodies of Shiga toxin 1 and 2, which were excreted by STEC, were produced and then attached to the magnetic beads. Afterwards, STEC isolation from non-pathogenic E. coli was examined and then attached to the magnetic beads. Afterwards, STEC isolation from non-pathogenic E. coli was examined. In terms of sensitivity and specificity, STEC 1CFU and 10CFU couldn’t be isolated but isolation ratio was higher than 80% at 10²CFU. The isolation ratio of STEC from other enteric pathogens (Salmonella, Vibrio, Shigella, EIEC, EPEC, ETEC) was higher than 80% in average. In particular, E. coli O157:H7 strain EDL933 was isolated by over 90%. Such findings demonstrated that a magnetic bead-based method is effective in isolating pathogenic microorganisms. This method will reduce laboratory animal passage if it is applied to virus, Borrelia and Rickettsia that cannot be easily isolated by conventional methods.
Efficacy assessment of novel anti-viral, anti-inflammatory and mucolytic agents using human airway epithelium (MucilAir™)

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The human airway epithelial cells play crucial roles in the pathogenesis of important respiratory diseases such as asthma, cystic fibrosis (CF), chronic obstructive pulmonary diseases (COPD) (Huang et al., 2009). Furthermore, due to the particular tropism of bacteria and viruses, sometimes, only human cell/tissue models are suitable for studying these human pathogens and for anti-viral and anti-bacterial drug development (Huang et al., 2011).

Using MucilAir™, a standardized human airway epithelium model both morphologically and functionally fully differentiated which can be maintained at a homeostatic state for more than a year, the following tests have been developed:

- Anti-inflammatory: Based on IL-8 release, this assay allows ranking anti-inflammatory drugs upon challenge with LPS.
- Anti-viral agents: human Rhinoviruses such as the type-C, extremely difficult to grow on other cell models, infect and replicate efficiently in MucilAir™; the replication is almost completely inhibited by novel anti-viral agents such as Rupintrivir. These results demonstrate that MucilAir™ is a reliable and powerful tool for anti-viral and anti-bacterial drug development (Tapparel et al., 2013).
- Mucolytic agents: using cilia beating frequency measurement, mucin secretion and mucociliary clearance as end-points, the effect of reference compounds and comparative study between different pathologies (COPD vs CF vs Normal donors) has been evaluated.

IL-13 induced asthma model in human precision-cut lung slices

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Novel therapeutic treatments are required for patients suffering from chronic respiratory diseases. Rodent model reflect some features of allergic asthma, however, human physiology is missing. To reduce animal testing and to overcome limitation of rodent models human precision-cut lung slices (PCLS) were chosen as an ex vivo tissue model. To mimic features of allergic asthma, e.g., airway immune response, mucus hypersecretion and airway hyperresponsiveness, human PCLS were stimulated with interleukin (IL)-13.

PCLS were prepared from human lungs and incubated with IL-13 for induction of inflammation, mucus production as determined by elISA. IL-13-induced bronchoconstriction was measured after methacholine (MCh) provocation and visualized by videomicroscopy. Specificity was proven by usage of IL-13 antagonists.

Human IL-13 induced the secretion of eotaxin-3 and TARC. Both cytokines were blocked by addition of inhibitors (anti-IL-13 or anti-IL-4Rα chain). Human IL-13 induced mucus hypersecretion (2-fold compared to control) in bronchial tissue. Strikingly, airway hyperactivity was induced here demonstrated by decreasing eC50 values for MCh from 180 nM to 47 nM and by an increase in maximal bronchoconstriction.

This study shows that human tissue mimics features of airway immune response, mucus hypersecretion and airway hyperreactivity, which can be used for drug development and preclinical testing.

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Research from basic science has remarkably changed the general perception of 3D organotypic tissue models. Precision cut lung slices (PCLS) display such a suitable ex vivo tissue model that maintains microanatomy and functionality of the respiratory tract. The model allows the investigation of effects of compounds and drugs directly on cytokine release and functional responses such as bronchoconstriction under similar experimental conditions in different species including human. The tissue can be stimulated with, e.g., chemicals, lipopolysaccharides, bronchoconstricting agents and disease-related proteins. Depending on the underlying immunology, lipopolysaccharides and proteins such as IL-13 induce an acute increase of pro-inflammatory cytokines and/or airway hyperresponsiveness. Effects of chemicals were shown to correlate with in vivo inhalation toxicity studies. We found that the tissue response is highly comparable with the in vivo response. In summary, PCLS can be used to model several features of lung injury, COPD and asthma ex vivo. The different tissue responses can be used for the prediction of toxicological endpoints and adverse health outcomes such as organ injury, respiratory sensitization and inflammation. The presentation will give an overview about the current use of lung tissue in inhalation toxicity but also state their use for drug research.

Responses of in vitro asthmatic human airway epithelial cultures to rhinovirus and poly(I:C)

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Human rhinovirus (HRV) is a common cold pathogen and inducer of asthma exacerbations. Human airway epithelial (HAE) cells are targets of HRV infection. To gain increased understanding of mechanisms linking HRV infection to asthma, we compared the response of submerged and ALI cultures to HRV, the effects of HRV and Poly(I:C) in HAE cultures, and evaluated differences in HAEs from asthmatic and non-asthmatics responses to HRV. Cultures from 6 asthmatic and 6 non-asthmatic donors were exposed to HRV or Poly(I:C). Gene expression and cytokine/chemokine secretion were determined at 1.5, 6, 24 and 48 h post-exposure. Genome-wide gene expression changes were analyzed using RNA-seq technology. HRV and Poly(I:C) induced significant responses in gene expression and cytokine/chemokine secretion. Responses were dependent upon culture condition and exposure agent. Poly(I:C) elicited stronger responses in submerged cultures, but was less effective in eliciting effects from ALI cultures. HRV elicited stronger responses in ALI cultures, but was less effective in eliciting effects from submerged cultures. Compared to other models, ALI culture systems challenged with HRV better recapitulate in vivo responses to viral infection. Evidence of differential expression in asthmatic vs. non-asthmatics was shown. This in vitro model may facilitate understanding of viral exacerbation mechanisms.

Intravital imaging of human lung tissue for simulation of severe lung infections

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Worldwide pneumonia is the most frequent cause of death due to infections and mortality rates remain constant since decades. Therefore, new therapeutic options for immune-modulation to control destructive hyper-inflammation are needed. However, the overwhelming majority of scientific data is based on mouse models and molecular knowledge of human pathogen-host-interactions is underrepresented.

Therefore, we established a human lung tissue culture model to investigate infectious processes directly in the human alveolar compartment. A great advantage of the model is that human pathogenic bacteria and viruses can now be investigated which are unsuitable in animal models. Next to determination of replication rates, cellular targeting and tropism as well as immune factor regulation we established overviews of functional molecular microscopy such as FRET/FRAP became possible.

Our recent publications, e.g., contributed to the understanding of alveolar type II cells during infection, showed the cellular replications niches of different types of Influenza A or even the alveolar damage of emerging SARS-like MERS-corona virus (Hocke et al., 2013a,b; Knepper et al., 2013; Szymanski et al., 2012; Weinheimer et al., 2012).

Taken together, the use of human (lung) tissue can serve for the simulation of human diseases and their analyses on a high technical level thereby allowing for the replacement of animal experimentation for the addressed hypotheses.

References


**Session VI-1b: In vitro disease models – Infection**

**Co-chairs**
Mario Fabri, University of Cologne, Germany  
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**Session VI-1b: Oral presentations**

**VI-1b-393**

*In vitro triple culture of inflamed human intestine as a model to investigate nanoparticle safety and efficacy*

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Nanoparticles offer several advantages to improve drug delivery to the intended site of action, which is promising for inflammatory bowel disease. Nevertheless, the interactions between nanoparticles and biological systems might be an important issue in disease conditions. Therefore, advanced and reproducible *in vitro* models that mimic intestinal inflammation are valuable as alternative model to animal tests (Leonard et al., 2010). Here we improved the 3D model of human inflamed intestine (Leonard et al., 2010), by using cell lines to have a more robust and reproducible system to assess particle toxicity and drug delivery efficacy.

THP-1 and MUTZ-3 were embedded in a collagen layer in transwell filter, with Caco-2 cells cultivated on top. This model was inflamed with IL-1β and particle toxicity and anti-inflammatory efficacy assessed through pathophysiological changes (epithelial barrier function and pro-inflammatory cytokines release). Au, TiO₂ and Ag particles, relevant to oral exposure, showed more realistic results regarding toxicity and inflammation in the 3D culture compared to the monoculture. Anti-inflammatory effect on 3D inflamed culture was reached with PLGA based nanoparticles and microparticles loaded with Cyclosporine A. Thus, the present model of inflamed human intestine is valuable to evaluate cytotoxicity and efficacy of engineered nanomaterials relevant to oral exposure as well as drug delivery efficacy.

**Reference**

**VI-1b-525**

*Regulation of pro- and anti-inflammatory human Th17 cell properties*

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Th17 cells have emerged as a new T helper cell lineage involved in the clearance of extracellular bacteria and fungi. A dys-regulated Th17 response, however, can induce severe tissue destruction and autoimmunity. Therefore, mechanisms must be in place to shield the host from immune-mediated damage.

We demonstrate that human Th17 cells transiently produce the anti-inflammatory cytokine IL-10 upon stimulation. Interestingly, IL-10 expression was accompanied by reciprocal down-regulation of IL-17, leading to a functional regulatory Th17 cell phenotype after the peak of the effector response. The ability of Th17 cells to express IL-10 was, however, restricted to certain antigen specificities. *Ex vivo* isolated C. albicans specific Th17 cells could not produce IL-10 in comparison to *S. aureus* specific Th17 cells. This was due to differential priming requirements of these Th17 cell sub-populations. IL-1beta instructed naïve T cells to develop into a pro-inflammatory non-IL-10 expressing Th17 cell subset. Th17 cell priming with *S. aureus*, however, was not IL-1beta dependent, leading instead to the generation of IL-10 producing Th17 cells with self-regulatory activities. This approach revealed that IL-1beta is a molecular switch for determining a pro- versus anti-inflammatory Th17 cell functions.

**VI-1b-581**

*Vitamin D-dependent antimicrobial pathways in human macrophages*

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T cell-mediated activation of macrophages is crucial for effective control of intracellular pathogens, such as mycobacteria. However, the mechanisms by which human macrophages kill intracellular pathogens are not well understood. We investigated the T cell-mediated activation of macrophage antimicrobial responses by studying primary immune cells isolated from human skin and blood. We found that activation of macrophages by T cell IFN-γ and/or CD40 ligand induces an anti-mycobacterial pathway dependent on the intracellular conversion of 25-hydroxyvitamin D (25D) into the bioactive 1,25-dihydroxyvitamin D (1,25D). 1,25D mediated activation of the vitamin D receptor results in the induction of antimicrobial peptide expression, as well as autophagy, which is required to overcome the phagosome maturation block in infected macrophages. Of relevance, vitamin D production is dependent on exposure to UV light and reduced in dark-skinned populations. In our model, sera from white individuals with sufficient amounts of 25D, but not sera from African-Americans with lower 25D levels, support the vitamin D host defense response. In summary, we could show that T cells activate a vitamin D-dependent antimicrobial activity in human macrophages and that cutaneous vitamin D synthesis and host defense pathways are possibly linked in humans.
A human in vitro allergy model showing allergen specific immune responses using house dust mite or grass pollen allergen

**Methods:** Antigen-presenting cells were generated from whole blood of healthy or allergic donors. APC were stimulated with house dust mite extract (HDM) or Phlp5 and co-cultured with autologous CD4+ lymphocytes in the presence of allergen and or immunomodulatory compounds. Effects on T cell proliferation and cytokine secretion were analyzed by \(^{3}\)H-thymidine incorporation and ELISA or Multi-plex analysis.

**Results:** Allergen-pulsed APC induced strong proliferation of the CD4+ lymphocytes. the presence of immunomodulatory plasma-cytoid DC (pDC) in the cocultures inhibited this response by about 50%. Proliferation of HDM-stimulated PBMC was dose-dependently reduced by pDC showing a significant deregulating functionality.

**Conclusions:** Allergen-specific T cell response can reproducibly be measured using the human in vitro allergy model showing regulatory properties of pDC. This model mimics regulatory mechanisms of allergic immune responses and might be applicable for efficacy testing of immunoregulatory cellular therapeutics or biomolecules with greater predictivity for the human situation.

**Session VI-1c: In vitro disease models – Skin**

**Co-chairs**

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Ellen van den Bogaard, Radboud University, The Netherlands

**Session VI-1c: Oral presentations**

**VI-1c-124**

**Modeling hypersensitivity of human skin towards UV radiation from the DNA-repair deficient genetic syndrome xeroderma pigmentosum**

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Sun exposure has been clearly implicated in premature skin aging and cancer development. The deleterious effects of UV radiation are exacerbated in patients with xeroderma pigmentosum (XP), a rare genetic disease caused by a deficiency in nucleotide excision repair (NER) of UV-induced mutagenic lesions. XP cells therefore represent a relevant model to study sequences of events leading to skin photaging and photocarcinogenesis.

Using keratinocytes and fibroblasts isolated from small skin biopsies of XP-C patients, the first XP-C organotypic skin cultures were generated (Bernerd et al., 2001). They could reproduce the NER defect in situ after UVB exposure and revealed yet undescribed defects in epidermal differentiation. Moreover, XP-C skin reconstructions unveiled the pro-invasive role of XP-C dermal fibroblasts, leading to the formation of epidermal invasions resembling early steps of neoplasia. Further characterization of XP-C fibroblasts revealed a photoaged-like phenotype, including an overexpression of MMP1 and a higher content of intracellular ROS (Frechet et al., 2008).

Reconstruction of XP skin in vitro thus represents a valuable three-dimensional model to study the impact of UV radiation in a context of photosensitivity. Furthermore, the possibility to reconstruct genetically corrected XP-C skins with restoration of DNA repair and cell survival after UV exposure now opens perspectives for therapeutic approaches (Warrick et al., 2012).

**References**


**VI-1c-282**

**PPAR agonists do not exhibit their beneficial effects in inflammatory skin diseases by upregulating FLG expression**

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Loss-of-function mutations in the filaggrin gene (FLG) are the major predisposing factor for atopic dermatitis (AD). Peroxisome prolifer-
Psoriasis is an inflammatory skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation affecting 1-3% of the global population. Availability of an in vitro psoriatic tissue model will facilitate drug discovery. In the current study, normal human primary keratinocytes and psoriatic fibroblasts were harvested and cultured to

\[ \text{References} \]


* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

**VI-1c-625**

**Approaching an in-vitro model for atopic dermatitis**

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The transferability of data generated in the animal appears to be limited (Hartung, 2013), human-based in-vitro disease models may allow for improvement in pre-clinical drug development. Reconstructed human epidermis/skin can make the step to skin disease models manageable which may improve the development of topical dermatics. We develop a dual approach to the signs and symptoms of atopic dermatitis skin which includes the knock-down of the filaggrin gene (Mildner et al., 2011; Küchler et al., 2011) and inflammation induction by cytokine exposure (Weindl et al., 2011). Filaggrin knock-down alters morphology, the order of stratum corneum lipids (Vávrová et al., 2014) and thus barrier function and response to irritants of reconstructed human skin (Küchler et al., 2011). Topical glucocorticoids improve the induced inflammation (Weindl et al., 2011). As nanoparticles can enhance the notoriously low skin penetration and induce drug targeting to defined skin strata, the atopic dermatitis model is now used for the detailed investigation of topical drugs including tacrolimus which is beyond the preferable range of skin penetration because of a high molecular mass.

**References**


**VI-1c-782**

**An in vitro reconstructed psoriasis tissue model for evaluation of drug therapeutics**

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Psoriasis is an inflammatory skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation affecting 1-3% of the global population. Availability of an in vitro psoriatic tissue model will facilitate drug discovery. In the current study, normal human primary keratinocytes and psoriatic fibroblasts were harvested and cultured to

\[ \text{References} \]


* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.
form a highly differentiated 3D tissue model. The tissue model was characterized for histological features, gene expression, and cytokine release patterns that are associated with psoriatic phenotype. Results showed that psoriatic fibroblasts can induce psoriatic phenotype in vitro with overexpression of HBD-2, psoriasin, elafin, and ENA-78, similar to the in vivo situation. Cytokine analysis showed increased release of IL-6 (7 fold), IL-8 (5.5 fold), and GRO-α (3.8 fold) compared to control tissues. Confocal microscopic evaluation revealed:

1) hyperproliferation of basal epithelial cells (Ki67 staining), 2) increased psoriasin, elafin, and CK16, and 3) reduced levels of filaggrin. Topical treatment of the tissue model with three over-the-counter psoriatic drugs decreased HBD-2, psoriasin, elafin, and ENA-78 gene expression. In conclusion, the in vitro psoriatic tissue model is anticipated to be a valuable tool to accelerate safety and efficacy studies of candidate therapeutics.

Session VI-1: In vitro disease models – Poster presentations

VI-1-009
Evaluation of in vitro inhibitory effect of Enoxacin on Babesia and Theileria parasites
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Enoxacin is a broad-spectrum 6-fluoronaphthyridinone antibacterial agent (fluoroquinolones) structurally related to nalidixic acid used mainly in the treatment of urinary tract infections and gonorrhea. Also it has been shown recently that it may have cancer inhibiting effect. The primary antibabesial effect of Enoxacin is due to inhibition of DNA gyrase subunit A, and DNA topoisomerase. In the present study, enoxacin was tested as a potent agent against the in vitro growth of bovine and equine Piroplasms. The in vitro growth of five Babesia species that were tested was significantly inhibited (P<0.05) by micro molar concentrations of enoxacin (IC50 values = 13.5, 7.2, 7.5 and 24.2 µM for Babesia bovis, Babesia bigemina, Babesia caballi, and Theileria equi, respectively). Enoxacin IC50 values for Babesia and Theileria parasites were satisfactory as the drug is potent antibacterial drug with minimum side effects. Therefore, enoxacin might be used for treatment of Babesiosis and Theileriosis especially in case of mixed infections with bacterial diseases or in case of animal sensitivity against diminazin toxicity.

VI-1-025
Inter-laboratory validation of an innovative huFcERIα-RBL-2H3 degranulation assay for in vitro allergenicity assessment of whey hydrolysates
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Background: Cow’s milk-derived whey hydrolysates are substitutes for allergic infants. Currently, huFcERIα-RBL-2H3 cells, sensitized with serum IgE from cow’s milk allergic patients, are used to assess in vitro residual allergenicity (van Esch et al., 2011). However, limited availability and inter-lot variation of sera impede standardization of safety testing using recombinant technology in degranulation assays.

Objective: An oligoclonal pool of chimeric (chu)Ige antibodies against bovine BLG was generated. Furthermore, an inter-laboratory ring trial was performed to investigate the reproducibility.

Methods: Six chimeric antibodies were generated comprising mouse variable domains and human constant IgE/K domains. These antibodies were tested for binding to the huFcERIα-RBL-2H3 cells and their subsequent degranulation with whey, BLG or whey-based hydrolysates with different hydrolysis grades. The ring trial was performed with five different hydrolysates.

Results: Anti-BLG chuIgEs sensitized huFcERIα-RBL-2H3 cells demonstrated degranulation upon cross-linking with whey, 18 kDa BLG, and 5-10 kDa hydrolysates, but not with a 3 kDa hydrolysate. The ring trial showed a good intra- and inter-correlation between four participating laboratories.

Conclusion: The huFcERI α-RBL-2H3-assay using BLG-specific chuIgEs is very robust and reproducible. In addition, these in vitro data obtained matched previous outcomes of in vivo allergy models, underscoring the potential predictive value of this huFcERIα-RBL-2H3-assay as alternative to animal studies.

Reference
**VI-1-192**

**An assessment of the cell stress response of lung epithelial cells exposed to cigarette smoke aqueous extracts**


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The ability of cells to adapt and survive environmental and physiological stress relies on activation of cellular stress responses characterised by increased oxidative stress, inflammation and apoptosis. These processes occur in the development of many tobacco smoke related diseases. As one of a suite of *in vitro* models for a comparative assessment of tobacco related products, an *in vitro* lung model of the cell stress response was developed. Human bronchial epithelial (NCI-H292) cells were grown to confluence in 96-well cell culture plates and exposed to cigarette smoke aqueous extracts (CSExaq: 0-67%) from a reference (3R4F), a reduced toxicant prototype (RTP) and an equivalent commercial control cigarette for 4 hours. The intracellular ratio of reduced to oxidised glutathione, caspase 3/7 activity and secreted IL-1β, IL-6 and IL-8 were then measured as oxidative, apoptotic and inflammatory endpoints respectively. Exposure to 3R4F CSExaq induced a concentration dependent increase in all cell stress response endpoints. RTP CSExaq (67%), induced significantly lower oxidative, apoptotic and inflammatory (IL-6 only) responses compared to the commercial control. This model is useful in the comparative risk assessment of reduced toxicant prototype cigarettes and with development could also be applied to the assessment of electronic nicotine delivery systems.

**VI-1-571**

**Human organotypic cancer model**

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Multiple xenocraft mouse models have been generated to understand cancer, with the disadvantages of less predictive, expensive or technically complicated procedures. Here we present an innovative *ex vivo* organotypic tumor invasion model using living human precision-cut lung slices (PCLS) and cancer cells.

An AdGFP transduced human breast cancer cell line MDA MB 231 was added to human PCLS over a period of one week. Viability assays show intact human tissue during the infection with the cancer cells. Growth curves and Ki67 staining reflect proliferation of cancer cells over the observation period time in human PCLS. Immune response and neoangiogenesis were determined by the cytokine markers VEGF, IL 10, IL 1beta and GM CSF. The decrease of the proinflammatory cytokine IL 1beta was linked to the number of MDA MB 231 associated macrophages in human PCLS. The model mimics cancer cell proliferation in the microenvironment of human tissue without using artificial substances. It provides the possibility to gain insights into functional local immune responses with human physiology background. The model can be adjusted to other cancer targeted organs. In terms of the 3R concept, this alternative model does not require any animal experiments and takes advantage of human tissue.

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**VI-1-568**

**A model of neuronal hyperreactivity in passive sensitized human organotypic tissue**

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Obstructive lung diseases are characterized by increased sensitivity of the airways (AHR) including peripheral neuronal hyperreactivity. Especially TRPV 1 positive non myelinated afferent C-fibers are believed to be involved in development of AHR. Local TRPV1 positive nerves are extensively co expressed with sensory neuropeptides. Local axon reflex response leads to the release of neuropeptides and provoke neurogenic inflammation including bronchoconstriction, vasodilation, inflammatory cell migration and mast cell degranulation. Using passive sensitized organotypic human precision cut lung slices (PCLS), it is possible to reflect partly asthmatic conditions. Capsaicin induced activation of the peripheral sensory neurons in passive sensitized PCLS leads to neurogenic inflammation including bronchoconstriction and mast cell degranulation. Bronchoconstriction was analysed by videomicroscopy using cross-sectioned airways in PCLS. Mast cell degranulation was determined by histamine ELISA and confocal microscopy. In contrast to *in vivo* asthma models in mouse, rat or guinea pig, *ex vivo* human PCLS predict human situation regarding peripheral nerve and neuropeptide composition, airway constriction in response to neuropeptides, local immune cell answer and airway mi-

croanatomy. Thus passive sensitized PCLS provide a suitable model to analyse features of obstructive lung diseases, like neuronal hyperreactivity, without the use of animal experiments.

**VI-1-578**

**Nucleus pulposus and annulus fibrosus cell isolation from human intervertebral disc**

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**Introduction:** To study mechanisms of intervertebral disc (IVD) degeneration, *in vitro* organ culture systems with live disc cells are highly appealing. Most disc cells are isolated from animal tissue. We established a relatively quick and easy protocol for isolation of nucleus pulposus (NP) and annulus fibrosus (AF) cells out of the IVD fragments.

**Materials and Methods:** Human intervertebral disc fragments can be obtained following discectomies. In sterile conditions, disc fragments are collected. The tissue is cut, grinded and partially digested with trypsin. After centrifugation, sediment is harvested and cells seeded in suspension, supplemented with special media containing high nutrient level. Characterization was made and sub-isolation of nucleus NP and AF cells followed.

**Results:** In appropriate environment, isolated cells retained viabil-
ity and proliferated quickly. Both NP and AF cell cultures were stable. Under standard culture conditions, cell proliferation and cluster formation was observed. Cell viability was 90%. The number of apoptotic cells and necrotic cells was positively correlated to cell seeding density.

Conclusions: The demonstrated isolation process is simple, quick and economical, allowing viable long-term organ culture. The availability of such a system permits study of cell properties, biochemical aspects and therapeutic candidates for human discs in a well-controlled environment.

VI-1-730

Low concentrations of cigarette smoke extract act cytotoxic on human alveolar epithelial cells type II

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Alveolar epithelial cells type II (AECII) play an important role in normal pulmonary function, host defense, and immune response. The human tumor-cell line A549 is the most popular model of AECII.

In the presented study, the effects of cigarette smoke extract (CSE) on human primary AECII, A549, and the adenocarcinoma-cell line NCI-H1975 were investigated.

Tumor-free lung tissue from patients who underwent lobectomy due to cancer was used to isolate AECII. CSE was obtained using a shell-less model. This model includes many advantages such as the simplicity of in vitro cultures as well as the relevance of the in vivo models in addition to a low cost. Three axes were developed.

X. Rebillard and P. Nirdé

VI-1-840

Application of in vitro skin models for cosmetic product efficacy: UV protection, skin lightening, skin hydration and anti-aging

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In vitro skin equivalent models offer the capability to screen for safety and efficacy of raw cosmetic materials and formulations intended for topical application. Here we describe three commercially available skin tissue models, EpiDerm™, EpiDermFT™ and MelanoDerm™ that are amenable to multiple applications of interest related to cosmetic product development and efficacy testing. EpiDerm™, produced from normal human keratinocytes, is widely used for assessment of irritation, percutaneous absorption, cytotoxicity, cytokine release and has recently been utilized for evaluation of skin hydration. EpiDermFT™ is a full thickness in vitro skin equivalent produced from primary keratinocytes and primary fibroblasts containing a functional barrier and fully developed basement membrane. This model is well suited for evaluating cosmeceutical endpoints such as UV protection (e.g., CPD analysis) and skin aging biomarkers related to extracellular matrix remodeling. MelanoDerm™, a tissue containing primary keratinocytes and melanocytes, can be used to evaluate skin lightening following treatment with topically or systemically applied cosmetic ingredients allowing for measurement of macroscopic darkening and melanin production. Utilization of these tissue models for cosmetic product testing can be highly valuable in streamlining product development efforts and reducing the use of animals for testing purposes.
Session VI-2: Use of stem cells in screening

Co-chairs
Mario Beilmann, Boehringer-Ingelheim, Germany
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Session VI-2: Oral presentations

VI-2-275

The DNT-EST: a predictive embryonic stem cell test for developmental neurotoxicity testing in vitro
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As the developing brain is exquisitely vulnerable to chemical disturbances, testing for developmental neurotoxicity of a substance is an important aspect of characterizing its tissue specific toxicity. Embryonic stem cells can be differentiated toward a neural phenotype, and this can be used as a model for early brain development. 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; Hayess et al., 2013). 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 in parallel, also measuring viability and proliferation. Here, we show that chemical testing of a training set comprising nine substances allows the formulation of a mathematical prediction model that can discriminate positive from negative DNT compounds with an in vivo – in vitro concordance of 100%. Based on these results our current work aims to establish three-dimensional cortical tissues from embryonic stem cells to better model the complexity of the central nervous system for in vitro neurotoxicity assays.

References

VI-2-532

Human stem cell-derived cardiomyocytes in cardiac safety research
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Induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) are increasingly used as a new source of cardiac cells for drug safety assessment. We investigated the effects of 20-60 reference compounds (mostly known to have cardiac effects) in 4 different types of stem cell-CMs, and using 5 different technologies, including two high content screens (HCS)-Ca²⁺ transient measurements, multielectrode Array (MeA), optical action potential, and xCelligence. We also compared data of 20-reference compounds in vitro-isolated rabbit wedge model. Our data suggested that 1) using hiPS-CMs with HCS screen technologies (Ca²⁺ transients and Optical AP) could be suitable to detect drug-induced QT-prolongation, shortening, and increase in beat rate; 2) xCelligence technology could be used to detect drug-induced chronic/delayed (days to weeks) cardiac effects in vitro; 3) hES-CMs and hiPS-CMs from different cell providers and different technologies could result in some different readouts. 4) The current HTS stem cell technologies do not differentiate causal mechanisms of certain different classes of drugs (e.g., Ca²⁺ channel blockers from IK₁,₅ channel openers), and additional lower throughput in vitro assays (ion channels) and in vivo assessments are still needed to clearly define MOA and ultimate risk position of new NME’s.

VI-2-254

Defining normal developmental dynamics for human in vitro neuronal differentiation – applications for setting a baseline for adverse outcome assessments
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Increasing reliance on in vitro models for developmental toxicity demands careful characterization of these models. We have previously adapted a human neural progenitor cell (hNPC) model to assess developmental neurotoxicity. To assess relevance for in vivo development, we differentiated hNPCs up to 21 days and evaluated changes in pro-
Neural stem cells as screening tools for developmental neurotoxicity

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Cell-based assays that model key cellular events have been proposed for high throughput screening of chemicals for developmental neurotoxicity. While *in vitro* systems cannot fully replicate the complex temporospatial development of the brain, neuronal cultures can recapitulate neurodevelopmental processes such as cell proliferation, differentiation, growth, and synaptogenesis. Neuronal cell lines and rodent primary neurons have been used as *in vitro* model systems; however, cell lines do not always recapitulate the phenotype of a primary cell, and rodent-derived neurons may not capture early developmental processes or potential species-specific effects in human cells.

Neural stem cells represent an alternative model system for studies of neurodevelopment *in vitro* and have several advantages including the property of self-renewal, the ability to generate the major cell types of the nervous system, and the availability of cells from multiple species including humans. We have used embryonic stem cell-derived neuroprogenitors, neuroprogenitors from fetal brain, and induced pluripotent stem cells in the course of evaluating cell-based assays for the neurodevelopmental processes of proliferation, differentiation, and functional maturation matched *in vivo* patterns.

Pathway analysis reveals that GO terms enriched among genes decreased through time are largely associated with proliferation, and stem cell maintenance. GO terms enriched among genes with significantly increasing expression through time are dominated by key developmental processes, including neuronal differentiation, migration, and synaptogenesis. Enrichment of several GO terms associated with forebrain development indicates that our culture conditions promote differentiation towards a forebrain identity. We compared *in vitro* pathway dynamics with pathway dynamics apparent in publicly available data from developing human brain tissue. Key processes important for the identification of AOPs of proliferation, differentiation, and functional maturation matched *in vivo* patterns.

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Session VI-2: Poster presentations

VI-2-040

**Genome-wide gene expression analysis by RNA-seq in murine embryonic stem cells**

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Although it is not yet possible to replace in vivo testing completely, we can refine and reduce the number of animals used today. Here, we focus on a tiered approach combining genome-wide gene expression analysis by RNA-seq in murine embryonic stem cells (mESCs) and development of mathematical models. We firstly exposed model chemicals including Bis(2-ethylhexyl) phthalate and p-dichlorobenzene in 1 or 28 days (daily dose) to mESCs, followed by total RNA were isolated, and deep sequencing analysis were performed. Our results indicate that non-coding RNAs (ncRNAs) (Tani and Torimura, 2013) as well as mRNAs respond to the model chemicals. Next, for each chemical, we established no observed effect level (NOEL) values (mg/kg/day) for the effects involving several targets with respect to 28 days repeated dose toxicity studies, using existing oral experiment data on mice. Then, we performed statistical comparisons between our in vitro data and existing in vivo data. In addition, we discussed the results with reference to the Adverse Outcome Pathway (AOP) concept. We propose that the novel tiered approach will allow interim decisions to obviate further animal testing.

Reference


VI-2-230

**Establishing an in vitro screening test for developmental neurotoxicity**

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According to US EPA OPPTS 870.6300 and OECD TG426, the chemicals evaluation of developmental neurotoxicity (DNT) is conducted with living animals, mostly rodents, which is time consuming, laboring and cannot satisfy with the requirement of testing so many chemicals. PSCs carry the characteristic of self-renew and differentiates into various types of cells in accordance with the stimulation of specific factors. Since the embryonic stem cell test (EST) may not fully suitable for DNT screening, it is necessary to develop a DNT screening method. Based on the mES, we tried to induce it to differentiate into neuron and glia cells by varied ways.

VI-2-507 *

**Cardiomyocytes from pluripotent stem cells – a promising alternative in drug assay**

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Organ specific stem cells of animal origin have potential application in drug assays and regenerative medicine. They are harvested from spent animals, cultured in vitro. Pluripotent stem cells used in the present study were derived from goat uteri containing undifferentiated fetus. The fetus were collected from slaughter house, sterilized, centrifuged and washed with Dulbecco’s PBS. The fluid containing the desired cells were washed in CR11 media and incubated at 38.5°C, 5% CO₂ and 90% RH in CO₂ incubator for a period of 7 days. Developed stem cell clones were mechanically disrupted and re-cultured in CR11 media with Lipopolysaccharide at the concentration of 2-8 µg/ml. The re-culturing was continued till 3rd passage. After that Lymphoma Inhibitory Factor (LIF- a media ingredient in CR11) was removed from the media and cells were allowed to get differentiated. Clones of beating cardiomyocytes like cells were observed in the culture plates on 28th day of the passaging. Transcript level of cardiac specific gene Nkx 2.5 was studied on these cells. Cardiomyocytes developed in the present study could be potential alternative to the use of lab animals for cardiac drug assays and toxicological studies. Further studies on these cells are progressing.

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VI-2-664

**Profiling of drugs and environmental chemicals for functional impairment of neural crest migration in a novel stem cell-based test battery**

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The EU-FP7 project “embryonic stem cell-based novel alternative tests” (ESNATs) aimed at establishing a test battery framework for developmental toxicity. Here, we present the overall test battery concept, suitable for a wide range of in vitro toxicity assays (Zimmer et al., 2014). A screen library of 28 compounds (pharmaceuticals and environmental toxicants) was selected and characterized for cytotoxicity as well as for clinical and toxicological data. To evaluate the feasibility of the test framework, the “migration inhibition of neural crest cells” (MINC) assay was chosen (Zimmer et al., 2012). Screening at the highest non-cytotoxic concentration resulted in 11 hits. To further understand the mechanisms underlying the observed inhibition, a microarray analysis was performed for some selected hits and the differentially expressed genes were analyzed by GO term enrichment analysis. This study confirmed the potential use of the MINC assay for the prioritization of substances belonging to different chemical classes. In conclusion, this feasibility study points out important design principles of a test battery for identification of reproductive toxicants. Our approach shows a potential strategy for the combination of assays available in different laboratories to provide more information both on the assays themselves and also on the compounds included in the screens.

References

Session VI-3: Human biomarkers
Co-chairs
Barry Hardy, Douglas Connect, Switzerland
Andre Schratzenholz, ProteoSys, Germany

Session VI-3: Oral presentations

VI-3-807
The automated FADU assay: an alternative method for monitoring DNA repair in human studies

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Our genome is constantly challenged by exogenous and endogenous damaging agents; therefore cells have developed DNA repair mechanisms to ensure genomic integrity. However, there are many influences that can compromise DNA repair resulting in DNA damage accumulation, which might lead to diseases, cancer or accelerated ageing. Therefore, establishment of alternative methods for monitoring DNA repair in human cells is of utmost importance for a wide variety of scientific fields. The automated Fluorometric Analysis of Alkaline DNA Unwinding (FADU) assay provides a reliable, convenient and objective method to quantify DNA breakage in vivo. In the context of a scientific multi-center cooperation, we investigated associations of subjective vitality with DNA damage as well as age, gender and genetic and environmental influence on DNA strand breaks. In another cooperative study we assessed DNA strand breaks and the immediate repair phase in blood cells from individuals with posttraumatic stress disorder before and after psychotherapy. Our results revealed that exposure to traumatic life events is associated with higher levels of DNA damage and we presented a proof-of-principle for the reversibility of DNA strand breakage after successful psychotherapy. In our study, gender and genetic background had no significant effect on DNA strand break repair.

VI-3-918
Use of alternative evidence in replacement research and safety assessment supported by OpenTox and ToxBank

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Data on SEURAT-1 Gold Compounds (SGCs) were obtained from the literature and organised and made available through the ToxBank wiki and data warehouse. The data integration included transcriptomics data from TG-Gates, assay data from PubChem, toxicokinetics data and parameters from the literature, and in vivo data resources. Interoperability between ToxBank components and other resources was based on service implementation of OpenTox standards. Analysis methods for Read Across, enriched meta analysis of multiple omics and functional data, background knowledge from GO ontologies and Kegg pathways, and pathway visualisation were developed and ap-
plied to the SGCs. We present an analysis case study for Doxorubicin based on publically available data examining the variation of pathway interactions as a function of dose and time. Using SGC examples, we discuss the differing information requirements and solutions for computational and expert-based components to Read Across and Weight of Evidence for the following contexts with consideration of the value and current limitations in Alternative-based evidence:

a) Hypothesis-driven mechanistic-based research
b) Predictive goal of an integrated testing strategy
c) Incorporation of evidence in safety assessment decision making.

The role of such methods in providing evidence and guidance supporting biomarker development will be discussed.

**VI-3-919**

**Human biomarkers of disease, from discovery to validation and test development**

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There are quite diverse phenotypic effects resulting from toxic interventions or pathological conditions, which are accessible for measurements with modern OMICS-technologies. They include a variety of nucleic acid species (RNA’s and DNA) and mass spectrometry (metabolites, proteins, lipids, sugars). Toxic effects depend on individual genetic, epigenetic and environmental predispositions and conditions.

Epigenetic and environmental effects are to a large extent present on the proteomic level. Proteins also show the most immediate molecular effects in time-resolved experiments. The kinetics of cellular responses requires special attention with regard to data types and formats, data models and ontologies and interface solutions for integration will be discussed.

Working with human in vitro models requires the establishment of kinetically controlled SOP’s for sample generation, processing and storage, meta data tracking frameworks (like, e.g., ISATAB). The aim is to define sets of biomarkers exactly describing molecular initiating events and the downstream key events which eventually cause adverse outcomes.

In terms of molecular biomarkers, in a first stage a thorough statistical analysis of raw data will reveal consistent quantitative data signatures plausible across samples and conditions. This is pivotal to exclude effects of contamination and unrelated biological activity. A clear definition of biological and data acquisition criteria will result in the selection of validated data sets.

In a second stage the whole validated data set or selected subgroups of biomarker candidates will be searched according to biological criteria. In the toxicological projects investigated so far (SEURAT-1, Reprotect) one of the hallmark was oxidative stress, contributing to a cascade of specific posttranslational modifications (oxidative, glycation), some of them directly accessible by mass spectrometry (e.g., N-formyl-kynurenin modification).

Obviously the number of pathways is relatively limited and these pathways are organized in flexible and redundant feed-back systems. Certain layers of omics analyses reflect better or worse the kinetics of reactions in these pathways of stress and escape responses.

Predictive modelling will require adequate incorporation of kinetic information and treatment of feed-back and feed-forward mechanisms.

**VI-3-920**

**The development, use and interpretation of in silico models to support the ICH M7 guideline**

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The International Committee on Harmonisation (ICH) has recently issued a draft guidance (currently out for public comment – termed step 2) that covers the qualification of mutagenic impurities (ICH M7). In the absence of carcinogenicity or bacterial mutagenes data for actual or potential impurities, an in silico structure-activity analysis can be performed to help understand whether a substance can be classified as having no mutagenic concern. To perform the computational structure-activity analysis, the guideline states that two complementary in silico methodologies should be used in the assessment. One should be expert rule-based and the second should utilize a statistical-based methodology. This presentation will outline how rule-based expert alerts systems as well as statistical-based QSAR models are developed and used to generate predictions to support the assessment of impurities as part of the ICH M7 guidance. The presentation discusses how the results can be combined to generate a consensus prediction as well as how these results can be used to support an expert opinion.

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**Session VI-3: Poster presentations**

**VI-3-005**

**Funding innovation to foster decreases in compound attrition and the 3Rs: a new process**

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Despite the considerable advances in science and technology in the biological space in the last two decades, research in pharmaceutical industry is still affected by a high rate of attrition of compounds during the drug development process. Similarly, our reliance on laboratory animals is high, despite the strong commitment of the application of the 3Rs principles in R&D processes. Attrition and 3Rs are inherently linked: in general, changes that would reduce compound attrition have also an impact on 3Rs.

A new process for identification and funding technological opportunities with an impact on attrition and the 3Rs has been set up in GSK. This presentation is aimed to illustrate the GSK model.
Proton magnetic resonance spectroscopy and recursive partitioning analysis of brain metabolism data from an Alzheimer’s disease cohort offers a replacement alternative for animal studies on disease prediction

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Fundamental research into Alzheimer’s disease (AD) relies on animal models for the investigation of disease mechanism and new therapeutics. Transgenic rodent strains are available (Kitazawa et al., 2012) with mouse models used for brain chemistry to memory loss studies (Murakami et al., 2011; Cassano et al., 2012). Also, micro-imaging methods have been used to study a transgenic rat AD model (Teng et al., 2011). A combination of a clinically and genetically well-defined human cohort, proton magnetic resonance spectroscopy (¹H-MRS) and pattern recognition analysis of cohort data (Londono et al., 2013) offers a replacement alternative to animal models that predict AD onset features. Seventy-five participants from a multigenerational AD pedigree were divided into; (1) carriers of the fully penetrant mutation in Presenilin-1 (n=44), and (2) non-carriers (n=31); seventeen carriers had mild cognitive impairment (MCI) or early-stage AD. ¹H-MRS was conducted on each participant, with brain metabolite data (e.g., choline/creatine ratio) for white and grey matter regions of the posterior cingulate gyrus and precuneus included with demographic data, prior to recursive partitioning analyses. Metabolite ratio profiles successfully discriminated carriers and asymptomatic carriers. Brain metabolites measured by ¹H-MRS are optimally sensitive and specific non-invasive biomarkers of subclinical emergence of AD caused by the PSEN1 mutation, achieved without pre-clinical animal experiments.

References

From transient transcriptome responses to disturbed neurodevelopment: divergent response patterns and epigenetic modifications triggered by the same drug
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Literature often assumes that toxicogenomics data reflect the immediate mode of action (MoA) of drugs. Alternatively, transcriptome changes could describe altered differentiation states as indirect consequence of drug exposure. We addressed this question in differentiating human embryonic stem cells exposed to TSA. Histone acetylation (primary MoA) increased quickly and returned to baseline after 48 h. Histone H3 lysine methylation of the neurodevelopmental regulators PAX6 and OTX2 was affected and remained persistent. These alterations correlated with neurodevelopmental defects and with changes in PAX6 expression, even with a drug washout after 3 days.

We hypothesised that drug exposures altering only acetylation lead to reversible transcriptome changes (indicating MoA) and altered methylation leads to irreversible developmental disturbances. Data from pulse-chase experiments corroborated this assumption. Short drug treatment triggered reversible transcriptome changes; longer exposure disrupted neurodevelopment. The disturbed differentiation was reflected by an altered transcriptome pattern. The changes were similar after a 4 days washout. We conclude that transcriptome data after prolonged treatment of differentiating cells mainly reflects the altered developmental stage of the model system and not the drug MoA.

Waves of gene expression as basis to define windows of sensitivity for developmental neurotoxicity
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In the field of neurodevelopmental toxicity there is an urgent need for appropriate in vitro tests. We established a human embryonic stem cell based test system. However, the major challenge of such a system is that gene expression patterns change already in untreated control conditions. Therefore, we hypothesize that not only concentration and duration of treatment matter but also the time window. We used microarray analysis to get (i) a closer insight into the underlying processes of neurodevelopment and (ii) to investigate drug effects for different
treatment scenarios. Gene expression analysis at different time points of undisturbed differentiation showed that gene regulation proceeds in a wave-like pattern. Using the HDAC inhibitors VPA and TSA as well characterized DNT compounds, we treated the cells for 6h/4d/6d. We found that developmentally regulated genes and drug regulated genes overlapped up to 90% at late time points but showed only a small overlap at early time points. This may allow differentiation between cell biological and developmental toxic effects. For phenotypic anchoring, gene expression changes were correlated with the capacity of treated cells to still form neural tube like rosettes.

### Session VI-4a: Absorption, distribution, metabolism (ADME)

**Co-chairs**

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Amin Rostami-Hodjegan, University of Manchester, UK

### Session VI-4a: Oral presentations

**VI-4a-424**

**SPECT-CT imaging to study the pharmacokinetics of radiolabelled antisense oligonucleotides (AONs) in vivo**

and M. L. H. Vlaming


**Aim:** For drug development, determining the biodistribution of compounds is extremely important. SPECT-CT imaging is a non-invasive technique, allowing repeated measurement of biodistribution in vivo. Currently, availability of dedicated non-invasive technologies to evaluate pharmacokinetics of DNA/RNA-based therapeutics, such as AONs, is still limited. We aimed to set-up and validate novel minimally-invasive methods to trace AONs in vivo.

**Methods:** An AON developed for Duchenne Muscular Dystrophy (DMD) treatment was radiolabeled with either 123I or 111In. The pharmacokinetics of [123I]-AON or [111In]-AON after subcutaneous administration (100 mg/kg) to Mdx mice, a model for DMD, were assessed with SPECT-CT imaging. Subsequently, tissues were collected, radioactivity counted and AONs quantified by an ELISA-based method.

**Results:** SPECT-CT imaging showed that both [123I]-AON and [111In]-AON could be used to determine in situ tissue levels of the AON up to 48 hours after administration. Scan data matched well with results from the invasive biodistribution.

**Conclusions:** Quantitative SPECT-CT imaging with radiolabelled AONs provides a powerful approach to non-invasively assess AON pharmacokinetics. Radiolabelling with 111In appears preferable for oligonucleotides. Imaging can significantly improve translation to humans, and reduce the number of animals used in preclinical drug development.

**VI-4a-539**

**Living cell as a tool in in vitro toxicity testing platforms**

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Toxicokinetic behaviour of most small organic xenobiotic chemicals is mostly determined by xenobiotic-metabolizing enzymes. Thus, in toxicity testing platforms, be it non-cellular or cellular system, it is important to ensure that xenobiotic metabolism is functional or incorporated and at levels analogous to those in vivo or at least useful for quantitative in vitro-in vivo extrapolation (QIVIVE). Although non-cellular testing systems with metabolic competence are widely used in in vitro toxicity testing, living human cells in 2D and 3D configurations are the most versatile tool for in vitro toxicity tests. However, cellular systems should be developed in a more detailed way than thus far, taking into account the early characterization of metabolism and other important kinetic processes, because the thorough characterization is a necessary for the validation and use of cellular systems for toxicological studies. In addition, because metabolism is not the only important factor to be incorporated into toxicokinetic predictions, a more comprehensive “systems toxicology” approach should be incorporated into any QIVIVE exercise.

**VI-4a-798**

**Gaining insight into xenobiotic biotransformation: the CYP induction in vitro method**


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Cytochrome P450s (CYP) are Phase I biotransformation enzymes that are frequently responsible for transforming both endogenous and exogenous compounds. EURL ECVAM used induction of CYP enzymes produced by exposure to xenobiotics to evaluate the metabolic competence of two human-derived in vitro hepatic test systems. The multi-study validation trial assessed the reliability and relevance of CYP induction methods to evaluate the functional induction of four CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) in two in vitro test systems: cryopreserved human HepaRG® cells (Andersson et al., 2012) and cryopreserved human primary hepatocytes (Richert et al., 2010; Alexandre et al., 2012; Yajima et al., 2014). The predictive capacity was assessed by testing 10 (HepaRG®) or 12 (cryopreserved primary hepatocytes) compounds and comparing the results to human in vivo CYP induction reference data. Since the CYP induction method is based on xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene, we predict that any class of compounds that can interact with such receptors can be used in the in vitro human CYP induction methods. Based on this project, OECD member countries accepted the development of a new test guideline as an addition to the OECD work programme.

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VI-4a-914
In vitro to in vivo extrapolation and reverse dosimetry
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The field of toxicology is currently undergoing a global paradigm shift to use of in vitro approaches for assessing the risks of chemicals and drugs, yielding results more rapidly and more mechanistically based than current approaches relying primarily on in vivo testing. However, reliance on in vitro data entails a number of new challenges associated with translating the in vitro results to corresponding in vivo exposures. A combination of in silico- and in vitro parameter estimation, together with pharmacokinetic modeling, can be used to predict the in vivo exposure conditions that would produce chemical concentrations in the target tissue equivalent to the concentrations at which effects were observed with in vitro assays of tissue/organ toxicity. This presentation will describe the various elements of IVIVE and highlight key aspects of the process including: (1) characterization of free concentration, metabolism, and cellular uptake; (2) conversion of in vitro effect concentrations to equivalent human exposures, and (3) potential complications associated with metabolite toxicity. Two examples of PBPK-based IVIVE will be described: a simple approach using whole hepatocyte clearance and plasma binding that is suitable for a high-throughput environment, and a more complicated approach for a case of metabolite toxicity. Recent efforts to improve the in vivo relevance of in vitro kinetic data will also be described.

VI-4a-916
The added value of physiologically-based pharmacokinetic in modelling the target tissue exposure for PKPD analysis
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Although the new generation of physiological-based pharmacokinetic (PBPK) models is now embraced by the pharmaceutical industry as well as regulatory organisations (Ho et al., 2014), the advantages of these models in estimating the local concentrations in various tissues over time has not reached its full potential. The drug safety or pharmacological effect is usually linked to the local exposure in the tissue. However, this is not directly measured. It is well known that variation in the local concentrations due to the effect of variable transport can blur the distinction between PK and PD variability. Various genetic variations in the functionality of uptake or efflux transporters have recently been discovered at blood brain barrier, hepatocytes, or proximal kidney tubule. PBPK models with “permeability-limited” organ models can account for transport-mediated uptake or efflux and assess the local concentrations (Neuhoff et al., in press; Hsu et al., 2014; Jamei et al., 2013).

Recently, Rose et al. (2014) reported the application of a PBPK model to predict the pharmacodynamics of roulavstatin patients with impaired OATP1B1-related activity. Accordingly, there is a compelling argument for using PBPK in projecting PKPD when drugs are subject to polymorphic transporter activity. This approach can provide information on sub-groups which are rarely studied in clinic.

References
Theme VI, Session VI-4b

Session VI-4b: Absorption, distribution, metabolism and excretion (ADME)

Co-chairs
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Session VI-4b: Oral presentations

VI-4b-313

Organ-like three dimensional test systems

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Hepatic metabolic clearance plays a key role in the transformation and the elimination of chemicals from the human body. In recent times various in vitro methods for human hepatic metabolic clearance/stability have been developed, employing different biological systems, monitoring either test item depletion or metabolite formation and including various test system configurations. This heterogeneity in in vitro clearance methods results in heterogeneity in in vitro clearance data. eURl eCV AM, acknowledging the importance of ADME information in the regulatory safety assessment of chemicals, is taking the initiative to develop harmonized standards for in vitro human hepatic clearance methods. These standards will be based on the results of (1) an evidence based literature research, (2) a test submission e-survey tool and (3) an expert workshop. The new standards aim to challenge the in vitro methods in relation to defined applications, to guide the end-user in using a harmonised reporting format and in providing estimations on the uncertainty of generated parameters. In this contribution, these emerging standards will be presented, along with the detailed workflow used to generate them.

References

VI-4b-672

Development of standards for characterising and describing in-vitro human hepatic metabolic clearance methods

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Hepatic metabolic clearance plays a key role in the transformation and the elimination of chemicals from the human body. In recent times various in vitro methods for human hepatic metabolic clearance/stability have been developed, employing different biological systems, monitoring either test item depletion or metabolite formation and including various test system configurations. This heterogeneity in in vitro clearance methods results in heterogeneity in in vitro clearance data. eURl eCV AM, acknowledging the importance of ADME information in the regulatory safety assessment of chemicals, is taking the initiative to develop harmonized standards for in vitro human hepatic clearance methods. These standards will be based on the results of (1) an evidence based literature research, (2) a test submission e-survey tool and (3) an expert workshop. The new standards aim to challenge the in vitro methods in relation to defined applications, to guide the end-user in using a harmonised reporting format and in providing estimations on the uncertainty of generated parameters. In this contribution, these emerging standards will be presented, along with the detailed workflow used to generate them.

References

VI-4b-711

New approach to predict human oral absorption using porcine intestinal tissue, abundance data and biorelevant matrices

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The predictive value of currently used models for oral bioavailability in humans is often insufficient, especially when food-drug interactions are evaluated. We aimed to design a new approach for optimal prediction of oral absorption. First, three different in vitro permeability models (Caco-2 cells, porcine intestinal tissue from re-used animals mounted in InteStine™, and human intestinal tissue mounted in Ussing system) were characterized with regards to apparent permeability (Papp), absolute expression levels of transporters and metabolic enzymes. Papp values determined using the porcine InteSTine™ system were in the same range as using human intestinal tissue, whereas data obtained with Caco-2 cells showed major differences. These differences could partly be explained by different abundance of transporters and metabolic enzymes in comparison with human intestinal tissue. Moreover, we showed a unique combination of porcine InteSTine™ system with luminal samples collected from a gastrointestinal model (TUM) studying the permeability of compounds in the presence of undiluted biorelevant samples without loss of integrity. The results show that porcine intestinal tissue mounted in InteStine™ system provides a good alternative for human intestinal tissue, and together with abundance data, biorelevant matrices and PBPK offers a unique combination for optimal prediction of oral bioavailability in humans.

References
Session VI-4: Poster presentations

VI-4-213

Porcine buccal mucosa as an in vitro route model for systemic drug delivery evaluation
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Purpose: Determination of drug permeability across the buccal mucosa barrier generally speeds up the drug development process, and models are needed for this purpose (Nielsen, 2002). The aim of the present study was to standardize the porcine buccal mucosa as an in vitro model (Mahalingam, 2007) for the examination of drug transport.

Methods: For this purpose, mucosal membrane, isolated from animals obtained from slaughterhouse were used. The membranes were then mounted in Franz diffusion cells for the in vitro permeation experiments. The permeation of caffeine was evaluated on fresh, -4°C stored, and frozen (-20°C and -80°C) tissues. For the freezing conditions, glucose and glycerol were evaluated as cryopreservatives. 14C-Mannitol was used as quality control.

Results: On fresh tissue the permeability coefficient (Papp) for caffeine showed reproducibility between the day 1 and the day 2. The very low Papp obtained for 14C-Mannitol confirmed the tissue integrity. There were no differences observed for caffeine cumulative transport, between fresh and -4°C stored tissue. By using -20°C frozen tissue, the cumulative transport was the same for each cryopreservative used.

Conclusion: Based on these results it could be stated that frozen or fresh the porcine buccal mucosa can be used as model for evaluation of drug transport (Hoogstraate, 1996).

References

VI-4-277

Characterization of monoamine oxidases, steroid-5a-reductases, sulfotransferases and glutathione S-transferases in reconstructed skin tissues and human skin ex vivo
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The use of reconstructed human skin (RHS) in preclinical development of topical dermatics and transdermal therapeutic systems requires detailed knowledge on the biotransformation capacity of the constructs. We investigated monoamine oxidases (MAO), steroid-5α-reductases (SRD5A) as well as sulfotransferases (SULT) and glutathione S-transferases (GST) in excised human skin, RHS (Phentin FT, Epiderm-FT) and in normal keratinocytes, HaCaT cells and fibroblasts.

Gene expression levels were determined by quantitative RT-PCR and MAO protein expression by Western blot analysis. GST activity was evaluated using 1-chloro-2,4-dinitrobenzene as a substrate.

MAO A, SRD5A type 1 and 3 were expressed in all matrices, whereas MAO B and SRD5A type 2 levels were higher in dermis than epidermis and hardly detectable in undifferentiated keratinocytes. In human skin and reconstructed tissues strong constitutive expression of SULT2B1b, SULT1E1, SULT1A1, GSTP1 and GSTT1 was found. GST activity was slightly higher in RHS compared to human skin.

Reconstructed tissues and human skin share a similar gene expression profile of the tested phase I and phase II enzymes and GST activity. Thus, RHS appear to be suitable for preclinical testing and toxicology studies with respect to biotransformation related processes.

VI-4-390

Influence of receptor fluid on dermal absorption of caffeine
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Dermal absorption describes the transport of chemicals from the surface of the skin into the skin and into the systemic circulation. An in vitro method, based on Franz cells, using preferably human skin is widely used for the investigation of penetration properties of substances. To ensure a correct interpretation of results it is important to investigate factors influencing this assay.

In the current study the influence of different receptor fluids on the penetration properties of caffeine was investigated. The study was performed in a dynamic flow through system using human skin. Caffeine was applied at the start of the study and receptor fluid was collected at various time points. At the end of the study all matrices (skin, strips, skin wash and receptor fluid) were analysed for the content of caffeine. The receptor fluids were water with 50% ethanol and DPBS supplemented with BSA (5%), DMSO (5%) or SDS (5%).

The results confirmed, that the receptor fluid used for dermal absorption studies in vitro has an influence on the penetration properties. The total absorption of Caffeine varied depending on the receptor fluid composition between 45% (DMSO and BSA) and 15% (50% ethanol and SDS).

VI-4-479

Microdosing of new biological entities (NBEs) in healthy volunteers: a safe and fast tool to predict clinical pharmacokinetics
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Aim: Microdosing, combined with Accelerator Mass Spectrometry (AMS), provides a safe approach to obtain first-in-human pharmacokinetic data, after very limited safety testing in animals. We investigated the microdosing approach to determine pharmacokinetics and safety of NBes, using recombinant human Placental Alkaline Phosphatase (hRESCAP, a therapeutic protein under investigation for clinical application) as a model compound.

Methods: hRESCAP and [14C]-hRESCAP were successfully, GMP-compliant, manufactured and characterized with in vitro methods. A microdose (53 μg) of [14C]-hRESCAP was i.v. administered to healthy volunteers, and safety and pharmacokinetics were assessed. Subsequently, increasing doses (414, 1240, and 5300 μg hRESCAP, including 53 μg [14C]-hRESCAP), were administered. [14C]-hRESCAP plasma levels were determined by AMS, total hRESCAP by an enzymatic assay.

Results: Single doses of 53-5300 μg hRESCAP, including 53 μg [14C]-hRESCAP, were well tolerated. Pharmacokinetic analysis indicated dose-linearity from microdose to therapeutic doses.

Conclusions: A microdose of [14C]-hRESCAP successfully predicted favorable plasma residence time of hRESCAP at therapeutic doses, and was a safe starting dose for a first-in-human study. Microdosing provides a safe and fast clinical development route for NBes like hRESCAP and can significantly reduce the number of animal studies.

Session VI-5: Oral presentations

Development of human airway tissue-based assay for respiratory absorption giving input parameters for PBTK modeling

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Airway epithelium is a prime entry portal of xenobiotics into the body. Knowledge of toxicokinetics is needed to estimate the possible range of target doses at the cell or tissue level that can be expected from realistic human exposure scenarios to inhaled compounds (Bur and Lehr, 2008). Our study evaluated the permeability property of an in vitro cell model of the human airway epithelium (MucilAir™) (Reus et al., 2014). The absorption of test items was assessed after apical or basolateral exposures, and the permeability rate (Papp) of the chemicals across airway epithelium was measured. A standard operating procedure was developed and its transferability and reproducibility was evaluated using 6 chemicals (propranolol, atenolol, nicotine, cadmium-chloride, cobalt-chloride and ammonium-hexachloroplatinate) in two independent laboratories. A panel of 30 compounds were further tested to evaluate the ability of the assay to rank relative permeability. A comparative permeability study between nasal and bronchial epithelium has been performed. The results showed generally: (i) a higher permeability of the airway epithelium for organic compared to inorganic compounds and (ii) a low transporter-mediated efflux involved in the permeability. The study indicates that this MucilAir™-based assay represents a promising
Design and characterization of in vitro models for human skin wounds

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In this study, a versatile skin wound model based on excised human skin is presented. This three-dimensional skin wound model with linear, rectangular wound geometry and defined tilted wound edges was created (Rizzo et al., 2012; Planz et al., in reply). Further, for non-invasive wound characterization, optical profilometry and Raman spectroscopy were introduced realizing a three-dimensional analysis of wound geometry and healing without tissue destruction (Kann and Windbergs, 2013). Virtual sectioning by these techniques provides a valuable alternative to invasive tissue histology. In direct comparison to established skin punch biopsies exhibiting variable wound size depending on the sectional plane, the linear shape of the novel wound model overcomes this problem, as visualized by profilometry scans and histological sections (Planz et al., in reply). In addition, the novel model provides a more realistic simulation of the human in vivo pathophysiology exhibiting tilted wound edges completely surrounded by intact tissue.

Even though rodent skin is more permeable and excised human skin is considered as gold standard for drug absorption testing, rodent wound models are frequently used (Scott et al., 1986; Ansell et al., 2012). To address this issue and provide an alternative to animal testing, this study presents a reproducible in vitro skin wound model based on excised human skin. Three-dimensional skin wounds with linear, rectangular wound geometry and defined tilted wound edges were created (Rizzo et al., 2012; Planz et al., in reply). Further, for non-invasive wound characterization, optical profilometry and Raman spectroscopy were introduced realizing a three-dimensional analysis of wound geometry and healing without tissue destruction (Kann and Windbergs, 2013). Virtual sectioning by these techniques provides a valuable alternative to invasive tissue histology. In direct comparison to established skin punch biopsies exhibiting variable wound size depending on the sectional plane, the linear shape of the novel wound model overcomes this problem, as visualized by profilometry scans and histological sections (Planz et al., in reply). In addition, the novel model provides a more realistic simulation of the human in vivo pathophysiology exhibiting tilted wound edges completely surrounded by intact tissue. The study presents a versatile in vitro model simulating human skin wounds which is reproducible as well as easily applicable and meets all requirements for valid testing of novel therapeutic systems at the site of action.

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Potential of mucosal in vitro models obtaining approval for drug products – case studies with Caco-2 and Calu-3 cells

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Drug development is growing more complex and time consuming. Furthermore, the generic market is growing and known drug molecules are formulated to improve therapy. Therefore, smart concepts should be integrated into the pharmaceutical development to avoid clinical studies to show bioequivalence of drug products. In vitro cell culture studies prove to be a fast and cost-effective tool to assess formulation influences on the bioavailability of a drug molecule. For orally applied drugs the BCS system can be used for class 1 substances. An example will be shown that this system can also be applied to other than fast releasing oral tablets as wells to non-class I substances to get approval.

The scientific base of the BCS system can also be applied to local acting substances, e.g., Corticosteroids against allergic rhinitis. Calu-3 cells as a robust mucosal model can be used to show bioequivalence of such products. An example of in vitro BE will be presented. The data show clearly that in vivo/in vitro correlation can be established and furthermore that the European Authorities accept in vitro also for the approval of drug products.

Application of in vitro human airway models for toxicology, drug delivery and disease modeling

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Organotypic in vitro human airway models offer the opportunity to screen for potential airway toxicity of nanoparticles and chemicals, screen the safety and efficacy of active pharmaceutical ingredients and formulations intended for inhalation delivery, and model airway infection by bacterial and viral pathogens. Here we present a survey of successful uses of a commercially available in vitro human airway model (EpiAirway™) for these applications. The EpiAirway™ model is produced by culturing normal human airway epithelial cells on microporous membrane inserts at the air-liquid interface. The model reproduces the organotypic, pseudostratified mucociliary phenotype of in vivo proximal airways. EpiAirway™ model tissues have functional tight junctions, in vivo-like barrier properties and in vivo-like xenobiotic metabolizing capabilities. Specific examples surveyed from the published literature include toxicological evaluation of tobacco products, optimization of nasally administered therapeutics, long-term host-pathogen interactions during bacterial (Haemophilus influenzae) infection, mechanisms of influenza virus airway infection, and rhino-virus infection of asthmatic compared to non-asthmatic tissues.

References
Modelling mucosal epithelia in state of inflammation

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For assessing the safety and efficacy of (nano)pharmaceuticals and other products, there is a need to study their interaction with mucosal barriers not only in healthy, but also in diseased state, i.e., when their barrier function might be impeded, especially in inflammation.

Regarding the oral route, a 3D model of the inflamed intestinal mucosa was developed by growing intestinal epithelial cells (Caco-2) on a collagen layer, comprising immune cells (dendritic cells: MZt-3; macrophages: THP-1). After stimulation with interleukin-1β, this model allowed the assessment of anti-inflammatory drugs and nanomedicines (Leonard et al., 2010, 2012).

Considering the importance of aerosol exposure/delivery to the lungs, we translated this approach to a model of the air-blood barrier, consisting of human alveolar epithelial cells (hAEPc), that differentiate into type I cells and show pronounced barrier properties (Daum et al., 2012). Autologous co-cultures of hAEPc and alveolar macrophages originating from the same patient can be stimulated with lipopoly saccharides (LPS) to study particle uptake and anti-inflammatory drug effects. However, in addition to these cellular elements of mucosal barriers, non-cellular elements, such as in particular mucus and surfactant, must not be neglected, as they act as utmost efficient clearance and diffusional barriers and may form a specific lipid-protein corona especially for nanoparticles deposited in the respective parts of the lungs (Kirch et al., 2012; Ruge et al., 2013).

References

In-vitro models of the oro-gastro-intestinal mucosa

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The oral cavity, although part of the aero-digestive tract, is still neglected in terms of risk assessment with respect to nanoparticle uptake (Teubl et al., 2014; Teubl et al., 2013a). If nanoparticles enter the oral cavity, either via oral products or inhaled materials, it is not clear, whether they first interact with the mucosae or are swallowed and end up in the small intestine. To learn more about this relationship ex-vivo and in-vitro models that closely mimic the oral cavity and the small intestine were developed in order to study the spatio-temporal aspects of nanoparticle uptake, as well as the intracellular localization in human epithelial cells and potential toxic effects.

For this, buccal/sublingual human cells (TR146) were incubated with an external (human/porcine) mucus layer, prepared by a film method (Teubl et al., 2013b). Mucin fibres adhered to the stratified squamous epithelium and the viability of the model was maintained for more than 48 hours. Nanoparticle uptake rates correlated well with data from ex-vivo permeability studies through porcine buccal tissue (Roblegg et al., 2012). In the small-intestine study, co- and triple culture models were established, mimicking the epithelium of villi (i.e., Caco-2 cells and goblet cells) and the follicle associated epithelium (i.e., Caco-2 cells, goblet cells and M cells), respectively (Schimpel et al., 2014). The data revealed that the mucus layer, which entirely covers the enterocytes, prevents penetration of conventional nanoparticles to the epithelial surface and that particle uptake in the small intestine mainly occurs via M cells.

References

Potential and limitations of reconstructed skin models in assessing pharmaceutical formulations

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In vitro methods are being validated and adopted by regulatory agencies for the use as full or partial replacements of animal experiments. To date, the best results for topical toxicity testing in vitro have been obtained with reconstructed human skin models (RHSM). Particularly for the determination of skin corrosion and skin irritation, RHSM are the most predictive tools in vitro. Their use is also included in the OECD Test Guidelines (TG 431 (OECD, 2004) and 439 (OECD, 2013)), in the EU regulatory framework for chemicals REACH (2006) and recently, also ICH guideline (ICH, 2013) implemented use of the RHSM tissues for phototoxicity assessment of the topically applied substances.

RHSM are usually seen as reliable tools in assessments of chemical hazard, however, their use can be extended also into the area of risk assessment process and preclinical safety evaluation if appropriate protocols are developed as demonstrated. Also in the area of medical devices, the reconstructed tissue models are considered as useful tools in the assessment of human skin irritation effects (Casas et al., 2013).

The presentation will review the use of the RHSM models for in vitro assessment of pharmaceutical formulations. Recently published data in the area of irritation, phototoxicity, skin penetration, skin metabolism, genotoxicity and wound healing will be discussed.

References


Session VI-5: Poster presentations

VI-5-281

Establishment of an intestinal model for oral vaccination testing

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The intestinal epithelium forms an impenetrable barrier for macromolecules and microorganisms, except the follicle-associated epithelium of the gut-associated lymphoid tissue where microfold (M) cells sample the intestinal lumen initiating an immune response. This entry site to the immune system is an interesting target not only for pathogens but also for the analysis and improvement of oral vaccines.

In this work, a functional intestinal in vitro model based on the adenoma carcinoma cell line Caco-2 co-cultured with dendritic cells derived from human blood on a three-dimensional matrix from acellular dermis as support for the proliferation of different numbers of keratinocytes and ADSCs. Our results show that 300,000 keratinocytes/cm² and 300,000 ADSCs/cm² plated at the same time are enough to create a full-thickness dermal-epidermal substitute.

References


VI-5-299

Full-thickness tissue engineered development using human keratinocyte and adipose tissue derived mesenchymal stem cells

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Tissue engineering is an emerging therapeutic strategy that has the potential to revolutionize the skin regeneration treatments (Xie et al., 2007). Three-dimensional epidermis has been previously reconstituted in vitro using keratinocytes and adipose tissue derived mesenchymal stem cells (ADSC) seeded on dermal substrates in an air-liquid (A/L) environment (Lu et al., 2012). ADSCs present immunomodulatory potential and are easily accessible (Du et al., 2010; Sheng et al., 2013). In addition, these cells have can differentiate into fat, bone, cartilage, and muscle under lineage-specific culture conditions (Tholpady et al., 2006). The aim of this study was to develop a three-dimensional full-thickness engineered skin substitute using irradiated glycerol-preserved acellular dermis as support for the proliferation of different numbers of keratinocytes and ADSCs. Our results show that 300,000 keratinocytes/cm² and 300,000 ADSCs/cm² plated at the same time are enough to create a full-thickness dermal-epidermal substitute.

References


VI-5-354

HulIFN-αN3 and 10% PBS holocene grain wash-out affect the glutathione and lipid peroxidation in CaCo-2 cells

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Purpose: HulIFN-αN3 and 10% PBS (Phosphate Buffer Saline) Holocene grain washout affects growth and apoptosis of CaCo-2 cells (Kestel et al., 2007; Filipić, 2009). The purpose of experiments was...
to measure the effect of HuIFN-α and 10% PBS washout of hologene grain on the on the Glutathione level and Lipid peroxidation in CaCo-2 cells.

Methods: HuIFN-αN3, HuIFN-α2a, HuIFN-α2b and 10% PBS Hologene grain washout were used. The cells were treated: (a) Cell control, (b) HuIFN-α + 10% PBS (1:1.1:2.2:1), (c) HuIFN-α, (d) 10% PBS. The AP50 inhibition test was used. Apoptosis was measured by “BioVision: Apoptotic cell isolation kit”. Glutathione and Lipid peroxidation were determined as described (Devasagayam et al., 2000).

Results: 10% Hologene grain washout AP50 activity can be enhanced by HuIFN-αN3, but not with the HuIFN-α2a or HuIFN-α2b. 10% Hologene grain washout showed 26.52% of apoptotic cells, while this % was increased to 49.85 with HuIFN-αN3. In the combination between HuIFN-αN3 and 10% PBS Hologene grain washout 2:1 the level of GSH was 24.9 ±2.4 nM/mg (70.2 ±3.2 nM/mg in control) and level of MDA 72.3 ±3.1 nM/mg (23.6 ±9.1 nM/mg in control).

Conclusion: HuIFN-αN3 and 10% PBS hologene grain wash-out synergize but not HuIFN-α2a or HuIFN-α2b in effect on Glutathione level and Lipid peroxidation.

References

Evaluation of serum growth factors in wound healing using a full-thickness in vitro human skin model

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Following skin injury, damaged tissue undergoes highly coordinated biological events to restore barrier function involving cross-talk between dermal fibroblasts and epidermal keratinocytes as well as their interaction with the extracellular matrix. A full-thickness in vitro human skin model (EpiDerm-FT™) was used to evaluate the role of serum growth factors in cutaneous wound healing. This model is constructed from primary keratinocytes and fibroblasts and contains a functional barrier and fully developed basement membrane. Small epidermal-only wounds (3mm biopsy punch) or full-thickness wounds (cauterizer burns) were induced in the tissue model and monitored histologically from day 0 to day 6. Addition of 2% human serum demonstrated an increased rate of epithelial healing and fibroblast accumulation which could be abrogated in the presence of an EGFR tyrosine kinase inhibitor or a TGF-alpha neutralizing antibody. Gene expression analysis of the wounded area showed temporally regulated changes in mRNA expression of basement membrane components, collagens and genes involved in extracellular matrix remodeling on days 2, 4 and 6 post wounding. These results demonstrate that EpiDerm-FT is a useful in vitro skin model for investigating dermal-epidermal interactions during wound healing as well as for the evaluation of new therapeutics in the dermal wound healing process.
**Theme VII – Ethics**

**Coordinators**
Herwig Grimm, Messerli Institute, University of Veterinary Medicine, Vienna, Austria
Roman Kolar, Animal Welfare Academy, Neubiberg, Germany
Katy Taylor, BUAV, London, UK

**Session VII-1: Ethical and normative aspects of human-based approaches**

**Co-chairs**
Brett Cochrane, Dr Hadwen Trust, UK
Jurgen Hescheler, Cologne, Germany

**Session VII-1: Oral presentations**

**VII-1-100**

**Creating safe spaces for ethical reflection by animal researchers**

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**Aim/Objective:** Those who work in animal research, whether inside the animal house or laboratory, confront ethical challenges. How they respond affects not only these people and their co-workers, but the animals they interact with and the quality of research undertaken.

(AALAS; Birke et al., 2007) Our work aims to assist researchers address these ethical issues in a safe and supportive environment.

**Methods:** Our methods are philosophical and sociological. In this paper we systematically identify the ethical issues confronting those who work with research animals, examine and critique existing attempts to tackle these issues, and articulate features essential to successfully addressing such challenges.

**Results:** From our research we develop an approach which emphasizes creating “safe spaces” in which researchers can reflect on and discuss the ethical issues they encounter as part of their work. For such an approach to be successful, it needs to be supported at several institutional levels and to be integrated into workplace and professional culture.

**Conclusion:** It is important for humans and the animals in their care that ethical issues raised by animal researchers be identified, discussed and addressed. The approach we develop in this paper outlines mechanisms to achieve this end.

**References**


**VII-1-312**

**The used of animals in research or alternative methods – pros and cons**

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The arguments which are used today in support of and against the use of animals in research are essentially the same as they were more than 100 years ago, the period when serious scientifically-based research began in the world. The history of the controversy over animal research is long and has taken place with either particular interest or indifference to the medical discoveries which are used to illustrate that without animal experiments the treatment would be ineffective or could not be used at all. The concept of alternatives comes from a publication in 1959 which recommended. Scientists look for replacements to animals – RePlACe. Reduce their numbers – ReDUCe. Relieve their pain, stress and discomfort – ReFINe. Legislative bodies and the majority of the public have accepted the alternative term, including many company scientists, the academic sphere until now has resisted and has preferred to pay more attention to reducing the pain, stress and discomfort of animals during research. Both, experiments on animals and alternative methods have a place in the research and later practical application in therapeutic use.

**VII-1-474**

**Use of fresh functional human tissue as a highly predictive alternative to animal research**

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High clinical attrition rates, particularly during phase II trials, have been partly attributed to an over-reliance on animal models. To address this problem, fresh human tissue is increasingly being used as a physiologically representative model; however, is it feasible that functional human tissue models can contribute significantly to
Practical and ethical issues with using stem cells in research

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In a five and a half-year multidisciplinary collaboration of leading European researchers in alternative testing, as well as representatives from regulatory bodies, the pharmaceutical industry and ethical advisors have developed a battery of toxicity tests using eSC lines. Throughout the project duration, ethical issues associated with the work undertaken in eSNAtS have been monitored. In particular, amongst others, the eSNAtS partner in charge of ethical issues, edinethics ltd., wrote public ethical guidelines on the use of hESCs for toxicity testing. Edinethics also created a “Democs Card Game”. This Card Game aims at engaging lay publics on the issues of the use of hESCs and their derivatives to test potential new medicines for toxic side-effects, as an alternative to testing them on animals. A hard copy of the game has been distributed to all eSNAtS partners as well as eC DG Research, the European Group on Ethics, Public Health and Food Safety of the European Parliament, and the European Medicines Agency. The primary purpose of the game is to get the public to play the game and think about the issues at stake. But it also produces qualitative and semi-quantitative information, which can be analysed.

Why animal suffering matters

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How we treat animals arouses strong emotions. Many people are repulsed by photographs of cruelty to animals and respond passionately to how we make animals suffer for food, commerce, and sport. But is this, as some argue, a purely emotional issue? Are there really no rational grounds for opposing our current treatment of animals?

The paper considers how animals have been traditionally defined as naturally slaves, non-rational beings, linguistically deficient, not moral agents, soulless, and devoid of the divine image. But, if true, these differences should require more, not less, moral solicitude since it fol-
A system for retrospective assessment of cumulative severity to identify targeted refinements

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The EU Directive requires retrospective reporting of actual severity at the end of procedures (Animal Procedures Committee, 2013), but continuous assessment of actual severity is recommended (http://bit.ly/U3BwGV). Ethical justification of procedures is balanced between harm to animals and benefit to society from knowledge gained. Levels of harm and cumulative severity are affected by how work is conducted in the context of application of the 3Rs, this includes elements of contingent and direct suffering. Implementation of the refinement loop (Lloyd et al., 2008) can reduce cumulative severity but assessment of welfare depends on measurement of interrelated parameters which vary according to species, the environment and procedure. Currently there are few tools to assess cumulative severity or even to recognize its existence. Assessment methods are required and the system presents a matrix (Honess and Wolfensohn, 2010) applied retrospectively using data collected as an intrinsic part of studies. It provides opportunities to identify key events which impact on welfare, and explain to lay observers how the harms may be justified by the research. The analysis shows thorough retrospective review enables continual assessment of the harm:benefit balance at ethical review, and how on-going refinements can be targeted at the specific elements that will improve the animals’ welfare and quality of life.

References

Openness and accountability of animal research: results of an expert forum

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In November 2013, a group of international experts in animal research policy (n=11) gathered in Vancouver, Canada to discuss openness and accountability of animal research. The primary objective was to bring together participants from various jurisdictions (United States, Sweden, Australia, New Zealand, Germany, Canada, and the United Kingdom) to share practices regarding the governance of laboratory animal use with emphasis on the governance process followed, methods of community engagement, and the balance of openness versus confidentiality. During the forum participants came to consensus on the need for: a) evidence-based metrics for evaluation and quality assurance to allow a “virtuous feedback” system for animal research, b) increased public access to information together with opportunities for stakeholder dialogue about animal research, c) a greater diversity of views to be represented on decision-making committees, d) a standardized, robust ethical decision-making process that incorporates some sort of societal input, and e) a declaration on transparency to promote increased openness of animal research.

Analysis of the ethical review process of projects to be funded under the European Union’s Horizon 2020 Framework Programme

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In 2012 and 2013, the European animal welfare community carried out a study on the ethical review of projects involving non-human primates funded under the European Union’s 7th Research Framework Programme (Sauer et al., 2013).

The study also determined how project proposals are assessed from an ethical point of view, and considered 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. It illustrated some common problems with the process of Ethical Review so a follow-on study was initiated.

Horizon 2020 started in 2014. This presentation will follow on from work carried out in the original study, looking at what changes there are regarding the process of ethical review under Horizon 2020 and what recommendations have been taken up in relation to the use of any animal for scientific procedures. It will consider what benefits, if any, there may be in improving the transparency and the accountability of animal use, how it acts as a driver for take up of the 3Rs and whether the requirements of Directive 2010/63/EU relating to the use of these animals are properly addressed.

Reference

Virtue ethics: an ethical approach for non-clinical drug development?

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For A. MacIntyre (2007), today’s ethics are characterized by fragments of morality and by disagreement. This is also true for the conflicting ethical standpoints concerning animal use in non-clinical
Drug development. This presentation shows how virtue ethics may complement ethical decision-making in non-clinical development.

**Approach:** Aristotelian and Thomistic virtue theory are applied to studies in non-human primates (NHPs) to highlight the need for awareness of the intrinsic relationship between virtues and a first person’s perspective in assessing a concrete situation. Proposals for safety studies in NHPs are used to illustrate the reductionism of moral status concepts which focus on single virtues.

**Results:** Ethical justification requires compassion to prevent suffering, justice to reflect respect for the animal’s intrinsic value (e.g., social rank) and the trust between NHP and trainer, and temperance and integrity for the harm-benefit analysis. The monitoring virtue prudence provides the basis for dynamic animal welfare and integration of the good of the animal and the human patient.

**Conclusion:** Virtue ethics outline a holistic approach for animal ethics committees, which, coupled with the 3Rs and basic principles, will obviate check-the-box ethics.

**Reference**

**VII-2:090**

**A patient tail**

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**Aim/Objective:** We propose a new means of Refining nonhuman animal use in research by reconstruing animals as patients.

**Methods:** Following Russow (1999), we argue philosophical reasoning can facilitate progress in debates about animal research. We therefore propose a thought experiment in which two cancer sufferers are compared: one a human patient in a clinical trial of a new medicine; the other an “oncomouse” used in testing that same product. Specifically, we compare differences in – ethical review, recruitment, consent, research methods, endpoints and outcomes.

**Results:** Significant differences in the experience of these cancer sufferers are identified, highlighting important ethical considerations. In response, we propose construing animals like “oncomouse” as akin to human patients in clinical trials. This approach assuages many ethical objections to animal research and arguably delivers data more relevant to human medicine. It does however have limitations which we identify and discuss.

**Conclusion:** Given regulatory norms and the societal value accorded scientific knowledge, animal experimentation will continue into the foreseeable future. What we propose is a Refinement in animal use which can deliver ethical and epistemological benefits to humans and animals, and contribute to work on the implications of using “humanized” animals in translational biomedical research (Davies, 2012).

**References**

**VII-2:149**

Animal rights and ethics in research: is it possible a non-speciest approach?

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The term *speciesism* denotes a different assign of values or rights to beings, depending on their affiliation to certain species. A discrimination that construct a hierarchical relationship between species, creating an uneven allocation of rights and prerogatives. In an anthropocentric speciesism point of view the human beings are on the top of a *moral status pyramid*. Therefore, the principle of equal consideration shall be applied only to human beings, reducing the nonhuman animals as objects.

The Human Rights construction derives from the notion that man as beings endowed with moral rationality has something that sets it apart from other species, and that something allows us to interact with nature and other animals in a relationship of domination.

Following this logic, it is intended here to confront this speciesist perspective on scientific research and raise the question: is possible/feasible a non-speciest approach in scientific research? In other words, the current state of art of scientific research allows an ethical approach that takes into account the moral status of nonhuman animals and the interests of the entire biotic community? This issue will be discussed by analyzing the potential of applying animal rights principles in scientific research.

**VII-2:184**

Openness and accountability of animal research: a focus group study with local stakeholders at a Canadian university

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The University of British Columbia (UBC) has recently been subject to complaints over the lack of publicly available information about animal-based research at the university. The local nature of the debate provided an opportunity to use UBC as a case study to explore people’s views on openness and accountability of animal research. Four homogeneous focus groups were conducted with UBC researchers (n=7), local animal advocates (n=8), UBC students (n=6), and UBC animal care staff (n=6). The facilitated conversations addressed one overarching question: “How can universities be better held accountable for their various animal research practices?” Within this question participants explored the concepts of openness, democratic decision-making and public engagement. Participants expressed a desire for greater openness of animal research. Publicly accessible lay summaries and annual UBC open houses were two of the suggested methods to increase openness. Most participants also welcomed bi-directional dialogue about animal research, rather than simply being provided information. That diverse stakeholders all expressed a desire for greater openness suggests that policy makers should now prioritize efforts to develop mechanisms for better sharing of information and for constructive dialogue about university-based animal research.
Trends in animal use at US research facilities

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Minimizing the use of animals in experiments is universally recognized by scientists, governments, and advocates as an ethical cornerstone of animal research. Yet, despite growing public opposition to animal experimentation, mounting evidence that animal studies often do not translate to humans, and the development of new research technologies, a number of countries – including Canada, Australia, Israel, the United Kingdom, and Germany – have reported increased animal use in recent years. In the United States (US) – the world’s single largest user of animals in experiments – a lack of readily available data on the species most commonly used in laboratories (i.e., mice, rats, and fish) have limited such analyses. Using information included in reports submitted to the government by the top 25 institutional recipients of National Institutes of Health research funds, this study analyzes their use of all vertebrate animals over a 15-year period ending in 2012. Despite institutional commitments and government policies to reduce animal experimentation, these data show the use of animals at these US facilities grew by 71% during this time period – driven primarily by increases in the use of mice, mirroring the increases in animal use seen globally.

1R is the new 3Rs

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Replacement of animal experiments is one of the new 3Rs, but this does not always result in complete substitution of animal use. It has become accepted by many in the research community that some aspects of animal use can be classed as “alternatives”. In particular, the use of species thought to either not experience pain or to have lower levels of sentience (e.g., invertebrates, fish), of animal parts (including tissues, embryos, serums) or of data from previous animal experiments. Some species are exempt from legislation designed to protect animals used in research, either entirely or for early parts of their lives, leading to accepted use despite the potential to experience pain and suffering. These methods are entrenched by regulatory bodies, making it more difficult to reach a time when no animal use will exist in research. Individual “replacements” are explored with discussion of the benefits and problems of their use. If animal testing is both scientifically and ethically invalid then the continued use of any animal or animal part should not be accepted. It is argued that replacement should mean the total elimination of animals and animal parts and that focus should be moved from 3Rs to a true 1R.

The curious language of animal research and how it jeopardizes the public trust

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The public trust is an essential element of the continued use of animals in biomedical research. Currently, the public largely supports the use of animals in research, but only when assured that (1) the use of animals is necessary and that (2) the animals are treated humanely. The scientific community has proclaimed both. However, the meaning, value, and truthfulness of these assertions – and with this, the credibility of researchers – are wholly dependent upon the way in which the key terms, in particular, “necessary” and “humanely,” are defined. Furthermore, “necessary” can only have meaning when the definition specifies “necessary for what”? Other provocative terms used in the context of animal care, such as “cruel” and “undue suffering,” also have definitions relevant to animal research and public support. This issue is not an argument for or against the morality of using animals in research; rather, the concern is whether the scientific community, when discussing animal research publicly, is using standard dictionary definitions for the most important terms. If not, the question of honesty arises, and being that the credibility of researchers and the public’s trust in the scientific research community is at stake, clearly the issue must be directly confronted.

Life after research: the psychological well-being of animals following release from the laboratory

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Recent years have seen increasing consideration for alternatives to the routine euthanasia of research subjects at the conclusion of all studies (Anon, 2003). As a result, a small but growing percentage of animals used for research are eventually released to live out their lives outside the laboratory (Carbone et al., 2003). In these cases, the notion of laboratory animal welfare extends well beyond the period of time the animal serves as an experimental subject. While the ideal for many of the companion animals released from the laboratory is to live out their remaining years with a loving family, some companion animals and all noncompanion animals (such as farm animals and chimpanzees) live out their lives in animal sanctuaries (Noon, 1999). The foremost concern for all of these animals is how well their lives fare after release from the laboratory, that is, their quality of life, psychological well-being, and enjoyment of life.

This talk will present a comprehensive view of the well-being of animals formerly used in research, including the peer-reviewed published reports as well as individual cases of the one-time laboratory animals now living in sanctuaries.

References
Animal experimentation: transparency and public concerns

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The public is scarcely informed about what transpires in animal research, and is rarely engaged in decision making. Some justify this state of affairs by describing the public as large, emotive and lacking experience-based expertise. Some researchers claim to be able to represent society at large, and to possess the relevant knowledge for decision-making with regard to science (Marks, 2013). Ethical concerns of the public often appear to be downplayed when making decisions about research priorities and projects. The purpose of this paper is to show how interested publics can gain access to relevant information so they are suitably educated in order to participate in decision making, and articulate their ethical concerns. In the first part of the paper I will give some insight into why the number of animals used in experiments continues to rise internationally, despite the integration of 3Rs Principle (Russell and Burch, 1959) into most applicable legislation (Directive 2010/63/EU) worldwide. The second part will identify methods of obtaining information pertaining to animal experimentation in seven countries including Germany, the UK and the US. The third part will analyse a questionnaire concerning transparency and ways of public engagement in animal research which was recently conducted with members of the interested public.

References

Emerging trends in alternative to animal experimentation

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Biomedical research and toxicological studies involving animal models have served the human race very well. However, these experiments are highly animal intensive. Therefore, toward reducing the animal cruelty, Russell and Burch (1959) put forth the principle of the Three Rs (Reduction, Refinement and Replacement), with the idea that alternatives to animals would play a vital role in reducing the numbers of animals used, as well as the level of their suffering during experimentation. Since then, research has come a long way in finding alternatives for various aspects of toxicological testing. However, animal models could not be replaced completely by non-animal research models. Despite this, several alternative research models, such as in vitro (cell culture), in vivo (lower vertebrates and invertebrates) and in silico (computational modeling) systems, are being employed by scientists and researchers to limit the numbers of animals used in experiments. Although the use of animals cannot be completely avoided, the initiative toward developing faster, cheaper and more-accurate methods is essential to ensure that the innumerable chemicals present in the environment can be evaluated. The development of alternative animal models and the subsequent interrogation of models, methods and data from all platforms, to better predict public health outcomes.

Reference

Attitudes on animal use shown by scientists at workshops in Taiwan

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taiwan law covers the use of animals in science and requires least pain and minimal numbers and ethical committee supervision. However, Taiwan has a cultural background very different from that in the UK, and it was of interest to assess attitudes to the use of animals in science by those involved in animal experiments and how that might change after a two day course on ethics, animal welfare and the 3Rs. A questionnaire presented 20 statements with an ethical content and asked for responses on a five-point scale, from strongly disagree to strongly agree. This was given to attendees at the beginning and end of each of two workshops run jointly by the RSPCA International and Taiwan’s National Laboratory Animal Centre. These workshops did not discuss specifically the statements used in the questionnaire. On some statements the initial responses were similar to those that might be expected in the UK, differed little between the 66 respondents, and did not change much. Other responses were variable, and in some, such as the routine use of single housing, there was a marked shift in attitude over the course of the workshops. This presentation will review the responses and what they indicate.

The harm-benefit analysis within the evaluation of animal experimentation projects. How to assess criteria like species, number of animals or death of animals?

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The Directive 2010/63/EU requires a harm-benefit analysis as a part of the project evaluation by the competent authority, which has to take ethical considerations into account. In Austria, the Animal Experimentation Act 2012 (TVG 2012) transposes this requirement into national law. As a specific feature, the TVG 2012 demands that applicants have to fill in a catalogue of criteria to objectively and standardize the harm-benefit analysis. We are developing this Austrian Catalogue of Criteria (ACC), which has to be used within the authorization process.

The ACC consists of different categories, including specific questions to the applicant. The answers are assessed, counted as factors in the harm-benefit analysis and reviewed considering the facts and justifications the applicant has to provide.
However, some criteria cannot be assessed easily. Given that the project has been planned accurately and the 3 Rs have been taken into account, the following questions still arise: Should the species play a role in the harm-benefit assessment? If yes, according to which criteria should the species be assessed? Is the number of animals relevant? If yes, what are high and low numbers? Should the death of animals count on the harm side in the context of Austrian legislation?

Acknowledgement: The project is funded by the Austrian Federal Ministry of Science, Research and Economy (reference number: BMWF-10.2400018- III/3/2012).

Using minipigs in pre-clinical studies in Russia

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References


In Europe, minipigs are widely used as an animal model in toxicology studies (Foster et al., 2010). The minipig presents a favourable profile as a non-rodent toxicology model, in terms of the similarity to man and also in terms of applicability to different study types. Minipig studies may better reflect human drug-induced toxicities than studies performed in traditional non-rodent toxicology models. It would be of particular value to gain a better vision of the potential utility of the minipig as a model for the safety testing of new biologics, where the minipig could potentially replace the use of non-human primates and dogs in the testing of some new products (Bode et al., 2010).

In Russia, mainly rodents are used for pre-clinical studies. Only a few organizations use minipig for biomedical research. Our organization (http://www.nc3rs.org.uk) adhere to the principles of 3Rs (Russell and Burch, 1959) and Directive 2010/63/EU. To improve the work in accordance with the principles of 3Rs 2 years ago we started breeding minipig as an alternative to rodents.

Minipig use in biomedical research will reduce the number of experimental animals. Using minipig for pre-clinical studies will improve the safety of drugs and will protect volunteers in phase 1 clinical trials.

VII-3a-104

Update from the AALAS-FELASA WG on harm-benefit analysis

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Aims/Objectives: Define the concept of harm-benefit analysis (HBA). Recommend how it can be implemented.

Methods: Literature review included papers from 1986 to 2013. References on cost/risk benefit from clinical trials were also included. A workshop on harm-benefit analysis where participants were introduced to one method of HBA was organized.

Results: Several approaches to HBA were identified including algorithms, graphic presentations and generic processes. All existing processes have been criticized. The definition of harm is based on a several factors influencing animal welfare. The 5 freedoms were suggested as a basis for harm assessment. Severity categories for harm are also defined. Subjective opinions cause problematic bias. To limit bias different modulating factors which aggravate and mitigate are defined for the HBA. Benefit domains include benefits for humans, animals, environment, knowledge and education. It was questioned if economic benefits alone can justify animal use. A similar approach to limit bias was applied to harm analysis. Examples of the working groups approach illustrations are included.

Conclusion: Several approaches to HBA are presented in the literature. The WG proposes a practical methodology to address HB analysis. Independent of method HBA must be systematic, transparent and verifiable. Further work-shops and training sessions are scheduled.

VII-3a-558

Harm-benefit analysis of animal experimentation: lack of conceptual clarity and underlying moral disagreement

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Harm-benefit analysis is now a required key element of the legal framework regarding animal experimentation in Europe and elsewhere. Despite its wide adaptation, however, there is a huge lack of clarity concerning what is meant by it; and there seem to be some genuine underlying ethical disagreements. We will highlight key challenges and discuss to what extent agreement can be reached over how to evaluate animal research.

Firstly, there is lack of clarity and potential disagreement over what to include in the benefit analysis and on how to evaluate benefits. One approach includes evaluating societal contribution (Bateson, 1986), whereas another focuses more narrowly on knowledge gain in relation to other work in the field (Smith et al., 2007). Including research purpose in the analysis will reveal disagreements about how to value different purposes. For example, some will attribute a lower value to obesity than to cancer research as a justification for using animals (Lund et al., 2012).

Secondly, the idea of weighing harms against benefits lends itself to different interpretations, in turn linked to ethical disagreements. The controversy over the non-renewal of Swiss neuroscience project licences because the “expected benefits to society were not sufficient to justify the burden to the animals” (Buchen et al., 2008) illustrates this tension.

References

VII-3a-649
Harm:benefit analysis – if no projects fail the test has the system itself failed?
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The conduct of an animal experiment in Europe is now only permitted following a positive harm:benefit analysis (HBA). However, HBA is new for many countries and is only done in a rudimentary sense by others. Using graphical representation of principles and real examples, we explore the animal protection view of some key theoretical issues relevant to the HBA and ask the question: is the system fails to reject any projects does this mean the system itself has failed?

Questions for discussion include, is the purpose of a HBA to apply the 3Rs or to evaluate an application after these have been applied? At what stage does the question of whether there is a better way (the “alternatives test”) need to be considered? Is the extent of harm being measured on the same level as the extent of benefit, i.e., at the level of the experiment? If quantification of harms and benefits is impossible, what is the best way to ensure the “right” answer? What is the value of single experts over committees? How do you ensure good representation of views in committees and does this matter? If regulators fail to reject projects what does this say about the process itself?
application of established approaches or the development of specific frameworks, such as the Endpoint Matrix. These issues will be discussed in this paper.

References

VII-3b-226
Ethical evaluation process: international approaches, issues and assessment
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Ethical evaluation of animal use is mandated in many geopolitical areas. Processes are based on institutional and/or government bodies. Such ethical oversight range from institutional committees, such as the American IACUC model to competent authorities at the country level. Differences exist also on functions, composition and authority of evaluation bodies. However, regardless of the differences in implementing procedures (engineering standards), the ethical principles and the desired outcome of the processes (performance standards) are similar.

AAALAC International categorized the findings (mandatory items and suggestions for improvement) identified during 671 site visits across the globe related to the ethical review process since 2011. Most frequent issues relate to Protocol Review Considerations (i.e., inadequate description of procedures), followed by Policies (more often categorized as a mandatory item). Other issues belong to categories such as Committee Composition and Participation; Oversight of Activities; Program Review and Facility Inspections; and Protocol Review Process.

Regardless of the existing process, the assessment of the ethical review efficacy should be based on the outcome: the actual implementation of the 3Rs and a culture of care at the institutional level. This performance based assessment can be performed internally by institutional committees and externally by competent authorities and assessment bodies. In September 2013, the National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals has devised a model procedure to ensure that “weighing of interests” is carried out correctly and uniformly (FFSVO, 2014). The model, which will be presented at the congress, provides an interpretation of the terms “strain” and “overriding interests” and leads users through the procedure in seven stages. It helps determine whether interventions are permissible in vertebrates, cephalopods and decapods, especially in the context of animal experiments, where weighing of interests is explicitly requested for each experiment. As condition precedent to the justification of animal experiments, any strain inflicted on animals must be limited to the indispensable minimum.

Reference

VII-3b-306
Project evaluation under Directive 2010/63/EU
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The Directive requires that all projects are subjected to a project evaluation and sets out in detail the elements to be considered, and the type of expertise which should be considered when conducting such an evaluation. The evaluation requires that a harm – benefit analysis forms part of the process, to assess whether the harm to the animals is justified by the expected outcomes, taking account of ethical considerations. In September 2013, the National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals

VII-3b-274
Dignity of the animal: weighing of interests in the context of the Swiss animal welfare act
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The Swiss Animal Welfare Act (SR 455, 2008) protects the dignity of animals. In the context of the act, the term “dignity of the animal” implies values beyond the mere wellbeing but is not considered an absolute asset. Therefore, respect for dignity does not preclude the imposing of strain on animals. However, potential strain must be justified by overriding interests. The relative importance of strain vs. interests is assessed by the “weighing of interests”. Because the animal welfare act does not provide detailed instructions, a study group of the Federal Food Safety and Veterinary Office has devised a model procedure to ensure that “weighing of interests” is carried out correctly and uniformly (FFSVO, 2014). The model, which will be presented at the congress, provides an interpretation of the terms “strain” and “overriding interest” and leads users through the procedure in seven stages. It helps determine whether interventions are permissible in vertebrates, cephalopods and decapods, especially in the context of animal experiments, where weighing of interests is explicitly requested for each experiment. As condition precedent to the justification of animal experiments, any strain inflicted on animals must be limited to the indispensable minimum.
The Austrian catalogue of criteria to objectify the harm-benefit analysis within the evaluation of projects using living animals

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According to the Directive 2010/63/eU (Article 38 (2) d) a comprehensive project evaluation comprising a harm-benefit analysis, taking into account ethical considerations, has to be carried out in Austria. As an outstanding feature, the Austrian Animal experimentation Act (tVG 2012, which has come into force on January 2013) demands that the applicant of a project has to fill in a catalogue of criteria. As a part of the application for project authorisation the catalogue has to be submitted to the respective competent authority.

Within a project at the Messerli Research Institute, we are developing the Austrian Catalogue of Criteria (ACC). This project is funded by the Austrian Federal Ministry of Science, Research and economy (BMWFW). the ACC has to be published not later than by the end of 2015 and to be used within the authorization process six month after publication.

The catalogue is based on scientific criteria in order to objectify and standardize the harm-benefit analysis. To provide a feasible methodology several methodological approaches have been integrated, amongst others a scoring and weighing procedure. The methodological structure of the ACC will be presented.

References
Session VII-3: Poster presentations

VII-3-055
Environmental impacts of animal research and testing
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The laudable trend among individuals, companies, and governments to decrease one’s environmental footprint has yet to fully address the significant impact of animal research and testing on the environment. The use and disposal of millions of animals globally from research and testing, and the associated use of chemicals and supplies, contributes to pollution as well as adverse impacts on biodiversity and public health. Environmental and human health hazards related to animal use occur as a result of the generation of chemical and biological wastes, energy usage, atmospheric release of incinerator gases and particulates, deposition of soil contaminants, and potential entry of waste and toxins into groundwater and drinking water (Taylor et al., 2008; Cubitt and Sharp, 2011; Office of Laboratory Animal Welfare, 2002; National Research Council, 2011; Chen et al., 2004). Investigations into these contributors to environmental harm will be presented. The environmental implications of animal use is another and to-date under-addressed reason for industries, government agencies, and other stakeholders to monitor and consider the need for and benefits of replacing animal use with increasingly available, non-animal alternatives that have fewer negative impacts. Science, human and animal well-being, and environmental sustainability together offer a cogent argument for accelerated development, validation, and use of non-animal research and testing methods.

References

VII-3-138
Analysis of animal experimentation oversight committee membership at US institutions
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In response to growing public concerns about the welfare of animals in laboratories stemming from high-profile exposés of abuse at research facilities, United States (US) authorities mandated the institutional animal care and use committee (IACUC) system to review, approve, and oversee vertebrate animal use and ensure compliance with federal regulations and guidelines. While minimum IACUC membership requirements set forth by federal bodies reflect equal representation of all interests – animal researchers, institutions, animals, and the public – there is no requirement in the US to maintain a balance between these interests. This study analyzed the overall membership of IACUCs at leading US research institutions. We found that these committees are comprised predominantly of animal researchers (62.5 percent); veterinarians, most of whom are involved in animal experimentation (17.1 percent); nonscientists (10.1 percent), and unaffiliated members (9.6 percent). This overwhelming presence of animal researchers and institutional interests dilutes input from the few IACUC members representing animal welfare and the general public, contributes to previously documented committee bias in favor of approving animal experiments, and reduces the overall objectivity and effectiveness of the oversight system.

VII-3-139
Great ape disgrace: how the US laboratory oversight system failed chimpanzees
J. R. Goodman1,2 and A. Chandna1
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The US remains the last industrialized country to conduct invasive biomedical experiments on chimpanzees. In recent years, significant public opposition has prompted a reappraisal of the scientific need for the controversial practice. In 2011, the Institute of Medicine released a landmark report concluding that “most current biomedical research use of chimpanzees is not necessary.” A subsequent report by the US National Institutes of Health (NIH) further determined that “research involving chimpanzees has rarely accelerated new discoveries or the advancement of human health for infectious diseases...” As a result, NIH cut funding for most of its invasive chimpanzee experiments, established new oversight mechanisms specific to chimpanzee use, and made plans to retire most federally owned chimpanzees in laboratories to sanctuaries. Until that point, NIH had widely funded, conducted, and advocated for experiments on chimpanzees. The findings of both IOM and NIH reports raise the question of how and why NIH was continuing to approve, conduct, and fund experiments on chimpanzees that were ultimately deemed “unnecessary.” By examining the approved applications for several of these now-defunct projects, we discuss how the scientific review and animal welfare oversight systems failed and ways to improve these systems for all animals going forward.

**VII-3-403**

**Germany: animal welfare representative legal action and its effects on animal experimenting**

*C. Baumgartl-Simons, C. Hohensee and C. Ledermann*

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In Germany, the decision as to whether the legal requirements for authorising animal experiments is more or less dependent on a subjective assessment, but the decisions made by applicants and authorities are by no means based on generally accepted scientific precepts. This statement especially applies to questions regarding available alternatives, the benefits and ethical justifiability of the animal experiment. For this reason, PARG has campaigned for the introduction of legal action and court examination of compliance with applicable animal protection legislation. To date, only those experimenting on animals could file law suits before administrative courts challenging animal welfare requirements deemed too stringent. No one could apply for a court examination of insufficient animal welfare requirements. Five of Germany’s 16 federal states have passed laws providing for representative legal action. Five more state parliaments are currently debating their introduction. In the states with animal welfare representative legal action, animal welfare organisations can be accredited by the state governments. The responsible authority forwards applications to them. In four weeks they can submit objections to the authority. If the authority does not take these objections into account, the accredited organisation can file legal action against the approved animal experiment at the administrative court.

**VII-3-726**

**German animal welfare act in breach with Directive 2010/63/EU**

I. Ruheld, K. Wagner and R. Kolar

Alternatives to the use of animals in research and testing, German Animal Welfare Federation, Animal Welfare Academy, Neubiberg, Germany

The German Federal Administrative Court recently announced an order (finalized on January 20, 2014) on the neurobiological experiments on primate brains of Prof. Kreiter at the University of Bremen. With this order, a preceding court decision by the Higher Administrative Court of Bremen was established as final and absolute and the last glimmer of hope to end the suffering of the primates in Bremen was extinguished. The court decision had claimed the experiments to be ethically justified. The Federal Administrative Court upheld the court decision and issued the order on the grounds that due to the phrasing of both the former and the current German Animal Welfare Act, authorities had no entitlement to assess the ethical justification of an experiment, but were obliged to approve an application if all formalities were complied with. The impact the order will have on the authorization of animal experiments and testing in Germany caused an outrage in the animal welfare community.

**VII-3-844**

**The “benefits” standard: how can it better protect animals?**

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Biomedical or toxicological experimentation on animals is usually accepted on the basis of its assumed or anticipated benefits to human health. When ethical justification of a study is required or requested, reviewers (and the general public) can usually be satisfied by a presentation of the costs (or harms) and benefits, comparing the moral weight of the study’s benefits to humans against the likely harms to the animal subjects. Because it is a consensus approach, underlying assumptions about the nature and moral status of humans and other animals are rarely questioned or challenged.

This presentation will briefly review those assumptions in order to show how the “benefits-to-humans” standard for animal experimentation will ultimately fail to protect animals without a more flexible interpretation of harms. Past efforts to refine harm assessment will be reviewed; new, practical approaches to such assessment will be explored; and ways to make our public communications about the ethics of animal research more exacting, educational, and enlightening will be discussed.
Assessing welfare – why and how

P. Hawkins, E. Lilley and M. Jennings
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Assessing the welfare of animals used in scientific procedures is important for several reasons. First, a good system for observing and monitoring animals, and recording observations, is necessary in order effectively to recognise signs of pain or distress. Any suffering that is detected can then be alleviated in good time, which benefits animal welfare. Second, unrelieved pain or distress leads to physiological and behavioural responses that can negatively affect data, so good welfare assessment and project evaluation. Examples of these will be presented.

norface: using a national consensus-platform to promote the 3Rs

A. Smith
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Differences in the perception of stakeholders regarding the harm and benefit of animal experiments led to an initiative in the late 1990s to increase dialogue and thereby identify areas where there was common ground. This resulted in the establishment of ecopa (European Consensus-Platform for Alternatives), an umbrella organisation which promotes the 3Rs by bringing together regulators, academia, industry and animal welfare organisations. Ecopa encourages the existence of national consensus platforms which do the same, among those the Norwegianorganisation Norecopa (http://www.norecopa.no).

A consensus platform can identify and address issues of national relevance to the 3Rs. The extensive use of fish in Norwegian research has led Norecopa to focus on welfare issues and severity classification for these species, but it has also arranged international consensus meetings on the care and use of wildlife and agricultural animals in research. Consensus statements from these meetings and Norecopa’s own guidelines for research act as useful checklists when planning activities to further advance the 3Rs.

National Consensus Platforms have access to resources and opinions from a wide range of sources. These platforms can generate 3R-tools which are relevant worldwide to welfare assessment, severity classification and project evaluation. Examples of these will be presented.
assessment benefits the science. Finally, assessment records are the basis for retrospective reporting of actual severity, a legal requirement under Directive 2010/63/EU. A robust protocol for assessing the welfare of animals day-to-day should include specific behavioural and physiological indicators for each species and procedure, to help judge the level and nature of any pain or distress – or whether welfare was good. Consistency in observations and judgements should be promoted by taking a “team approach”, including input from staff with a range of expertise and priorities, coupled with adequate training in animal biology and behaviour and in the assessment protocol itself.

This presentation will set out available guidance on assessing laboratory animal welfare, from the European Commission and other bodies, including examples and recommendations for good practice.

VII-4-635

The case for refinement: making prospective and retrospective assessment of the severity of procedures count for animal welfare in biomedical research

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Scientists conducting animal experiments work under a social licence, which warrants its scientific and ethical justification, and a commitment to the 3Rs. To uphold these standards, a set of external (legislation), internal (community-centred) and mixed (ethical appraisal) regulatory systems are commonly in place. However, animal experiments often fail to yield valid results, due to methodological errors and/or unaccounted biases (Perrin, 2014; Macleod, 2011). Also, many studies still allow animals reaching severe end-stages or death, even when ethically approved (Franco et al., 2012; Franco and Olsson, 2012). The regulatory framework set by the 63/2010/EU directive can help improve the relevance of protocol appraisal, through mandatory prospective and retrospective assessment of the severity of procedures. These provide an opportunity for a community-centred, case-by-case, reflection on the welfare impact of experiments and on how it can be alleviated. While prospective assessment calls upon scientists to consider all potential sources of animal suffering and means to address them, retrospective assessment allows for an evidence-based reappraisal of the impact of experimental procedures and refinement measures. This presentation will stress the necessity to make such data available to inform subsequent studies, as it can allow to more accurately predict adverse outcomes; develop early, scientifically sound humane endpoints; improve prospective severity assessment; and identify opportunities for refinement.

References

Session VII-4: Poster presentations

VII-4-410

Emotional pain: why it can matter more to animals than physical pain

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The last two decades have seen remarkable advances in our understanding of well-being studies, Best Friends Animal Society, Kanab, Utah, USA

Physical pain is usually a sign of actual or impending tissue damage, whereas emotional pain, as a form of pain has strong empirical support (Eisenberger, 2012). Current evidence suggests that in the course of brain evolution, the brain systems that underlie specific types of emotional pain developed as a form of pain has strong empirical support (Eisenberger, 2012). Current evidence suggests that in the course of brain evolution, the brain systems that underlie specific types of emotional pain developed by co-opting brain circuits that mediate the affective component of physical pain (Panksepp et al., 1997) and that in present day mammals physical and social pain rely on the same neurobiological substrates (Kross et al., 2011). Separation distress and loneliness, for example, are now known to be regulated largely by endogenous opioids (Benton et al., 1988). More generally, and importantly, current evidence demonstrates that emotional sufferings in animals and humans often weigh more heavily in decision-making, i.e., matter more to the individual, than physical sufferings (Anil et al., 2005).

This talk will present the current knowledge regarding emotional pain and suffering; clarify the essential distinctions between emotion, stress, and distress; and explain the importance of these issues for laboratory animal care.

References

VII-4-412

The complexity of animal well-being: advances in our understanding of quality of life, welfare, and happiness – and why it matters in animal research

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For the objective of maximizing animal well-being, the near-exclusive emphasis on the alleviation of suffering (also commonly viewed as minimizing stress) is an incomplete approach, and attention to promoting positive mental states is recently recognized as also impor-
tant (Boissy et al., 2007). However, this well-being issue is relatively simple when compared to the much more complex questions that arise when the various well-being constructs – quality of life, welfare, psychological well-being, and happiness – are applied to animals. Current research is revealing the relevant similarities – and crucial differences – in these constructs between animals and humans (Weiss et al., 2011), with compelling evidence in nonhuman primates and other species for (1) the coexistence of short-term, momentary affective states (e.g., transient happy feelings) and long-term, relatively stable mood states (e.g., global happiness or subjective well-being) (Weiss et al., 2006), (2) correlations between long-term well-being and personality traits (Weiss et al., 2002), and (3) the health benefits of positive dispositions (Glickman et al., 1997).

This talk will present the current understanding of quality of life, well-being, welfare, and happiness in animals; the evidence for long-term affective states and their relationship with animal personality; how these states in animals compare with those in humans; the beneficial health effects of positive mental states; and recent advances in evaluating well-being states.

References

Session VII-5: Benefit evaluation
Co-chairs
Marlies Leenaars, UMCN, The Netherlands
Malcolm Macleod, University of Edinburgh, UK

Session VII-5: Oral presentations

VII-5-087
An analysis of the use of dogs in predicting human toxicology and drug safety
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Dogs remain the main non-rodent species in preclinical drug development. Despite the current dearth of new drug approvals and megapipelines, this continues, with little supportive evidence of its value or necessity. To estimate the evidential weight provided by canine data to the probability that a new drug may be toxic to humans, we have calculated Likelihood Ratios (LRs) for an extensive dataset of 2,366 drugs with both animal and human data, including tissue-level effects and Medical Dictionary for Regulatory Activities (MedDRA) Level 1–4 biomedical observations. The resulting LRs show that the absence of toxicity in dogs provides virtually no evidence that adverse drug reactions (ADRs) will also be absent in humans. While the LRs suggest that the presence of toxic effects in dogs can provide considerable evidential weight for a risk of potential ADRs in humans, this is highly inconsistent, varying by over two orders of magnitude for different classes of compounds and their effects. Our results therefore have important implications for the value of the dog in predicting human toxicity, and suggest that alternative methods are urgently required.

VII-5-243
Lack of clinical results: are animal experiments still ethically acceptable?
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The ethical acceptability of animal experiments is to a great extend dependent on the anticipated (health) benefits for humans. After decades of performing animal experiments, it is useful to do retrospective research into the extent in which animal research has archived these benefits. The results until now show that only a small percentage of data from animal research reaches the clinic, which constitutes a threat for the ethical acceptability of animal research. There seems to be two reasons for this. First the methodological quality of the experiments is too low. Second there is an intrinsic conflict between the reductionist character of preclinical research and the complex nature of clinical treatment. For example working with young, healthy, inbred animals of one gender may produce interesting scientific results which, however, have little relevance for the patients of different (genetic) background, age, gender, etc. Ways must be found to include the complexity of the patient into the research, instead of “reducing it out”.

In the scientific field these problems have been recognized and ways to address them have been figured out, but in a scattered and not systematic way. In order not to lose credibility, action of the scientific community is needed.
Added value of research synthesis in laboratory animal science

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To fully implement the principles of humane science, it is essential to ensure that animal studies are of the highest possible relevance and quality. Methodology and reporting of animal studies is currently inadequate and improvements are urgently needed (Anon, 2014). Research synthesis may be helpful to improve this situation. Recently, systematic reviews of animal studies, as a methodological approach of synthesis of evidence, were introduced within laboratory animal science. For example, in 2011 the Montréal Declaration on the synthesis of evidence to advance the 3Rs principles in science was adopted at the 8th World congress on Alternatives (Leenaar et al., 2012). The potential benefits of systematic reviews of animal studies are: stimulating better science, leading to better informed ethical review, helping to achieve the Three Rs, and improving translational transparency to inform clinical trials. The number of systematic reviews of preclinical animal studies (Ritskes-Hoitinga et al., 2014) is growing. Education and training is already available and needs further development and widespread distribution worldwide, just as tools and guidelines need to be developed further (Van Luijk et al., 2014). An overview of activities on systematic reviews of animal studies within laboratory animal science, the current state of affairs and what is needed for further progress will be presented.

References

Does interpretation of the principle of reduction by ethics committees lead to underpowered studies?

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When animal use in science cannot be replaced, there is an ethical imperative to use the minimum number of animals consistent with the study’s objectives. Determining the right number of animals should be the shared goal of investigators and animal ethics committees. However, this is not easy: if too many animals are used, then their lives and other resources are wasted; and if too few animals are used, then results may lead to a type II error. It has been suggested that few animal ethics committees ask for an increase in numbers when the proposed experiments are underpowered, with the implication that a narrow interpretation of “Reduction” is trumping optimal experimental design. We wanted to assess how the principle of Reduction is interpreted and sample size calculations evaluated by Canadian animal care committees (ACCs). We surveyed all ACCs in Canada to determine: 1) if ACCs utilize a priori sample size calculations when evaluating proposed experiments; 2) how ACCs interpret Reduction; and 3) if other considerations (e.g., costs) are included in the evaluations. We will discuss whether the results show that ACCs’ interpretation of Reduction is indeed discouraging optimal experimental design.

Why considerations of rigour should be central to the ethical review of experiments using animals

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Research regulators are now quite good at ensuring that adequate attention is given, in the design of experiments, to the principles of refinement and replacement. Attention to reduction, combined with limited resources, mean that in many fields experiments are too small reliably to detect biologically important effects.

The focus on regulation from the perspective of the experimental animal has led to a failure to consider another, fundamentally important consideration. It simply cannot be ethical to conduct animal experiments which are at such high risk of bias that their results are of little value.

In a random sample from PubMed 20% of in vivo studies reported randomisation, 3% reported the blinded assessment of outcome, and none reported a sample size calculation. 68% of in vivo studies from 5 leading UK universities in 2009-10 did not report any of 4 measures to reduce the risk of bias and only one reported all 4 (Nagel et al., 2011).

Considerations of the ethical status of experiments using animals cannot have legitimacy unless they consider the scientific rigour of those experiments; and that, at least for research which is published, the available evidence suggests that this is either not being done at all or not being done well.

Reference

A structured approach for using preclinical studies to assess risk/benefit in trials and animal studies

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Moral evaluation of risk/benefit in animal studies of new drugs and early phase human trials requires assessing the clinical promise of a candidate intervention using preclinical evidence. Yet there is little to guide ethics committees or investigators making these assessments. In what follows, we draw on published guidelines for preclinical study design to develop a structured process for assessing the clinical promise of new interventions. In the first step, reviewers gather all relevant
preclinical studies, assess the magnitude of treatment effects, and determine clinical promise in light of various threats to valid clinical inference. In the second step, reviewers adjust assessments of clinical promise from preclinical studies by examining how other agents in the same reference class and supported by similar evidence have fared in clinical development. Assessments of clinical promise can then be fed into moral evaluation of risk and benefit in early phase trials and animal studies. Though our approach has limitations, it offers a systematic and transparent method for assessing risk/benefit for studies involving novel interventions.

Session VII-5: Poster presentation

VII-5-307

**Benefit evaluation within project evaluation under directive 2010/63/EU**

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The Directive requires that all projects are subjected to a project evaluation, which includes that a harm-benefit analysis forms part of the process, to assess whether the harm to the animals is justified by the expected outcomes.

In September 2013, the National Competent Authorities for the implementation of Directive 2010/63/EU endorsed recommendations prepared by an Expert Working Group convened by the European Commission to develop guidance on Project Evaluation.

The recommendations include guidance on the assessment of benefits, both direct and indirect. Issues to consider include, for example – What will be the benefits of the work? Who will benefit from the work? How will they benefit – impact? When will the benefits be achieved?

The weighing of non-comparable, sometimes abstract benefits arising from different types of research programmes is very difficult to perform objectively.

The “importance” of work is a subjective judgement changing with time and place depending on a number of variables such as culture, environment, economic situation, acquired knowledge, emerging unsolved scientific problems and ethical values.

His emphasises the need for a unique, case-by-case evaluation of the importance and magnitude of benefits for each proposed project.
Can facial expression identify pain responses in rhesus macaques (Macaca mulatta)?

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Successful captive animal management is dependent on addressing welfare needs, including the recognition and alleviation of pain. Facial behaviour is an effective method for identifying pain in humans and rodents, but its use is yet to be evaluated in rhesus macaques. We utilised the Macaque Facial Action Coding System to determine whether facial action units differ in the pain and non-pain state in this species. Video footage was collected opportunistically from animals undergoing potentially painful research procedures and coded for facial movements and general behaviour in four conditions: pre-procedure, post-procedure, pre-analgesia and post-analgesia. Facial action units were analysed using a discriminant functions analysis to identify dimensions that map closely with expected levels of pain. Two dimensions were identified – the first fit closely with predicted pain, and the second was likely associated with arousal. Several behaviours were affected by study condition including cage manipulation, focused vigilance, and bipedal standing, however facial movement mapped more closely with expected pain levels than did general behaviour. While this study is the pilot of on-going research, we conclude facial expressions are useful indicators of pain states in rhesus macaques, and training staff to recognise facial expressions would promote refinements in husbandry and veterinary care.

Refining the use of non-human primates in research: the NC3Rs as a catalyst for change

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For 10 years the NC3Rs has led the application of the 3Rs to non-human primate (NHP) research. Working with the pharmaceutical industry, we have identified opportunities to reduce NHP use in studies of abuse potential, assessment of candidate therapeutics, and testing of monoclonal antibodies. Our guidelines on NHP accommodation, care and use, adopted by the major UK public funders of bioscience, and our involvement in the funders peer review processes, have raised animal welfare standards internationally. We have invested £1.8M in research and training to refine techniques used in behavioural neuroscience and to develop better methods for assessing pain and distress in NHPs. Working with scientists, veterinarians and animal technicians, we have published in the scientific literature detailed guidance on refining many aspects of NHP care and use (e.g., weaning, transport, training, food and fluid control protocols, rehoming). We have developed an interactive web resource on marmoset care. Our annual Primate Welfare Meeting continues to be a key event for showcasing the latest research in NHP welfare and sharing of best practice. This presentation will review advances in refinement made as a result of our investment and collaborative work, and identify areas where further research and changes in practice are required.
Vll-1a-761
Development of resources for refining research primate use: APV primate retirement guidelines
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The Association of Primate Veterinarians (APV) plays a key role in refining primate use and enhancing primate welfare by developing, educating, and disseminating best practices for nonhuman primate care and management. APV has developed and published a number of guideline documents to assist veterinarians, researchers, and animal ethics committees to enhance animal well-being. Subcommittees of subject experts from within and outside of APV develop draft guidelines. Guidelines undergo several rounds of peer and expert review prior to being adopted but are considered living documents subject to updates, as new information becomes available. To date, completed documents include food restriction guidelines, humane endpoint guidelines, social housing guidelines, and jacket use guidelines. Other documents under development include guidelines for fluid regulation, blood collection, restraint, cranial implant care, female reproductive laparoscopic surgery, male reproduction (semen collection), and infusion administration. A recent project has involved developing draft guidelines to assist facilities interested in permanently retiring non-human primates from research use. These guidelines cover selection of the prospective retirement facility, developing an institutional process, preparation of the animal, and preparing the staff and research team for primate retirement. Consideration of these issues when retiring research primates will be addressed.

Vll-1a-897
Nonhuman primate use in North America: overview, trends and important considerations for the future
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The use of non-human primates in North American laboratories will be reviewed and discussed. Invasive studies on chimpanzees are finally coming to an end but the use of other non-human primates has increased over the past fifteen years despite increasing questioning about the ethics of such use. Statistics available for Canada and the United States since 1999 show significant increases in the number of primates in laboratories (both used and held or bred for research) as well as the number of NHPs imported to the U.S. for research and breeding. In addition to a discussion of the overall statistical trends in NHP use, we will: (1) give an overview of NHP use and funding at the eight National Primate Research Centers in the U.S. (which are among the largest NHP labs in the US and house approximately 25% of the total US NHP lab population); (2) explore the reasons behind the end of chimpanzee use worldwide and what that means in terms of a critical examination of the use of other NHPs; and, (3) discuss where efforts should be focused to increase humane treatment of NHPs and decrease their numbers in laboratories.

Session VIII-1b: Non-human primate use and welfare

Co-chairs
Jaco Bakker, BPRC, The Netherlands
Melanie Graham, University of Minnesota, USA

Session VIII-1b: Oral presentations

Vll-1b-092
Refinement of non-human primate disease models and procedures at the BPRC
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Non-human primates are still needed in research on serious human life-threatening diseases. We also have the moral obligation to treat these animals in the best way as possible. The BPRC is involved in non-human primate studies but has also implemented an active and expanding programme in accordance with the principles of reduction, refinement, and replacement in non-human primate disease models. Besides 3R-research on alternatives, the BPRC also focuses on refinement with respect to animal husbandry and care. New housing concepts, positive reinforcement training of the animals and veterinary practices have reduced stress associated with routine husbandry and with experimental studies. Extensive environmental enrichment programs in both breeding and experimental facilities have been developed and implemented. New techniques like telemetry and non-invasive imaging methods are essential to further reduce the number of animals in studies. Continuous training of staff ensures ongoing improvement. Several examples of refinement involving non-human primate disease models, housing conditions and PRF-training procedures at the BPRC will be discussed. The new developments at the BPRC over the past years have enhanced the well-being of these highly complex and social animals.
A holistic approach to refinement improves medical management and safety in nonhuman primate disease models

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Nonhuman primates (NHPs) are used only in experiments when there are no suitable alternative methods or species. Their biological complexity closely mimics the human clinical patient adding unique translational value especially in infectious disease, brain injury, and immunology. The therapeutic need is high for clinical patients in these categories who experience a serious reduction in measured quality of life parameters. Likewise, it’s logical to assume there is a substantial burden on the animal considering disease state and management plus necessary monitoring for safety and efficacy trials. The application of refinement can shift the harm-to-benefit ratio, limit experimentally induced complications, and avoid additive effects. We present an overview of our experience with refinement in the complicated NHP model of diabetes including cooperative handling (training), enrichments, minimally invasive instrumentation, and advanced clinical care strategies. The impact of these techniques on morbidity and mortality led to a reduction in overall animals used, illustrating also the interplay between the 3Rs. The scientific value was clearly demonstrated by a significant reduction in confounding in stress sensitive parameters. This holistic refinement approach unequivocally benefits our animals and can enhance predictive value of the model, presenting an opportunity to engage scientists in a meaningful way with the 3Rs.

The use of advanced imaging to refine vaccine and therapeutic studies with non human primates

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Following successful quantification of disease using MR scanning of TB infected lungs (Sharpe et al., 2009, 2010) and in-life proof of principle studies using MRI and CT scanning, macaques infected with tuberculosis by aerosol challenge have been successfully scanned using a mobile high containment pod to avoid contamination of the tuberculosis by aerosol challenge have been successfully scanned. Scans have successfully identified lesions at very early stages of disease before onset of clinical signs and before disease detection by conventional X-radiography. Results of these early infection, low dose studies have ethical implications in refinement and reduction that come from the ability to detect early stage disease following administration of realistic low doses of the pathogen. Use of in-life scanning allows assessment of vaccine or therapeutic efficacy without the need to progress to stages of disease when clinical signs such as cough and weight loss become apparent. The use of scanning allows studies that can define progression of disease and spread to other organs such as the spleen, liver or kidneys in individual animals without the need for serial sacrifice of greater numbers of animals. The use of this technology will allow refinement and reduction in any infectious disease model requiring the use of non-human primates.

Simultaneous PK/PD: automated blood sampling and CV telemetry in conscious nonhuman primates

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Preclinical cardiovascular safety studies normally entail the use of radiotelemetry in large animals such as nonhuman primates (NHPs), while keeping animal welfare a priority. Challenges with the use of NHPs include signal interference during procedures requiring animal manipulation and increases in stress parameters. To address these issues and improve animal welfare, we conducted a proof of concept study whereby automated blood sampling (Culex-L) was coupled with digital radiotelemetry (DSI). Improved sensitivity from this combination allowed for a reduction in animals and improved correlation between the pharmacokinetics and pharmacodynamics (PK/PD). Four female NHPs implanted with telemetry devices and intracarotid catheters were dosed with moxifloxacin (Moxi), physiological parameters recorded, and blood sampled either manually (Man) or via the Culex-L. During sampling, plasma was collected several times between pre-dose and 24 hours post-dose. Challenges encountered included missed plasma samples and occasional telemetry data dropout. These challenges were resolved by revising component connections and by adjusting tracceiver placement. This study established the feasibility of collecting simultaneous blood samples by the automated Culex-L system while recording physiological measurements via radiotelemetry in unrestrained NHPs. This enhancement in methodology resulted in refining the study while reducing the number of animals involved and therefore, improving animal welfare.
also show in vivo data how these new adjuvants can contribute to the refinement of non-human primate studies and animal welfare.

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Workshop report: ‘Alternative methods for the use of non-human primates in biomedical research’

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The experimental use of non-human primates (NHP) in Europe is tightly regulated and is only permitted when there are no alternatives available. As a result, NHP are most often used in late, pre-clinical phases of biomedical research. Although the impetus for scientists, politicians and the general public to replace, reduce and refine NHP in biomedical research is strong, the development of 3R technology for NHP thus poses specific challenges. Last spring, a workshop on “Alternative methods for the use of NHP in biomedical research” was organized within the international exchange program of EUPRIM-Net II, a European infrastructure initiative that links biomedical primate research centers.

The workshop included lectures by key scientists in the field of alternatives as well as by experts from governmental and non-governmental organizations. Furthermore, parallel sessions were organized to stimulate discussion on the challenges for advancing the use of alternative methods for NHP. Subgroups voted on four statements and together composed a list with opportunities and priorities. I will present a summary of the voting and discussion sessions and end with recommendations on 3Rs development for NHP specifically. These include technical, conceptual as well as political topics.

Session VIII-1: Poster presentations

VIII-1-359

European Primate Network EUPRIM-Net II: advancing 3Rs and international standards in biological and biomedical research

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Biological and biomedical research is critical for our understanding of human and animal physiology and medical progress. Where no alternatives exist, this research necessitates the use of non-human primates (NHPs) for the foreseeable future. The EU-funded research infrastructure project EUPRIM-Net brings together ten European publicly-funded primate centers, to promote highest standards using these animals for top-level research. Under the project, the primate centers’ infrastructures and expertise are integrated to provide critical services, training and advice to scientific institutions within and outside Europe conducting primate research and to zoological gardens keeping primates. The activities are divided into Network-, Access- and Research Activities all aimed at promoting animal welfare and the 3Rs. Directive 2010/63/EC foresees various animal protection and welfare measures reflected in EUPRIM-Net’s activities. The Network Activities are about Education and Training, Best Practice and Veterinary Care, Positive Reinforcement Training (PRT) and Animal Behavioural Management. These activities are supported by Research Activities on Diagnostics and Diseases, Telemetry, and Alternative Methods. Moreover, EUPRIM-Net offers – via an easy online system (http://www.euprim-net.eu) – access to primate material (BioBank) and to primate-based animal models of severe human diseases (PRIMOCID) thus contributing to improving the 3Rs.

VIII-1-360

A cage-based behavioural testing system for NHP – Towards a cognitive neuroscience without restrained animals

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In conventional setups for experiments in cognitive neuroscience, the animals are usually restricted in several ways. For monkeys, movement restriction of the head is common practice when recording neuronal activity by electrophysiological or imaging techniques. However, technological progress especially of wireless techniques allows for recordings without major movement restrictions. We developed a cage-based system monkeys can interact with in an unrestrained and self-paced manner. It utilises a computer controlled touch screen and rewarding system, allowing for setting up various cognitive tasks and recording behavioural data whilst a monkey is interacting with the system. A future addition of miniaturised wireless devices for neuronal recordings, might enable cognitive neuroscientific experiments on unrestrained, freely moving monkeys not separated from their social group. We intend to include an identification system in order to individually assign cognitive tasks depending on the monkey that approaches the device. These refinements of classical setups might result in less stress for the animals and a positive impact on their learning progress. First experiences support the notion that with this system the natural curiosity of monkeys can be stimulated just as if it is part of the environmental enrichment.
Using an automated system of positive reinforcement training to refine the process of behavioural training and reduce the need for food control in rhesus macaques

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Behavioural neuroscience research requires subjects to perform repetitions of specific behaviours for food/fluid reward. Some animals fail to perform at a sufficient level, limiting the amount of data that can be collected from each animal and increasing the number of animals required for each study.

We have implemented an automated positive reinforcement training system (a button press task with variable levels of difficulty using a fluid reward) at the breeding facility Centre for Macaques (CM) and Newcastle University to pre-screen animals for selection and refine training protocols.

We found that animals learned 1- and 4-choice button tasks within weeks of home cage training, with some inter-individual differences. High performance levels (~200-300 trials per 60 min session at ~80% correct) were obtained without food or fluid restriction. Moreover, training quickly transferred to a lab-based version of the task.

Preliminary evidence suggests that animals that acquired the task at CM subsequently performed better in early sessions at Newcastle University. Therefore, it might be possible to use the automated system at CM to pre-screen animals for suitability for behavioural neuroscience research, thus potentially reducing animal numbers required for studies, refining training protocols and minimising requirements for food/fluid control.

Work supported by the NC3Rs.

Optimisation of preclinical use of Non-human primates (NHP) by species-specific Monocyte activation test (MAT)

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Pyrogenicity (fever induction) might be due to drug contamination or product characteristics (immune activation). Vaccines are a typical case for drugs with immune-stimulating potential. The platform technologies (combining known antigens with new adjuvants) increase the speed of vaccine development, accelerated and predictive safety tests are needed.

Non-human primates (NHP) are frequently chosen as a preclinical model (test species) for drug candidate testing before the first application in humans. The whole blood of NHP is typically investigated for biomarkers. In this study we compared four NHP-species to humans in vitro by species-specific MAT-versions with fresh whole blood. Compared to each other the NHP-species exhibit a different reaction pattern towards LPS (Endotoxin), but to some extend comparable to humans (depending on the chosen readout). We conclude that NHP-specific MAT are suitable for a first assessment of pyrogenicity of drug candidates or new platforms in the preclinical phase. The use of NHP-fresh blood for in vitro experimentation might lead to a diminished (Reduce) and optimised (Refine) use of NHP in preclinical testing. A complete replacement of animal tests in the preclinical phase seems unlikely in the near future.

Session VIII-2a: Best practice welfare approaches – Mouse

Co-chairs
Robert Hubrecht, Universities Fed. Animal Welfare, UK
Joanne Zurlo, CAAT, USA

Session VIII-2a: Oral presentations

Contribution of animal care and welfare to 3Rs in the pharmaceutical industry

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Animals continue to play a small but vital role in the discovery and development of new medicines and vaccines. We use non-animal methods when available, but there is no current replacement for a whole living organism; it is vital to understand how a medicine will interact with our internal system, whether it will be effective and whether it is safe. The provision of healthy, well cared for animals is essential for success of these studies, but we go beyond the basic requirements. In addition to ensuring that our animals receive appropriate housing, food and water we also strive to better understand their needs, to refine their care and environment and hence improve their well being.

This presentation discusses some of the welfare initiatives and resulting refinements in care and consequent changes to the scientific
studies achieved at GlaxoSmithKline over the past few years. These initiatives include changes to housing paradigms, refinement of experimental techniques, reward systems for new ideas and setting up a global community of practice to discuss and effectively disseminate refinements to animal care. All of this operates within a governance system which ensures implementation of the GSK core principles worldwide.

VIII-2a-284

Refinement and experimental design in publications: a longitudinal case study of ALS research

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Amyotrophic Lateral Sclerosis (ALS) is a human neurodegenerative disease of rapid progression for which no effective treatment exists, resulting in progressive paralysis and death. Similar clinical signs cause distress to research animals modelling the disease. From a 3Rs perspective it is desirable to implement refinement measures such as humane endpoints and housing adaptations. To evaluate whether measures of refinement and experimental design optimisation were reported, published studies on murine ALS models were analysed (n=267) (Franco and Olsson, 2012). Studies were classified according to a scale of severity, based on the disease stage that animals reached. Studies published in 2005, 2009 and 2011 were included to cover the period in which guidelines to improve pre-clinical ALS research were introduced (Ludolph et al., 2007, 2010).

Report of compliance with regulation for animal care and use increased over the years reviewed (p<0.01), however the severity level remained unchanged (62% of studies implemented a late stage endpoint). Preclinical studies were reported with significantly higher level of detail than proof-of-concept studies throughout the years. Compared with Huntington’s disease, a similar neurodegenerative disease model for which no specific research guidelines exist, ALS research used less severe endpoints with humane endpoints reported in 90% of publications.

References

VIII-2a-666

Welfare in the research environment: is what we have as good as it gets?

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Ensuring high standards of welfare for animals kept and used for research purposes requires a multifaceted approach that addresses the animal, its environment and husbandry and research procedures. The environment must meet the animals’ needs in terms of appropriate environmental controls, and housing provisions including enrichment where this is appropriate. The animal itself may also be modified to improve welfare, by selection for strains/breeds that do well in such conditions; by ensuring appropriate conditions during development and, where necessary, provision of training and habituation regimes. Procedures and techniques can also be refined to minimize stressful events such as capture and restraint. Nonetheless, there are still many gaps in our knowledge. The principle of enrichment is, for example, widely accepted, but the implications of strain, age, stocking density, environmental changes, and the special needs of animals under procedures are under-researched and the requirements of some groups such as fish and cephalopods have received little attention. High quality rather than ad hoc research is necessary for informative results and to provide credibility to the field. Best practice is most likely to be achieved when there are good links between those who use animals in research and the animal welfare science community.

References
PhenoWorld: increased welfare in a new paradigm for housing and evaluation of rodent behaviour

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Finally, both behaviors are reduced by pain and stress, therefore, in full as an early sign of dysfunction and to monitor disease progression. Their use has been proved as parameters in models of psychiatric disorders or to monitor sickness behavior following infection. Sucrose preference test revealed an increased hedonism and forced swimming test indicated lower immobility time for PhW living animals, proving an increased well-being of those animals. Light/Dark test revealed that PhW living animals were less anxious than standard housed animals. We have shown that this new ethological refined/enriched paradigm – the PhenoWorld – provides an improved welfare condition for rodents compared to standard cages.

Session VIII-2b: Best practice welfare approaches – Mouse

Co-chairs
Michael Walker, University of Guelph, Canada
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Session VIII-2b: Oral presentations

Burrowing and nest building behavior as indicators of well-being in laboratory mice

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The assessment of pain, distress and suffering, as well as evaluation of the efficacy of stress-reduction strategies, is crucial in animal experimentation but can be challenging in laboratory mice. Nest building and burrowing performance, observed in the home cage, have proved to be valuable and easy-to-use tools to assess brain damage or malfunction as well as neurodegenerative diseases. Both behaviors are used as parameters in models of psychiatric disorders or to monitor sickness behavior following infection. Their use has been proposed in more realistic and clinically relevant preclinical models of disease, and reduction of these behaviors seems to be especially useful as an early sign of dysfunction and to monitor disease progression. Finally, both behaviors are reduced by pain and stress. Therefore, in combination with specific disease markers, changes in nest building and burrowing performance may help provide a global picture of a mouse’s state, and thus aid monitoring to ensure well-being in animal experimentation.

Improving laboratory mouse welfare and reducing animal numbers through mixed-strain housing

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All common identification methods for mice can impair animal welfare (Dahborn et al., 2013) and many experiments utilize homogenous populations that inadvertently contribute to low external validity and poor reproducibility (Wuerbel, 2000). This work aimed to validate mixed-strain housing as a way to remove the need for marking; heterogenize the study population; and utilize a more statistically powerful experimental design. We raised 3–4 week old female C57BL/6, DBA/2, and BALB/c mice in single-strain or mixed-strain trios. At 3–5 months of age mice were assessed for 26 different variables spanning behaviour (e.g., stereotypies), physiology (e.g., blood glucose), and haematology (e.g., white blood cell counts). Single- and mixed-strain housed mice did not differ in any measured variable. Several strain differences emerged (e.g., faecal corticosterone metabolites): all were in the expected direction (e.g., Harizi et al., 2007). Furthermore, there were no interaction effects between strain and whether mice were in single- or mixed-strain trios. Mixed-strain housing also reduced inter-individual variation across all variables (p<0.0012). Mixed strain housing did not modify strain-typical phenotypes nor introduce any Laboratory environment in which rats are housed contribute for an impoverished ethological evaluation and reduced welfare of the animals. We used a new concept for housing and behaviour analysing rodents – the “Phenoworld” (PhW), in which groups of 6 rats lived in a socially and physically enriched environment, and had their feeding, locomotor activity, sleeping and social behaviour automatically monitored.

Home-cage behaviour analysis of animals housed in the PhW, as compared with standard housing revealed the new housing system promotes increased welfare as shown by the increased levels of sleep in the resting phase of the light/dark cycle and by the performance of species specific behaviours such as hopping and climbing. Sucrose preference test revealed an increased hedonism and forced swimming test indicated lower immobility time for PhW living animals, proving an increased well-being of those animals. Light/Dark test revealed that PhW living animals were less anxious than standard housed animals. We have shown that this new ethological refined/enriched paradigm – the PhenoWorld – provides an improved welfare condition for rodents compared to standard cages.
new welfare concerns, thus is therefore a potentially valid experimen-
tal paradigm to improve welfare and reduce animal numbers by im-
proving statistical power and increasing external validity.

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VIII-2b-288

The scientific and welfare benefits
of the right environmental
enrichment for laboratory mice

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Environmental enrichment is most impactful on the wellbeing of an animal when it is biologically relevant, meaning that the item or substrate addresses some behavioral need. Enrichments are especially beneficial if they allow control over the environment or a stressor. One of the few enrichments that meet these criteria for mice is nesting material. Mice under normal laboratory temperatures are thermally stressed, which can compromise aspects of physiology from metabolism to behavior. Over a series of experiments with 3 strains of mice, a nesting material was validated in terms of reducing a physiological stressor with natural coping behavior, improving welfare, and demonstrating end user benefits. Under recommended temperatures all mice preferred 6-10g of nesting material. When provided with 8g, nesting material was shown to reduce radiative heat loss, food consumption, non-shivering thermogenesis, pup mortality, and increase reproduction. One drawback to this amount of material is that mice build fully enclosed nests which prevent observations by animal care staff. Although the mice are not directly visible, nesting behavior shows promise as the basis of an assessment tool for recognizing pain, illness, and distress. An overall reduction in stress, by creating a unique microenvironment, can improve laboratory mouse welfare and scientific outcomes.

VIII-2b-475

The Mouse Grimace Scale (MGS): a clinically useful tool?

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Medical research has a heavy and continuing demand for rodent models. Behavioural assessment of pain in such models can be highly time consuming (Roughan, 2003; Miller, 2012) thus limiting the number of models and analgesics that can be studied. Facial expressions are widely used to assess pain in infants, and recently the mouse grimace scale (MGS) has been developed (Langford, 2010). The MGS has shown to be a coding system with high accuracy, repeatability and reliability requiring only a short amount of training for the coder (Langford, 2010). This system therefore has the potential to become a highly useful tool both in pain research and in the clinical assessment of mouse pain.

To date, the MGS has only been used as a research tool; however there is increasing interest in its use in cage-side clinical assessment. Here, we aim to assess the variability in baseline MGS scores between cohorts, sexes and strains. Establishing the presence of a consistent baseline MGS score could lead to a valuable clinical pain assessment tool for mice when prior (baseline) information from the individual mouse may not be available as a comparator. Additionally, the effects of isoflurane anaesthesia on MGS scores will be assessed.

* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

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VIII-2b-684

A global pharmaceutical company initiative: an evidence-based approach to define the upper limit of body weight loss in short term toxicity studies

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This abstract is submitted on behalf of the NC3Rs Acute toxicity Working Group. Short term toxicity studies are conducted in animals to provide information on major adverse effects typically at the maximum tolerated dose (MTD). Such studies are important from a scientific and ethical perspective as they are used to make decisions on progression of potential candidate drugs, and to set dose levels for subsequent regulatory studies. The MTD is usually determined by parameters such as clinical signs, reductions in body weight and food consumption. However, these assessments are often subjective and there are no published criteria to guide the selection of an appropriate MTD. Even where an objective measurement exists, such as body weight loss (BWL), there is no agreement on what level constitutes an MTD. A global initiative including 15 companies, led by the NC3Rs, has shared data on BWL in toxicity studies to assess the impact on the animal and the study outcome. Information on 151 studies has been used to develop an alert/warning system for BWL in short term toxicity studies. The data analysis supports BWL limits for short term dosing (up to 7 days) of 10% for rat and dog and 6% for non-human primates (Chapman et al., 2013).

Reference
Microenvironment in reusable and disposable individually ventilated cages: a comparative study

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The aim of this study was to compare 3 IVC cages (two disposable, one reusable) in terms of microenvironment quality (inha-cage NH3 gas), bedding weight, temperature, relative humidity, and in relation to early indicators of poor welfare in healthy mice: body weight, food and water intake (Burman et al., 2014; Vogelweid et al., 2011; Silverman et al., 2008).

30 C57Bl/6 and CD1 male mice, 5 week old male mice were housed in groups of five in Innocage IVC Mouse (disposable Universal Euro II Type Long), SUMC (Single Use Mouse Cage) and GM500 (reusable polysulphone Euro II Type Long) for six weeks. Intracage ammonia level, temperature and relative humidity were recorded every two weeks for three times. Mice and bedding at cage change were all weighed every two weeks, moreover food and water intakes were calculated weekly. Descriptive statistics and one-way ANOVA showed different performances of the 3 IVC systems in the maintenance of the microenvironment quality with the worst ammonia level in Innocage system. Bedding weight increased after two weeks in all cages, but the weight of bedding collected from Innocage system was steadily the highest. Water and food intake as well as mice growth curves were comparable and consistent with the strain features.

References

Husbandry and enrichment for the recently protected common European cuttlefish (Sepia officinalis)

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Cuttlefish are prolific inkers and can easily damage themselves when threatened by jetting into the sides of aquaria; these injuries rarely heal. Enrichment can reduce some forms of damaging behaviour but may have negative effects on water quality. We performed welfare assessment experiments, investigating which environments reduce stress indicators, whether the colour of equipment/clothing has an effect on their behaviour, what types of enrichment they used, if facsimiles of their natural habitat would be accepted and various aspects of group living.

We found certain practices significantly reduce the number of stress responses, along with inking and other damaging behaviours, also, welfare can be increased by enrichment that will not detrimentally affect water quality. This is the first study of its kind regarding welfare for these protected, complex and intelligent animals and we provide numerous evidence based recommendations for their welfare in captivity.

References
Vaccination in lambs – an animal welfare issue?

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Vaccines are important to improve animal health (Morton, 2007; Roth, 2011). In addition, vaccination is an easy and effective way to reduce pain or suffering and decreases the use of antibiotics (Singer et al., 2003). But, there are also reports about adverse effects. Local reactions at the injection site are a potential animal welfare issue, especially in sheep. The current concepts for clinical studies to evaluate side effects need a considerable number of animals due to pathological examinations performed at different points of time after vaccination. This study tested magnetic resonance imaging (MRI) as a non-invasive tool to evaluate local reactions after vaccination in live lamb. Totally, 32 Merino lambs were vaccinated and scanned at day 1, 3, 8, 15, 22 and 29 after vaccination. Extensive inflammatory reactions could be identified (inflammatory volumes up to 35 cm³). 27 lambs developed abscesses at the injection site. 50% of these animals underwent a pathologic examination, which reappraised the MRI results. It became apparent that vaccination with licensed products – even under experimental conditions – may cause severe local reactions. MRI seems to be suitable to investigate injection site reactions in live lambs. Using MRI for safety testing may help to avoid such welfare issues in practice.

References


Refinement in pain relief in sheep fetal surgery

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Pregnant sheep are commonly used as a model to study placental and fetal hemodynamics. Such studies require major surgeries and adequate pain relief. Although there is some evidence that fetuses do not feel pain, pain alleviation during and after surgical procedures is critical. The aim of this study was to determine fentanyl concentrations in plasma of pregnant sheep and fetuses, in order to refine the pain alleviation program in sheep surgery. Blood samples were taken from animals that were part of an experiment aiming to improve the outcome of the newborns suffering from placental insufficiency. Altogether 21 Aland landrace sheep and one fetus from each sheep were sampled. Fentanyl was administered intravenously to the ewes during the operation. Transdermal fentanyl administration was started perioperatively and continued postoperatively. Maternal and fetal blood samples were collected simultaneously during surgery and fentanyl concentrations were determined with LC-MS. The intraoperative fentanyl concentrations ranged between 0.3 and 0.6 ng/ml, a level considered to be analgesic (0.2-2.0 ng/ml). The fetal/maternal ratio varied between 0.41 and 0.79. However, the assessment of the pain relief must be based on the responses of the fetus and the ewe during the operation and behavior of the ewe postoperatively.
VIII-2-202

Putting animal welfare principles and 3Rs into action

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Promoting good science and animal welfare, and increasing understanding of how the two are intertwined, is an essential enabling of high quality research and 3Rs achievements. The progress we make in this area is one step in enhancing benefits for patients. This poster presents a non-exhaustive inventory of examples from the pharmaceutical industry and its collaborations (http://www.efpia.eu/topics/innovation/animal-welfare);

- Beyond policy principles. Since the late 80’s, the European Federation of Pharmaceutical Industries and Associations has an expert group fostering exchange of information and good practice within and across sectors, and promoting development and uptake of 3Rs approaches;
- Many 3Rs methods are a result of joining forces and sharing information. Examples include dried blood spot for toxicokinetic studies, refinement of short term toxicity studies based on body weight loss, and education tools on training animals;
- Beyond compliance. Pharmaceutical companies strive to improve research practice beyond legal requirements through monitoring and auditing research, review and best practice sharing, training on requirements of lab animal legislation;
- Continuous research efforts. Whether aimed at more predictive science or at 3Rs and welfare (IMI – Innovative Medicines Initiative, http://www.imi.europa.eu);

As research paradigms evolve and industry continues its efforts, more dramatic improvements can be expected in the future.

VIII-2-305

Refinement in German animal research applications

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The German Animal Welfare Act (TierSchG, 2006) and Directive 2010/63/EU require researchers to take all practicable steps to minimise pain, suffering and distress (Article 4 (3)). For the first time ever access was granted to animal research proposals that were submitted all over Germany in 2010. They were assessed to determine how effectively these legal obligations were being met. In over 500 research applications involving procedures in which mice and rats underwent recovery surgery Refinement methods, particularly the use of intra- and postoperative analgesia, humane endpoints and health score sheets as well as the monitoring frequency of the animals’ health status were evaluated.

This detailed survey indicated that approximately 19% of the animals who underwent severe procedures did not receive any postoperative analgesia. This presentation will include a range of examples of severity classification and will discuss potential ways to reduce the severity and improve Refinement.

References


VIII-2-304

Severity classification in German animal research applications

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Directive 2010/63/EU (1) aims to enhance transparency in animal research and requires researchers to classify the severity of procedures prospectively in research applications (Recital 22). This requirement was present in Germany prior to introduction of the revised Directive. For the first time access was granted to over 500 animal research proposals that were authorized in Germany in 2010. These involved procedures in which mice and rats underwent recovery surgery. They were examined to determine how researchers estimated the severity of procedures. Their judgements were compared to the guidance given in Annex VIII of the Directive (2010/63/EU) and to the recommendations of the Expert Working Group on a Severity Assessment Framework (NCA, 2012; EC, 2013). The researchers’ use of Refinement was also taken into account in the severity assessment.

The majority of the researchers (63%) underestimated the severity of their procedures. Estimated severity was also often higher than necessary since possible Refinement methods were not always applied. The use of analgesics for example was not routine – 19% of animals that underwent severe procedures did not receive any postoperative analgesia. This presentation will include a range of examples of severity classification and will discuss potential ways to reduce the severity and improve Refinement.

References


India frames guidelines for re-use and rehabilitation of dogs and non-human primates in research and testing

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India has boldly come forward to frame necessary guidelines which limit the use of dogs and non-human primates in testing and research. The CPCSEA, the statutory body of the Government of India, which regulates use of animals in experimentation, has formulated guidelines that define a time limit for which dogs and primates can be tested and/or housed in laboratories. The concept of Rehabilitation, has been recognised in India as the 4th R as early as in 2001 (Pereira et al. 2004; Pereira and Tettamanti, 2005), and evolved as an official policy of the CPCSEA in 2004 (Anon.). The guidelines are based on the premise that animals in laboratories undergo psychological, physiological and physical trauma, not just from the interventions made on them, but also from solitary confinement, lack of natural conditions, caging and absence of appropriate social interaction. The guidelines define “re-use” and “rehabilitation” and have limited use of dogs/primates up to a maximum period of 3 years. Repeated use of dogs/primates in regulated procedures is allowed only after liver and kidney function tests confirm that the animal is normal (Fentener van Vlissingen et al., 1997). The guidelines cover the use of dogs/primates in PK studies, telemetry studies, high pain/distress studies, basic bio-medical research and breeding.

References

Determination of the optimal dose of benzocaine hydrochloride in euthanasia of frogs (Rana catesbeiana)

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A new guideline concerning laboratory animal euthanasia has been created recently in Brazil. Concerning frogs we had been used a physical method and nowadays only chemical methods with benzocaine or tricaine have been approved and the optimal dosage and the time need to be stablished to induce the euthanasia according to our needs. The aim was to study the best optimal dose of benzocaine hydrochloride to induce euthanasia in bullfgrots to use this method in our University and offer this information to other institutions. In this study we used 10 frogs and the benzocaine was diluted in 50 ml of ethanol, in concentrations of 100, 200, and 300 mg/l of water. The loss of all reflexes and the time of induction was registered. The results were 34 minutes in concentrations of 100 mg/l, 22 minutes with 200 mg/l and 18 minutes with 300 mg/l to induce the euthanasia. These preliminary results demonstrated that euthanasia can be induced using 300 mg/l in 18 minutes. Now we will continue our study increasing the dose to try to decrease the time of induction and compare with tricaine to prove that benzocaine is as good as tricaine and the best due to costs.

Assessing physiological and behavioral responses over time in laboratory beagles

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To assess adaptation to their laboratory environment, twelve young, naive beagles (6M+6F) and 14 (7M+7F) older, more experimentally experienced Marshall beagles were observed using a standardized procedure: 1) dogs were placed on an exam table and fitted with a
non-invasive Polar® heart-rate monitor; followed by 2) a 5-min observation with dogs standing/sitting on the table lightly restrained by a technician; 10 min after the procedure, 3) a saliva sample was collected for cortisol determination.

Beagles showed variable behavioral responses like licking lips, paw lifting, tail between legs and sniffing. ‘Experienced beagles’, showed more tail between legs during the 5-min observation (p<0.05). We found a large individual variation in heart rate variability and in both morning and post-observation salivary cortisol values, but no significant associations with age, gender or experience.

The beagles’ heart-rate responses over time showed four distinct patterns: 1) a high heart rate slightly decreasing; 2) a steeply decreasing heart rate; 3) a fairly constant heart rate; 4) a low heart rate slightly increasing. Further research is needed to verify whether these responses are indicative of the individuals’ adaptive capacity in those circumstances, and if they may be useful indicators for estimating potential welfare risk in laboratory beagles.

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VIII-2-460

**Refinement strategies to improve the survival rate in mouse models for influenza A virus infections**

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Mouse adapted influenza strains have been used to increase the susceptibility to Influenza A (IA) viruses in this model. Mice infected with these viruses could show severe clinical signs, including ruffled coat, depression, anorexia, dehydration, hypothermia, body weight loss exceeding 20% and eventually death. The aim of this study was to investigate possible refinement strategies to improve the survival rate of mice infected with a mouse adapted IA virus. From day 3 to 15 post-infection animals were monitored twice a day and treated with the increase of room temperature at 25°C, and the rehydration with 1 ml of physiological saline (38°C) subcutaneously when 15% body weight loss was reached. However, mortality and survival rate did not show any significant improvement in comparison with previous routine management. Moreover, we proposed the administration of a hydro-gel or corn mush to stimulate food intake, and the early administration of a non-invasive Polar® heart-rate monitor; followed by 2) a 5-min observation with dogs standing/sitting on the table lightly restrained by a technician; 10 min after the procedure, 3) a saliva sample was collected for cortisol determination.

Beagles showed variable behavioral responses like licking lips, paw lifting, tail between legs and sniffing. ‘Experienced beagles’, showed more tail between legs during the 5-min observation (p<0.05). We found a large individual variation in heart rate variability and in both morning and post-observation salivary cortisol values, but no significant associations with age, gender or experience.

The beagles’ heart-rate responses over time showed four distinct patterns: 1) a high heart rate slightly decreasing; 2) a steeply decreasing heart rate; 3) a fairly constant heart rate; 4) a low heart rate slightly increasing. Further research is needed to verify whether these responses are indicative of the individuals’ adaptive capacity in those circumstances, and if they may be useful indicators for estimating potential welfare risk in laboratory beagles.

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VIII-2-553

**Update on best practice approaches to the welfare and husbandry of fish, cephalopods and decapods**

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Aquatic species are increasingly used in research and testing, in such diverse areas as toxicity testing, environmental monitoring, comparative medicine and genomics. According to the latest (2011) EU statistics, fish species account for 12% of the 1.15 million animals used in research and their numbers have increased by nearly 30% since 2008. The new EU Directive 2010/63/EU now covers cephalopods, and several countries regulate the use of decapod crustaceans in their domestic legislation.

Most guidelines on animal care and use are, however, heavily biased towards terrestrial vertebrates and are often of little value when addressing the welfare and husbandry of aquatic species. The paucity of specific guidelines for aquatic animals reflects the general lack of scientific knowledge of their welfare and husbandry needs, complicated by the large number of species involved (e.g., guidelines for “fish” would not be feasible as there are over 30,000 species).

This presentation will describe current legislation regulating the care and use of laboratory animals in Europe as it applies to fish, cephalopods and decapods. It will also present ongoing work to supplement current legislation with good practice guidelines on welfare, refinement and husbandry. The role of non-governmental organisations in this process will also be discussed.

VIII-2-562

**Use of Eclipta prostrata extract prevents tissue damage induced by snake venoms immunization in horses during antifodic serum production**

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**Introduction:** Treatment of snake venomous accidents is a medical and veterinarian challenge. Venoms from these snakes induce edema, hemorrhage and necrosis. Serum obtained from hyperimmune horse plasma is the main treatment of the snake bites. The serum production involves the subcutaneous injection of snake venoms in horses. Although these injections do not cause any systemic repercussions, it promptly causes local edema and tissue damage and in some cases abscess, compromising the animal health. The reduction of these local lesions, without changing the immune response of these animal to the venoms components is a challenge for the serum production centers.

**Objective:** Add the Eclipta prostrata (EP) crude extract to the snake venoms on the horse immunization process at Vital Brazil farm with the goal of reducing the local tissue damage.

**Methods:** The EP was added to the venoms and injected subcutaneously in horses.

**Discussion:** The EP reduced in 50% the edema and 100% the abscess formation rate caused by the immunization process in horses compared to the control group.

Our results show that the EP are able to reduce the local tissue damage caused by snake venoms in the immunization process improving the animal welfare and the serum production.
Decreased use of experimental animals through optimization of experimental design for efficacy studies

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The aim of this study was to explore whether asymmetric experimental designs can reduce animal numbers without affecting statistical power of the study.

Data from several lung fibrosis studies in mice were combined to estimate expected outcomes for newly planned studies. Classically, these studies comprise a PBS control group, a bleomycin-induced (bleo) group, and several treatment groups with equal animal numbers. Instead of comparing all groups to each other, comparisons of interest were defined in advance: bleo versus PBS control, and bleo versus k treatments (in total k + 1 comparisons). Since the bleo group appears in all comparisons, changing the size of this group will have most effect on the statistical power. The optimal asymmetric design achieves the desired statistical power for each planned comparison with the minimum of animals needed.

We calculated that the best asymmetric designs required about 20-25% less animals than the best symmetric design. This demonstrates that substantial reduction of animal use is possible by smarter choices on group size without loss of power. To aid researchers to optimize their experimental design, we developed a freely available sample size calculator (available at http://www.tno.nl/3R) to compare symmetric and asymmetric designs.

Animal welfare issues in air transportation and security services

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Pet animals are often transported by domestic and international flights as per IATA regulation. Major constraints of pet animal transport via air are: 1) Sudden exposure to entirely different climatic condition makes them stressed. 2) Confinement of animals to the new specified and strange container makes them aggressive and off feed creating starvation and dehydration for more than a day. 3) During air transit they get exposed to different atmospheric pressure gradient. 4) Darkness air cargo may make them stressful. 5) On long transport, the container may not be cleaned properly and animal is forced to stay in unhygienic environment. All together air travel can be stressful to dogs and cats. However, proper planning and care before transit, acclimatizing them to the specified crate, feeding on high energy, high fat food before travel, health check-up by a veterinarian and administration of immunity boosters make their travel more comfortable. Dogs are often used by customs and other security agencies to detect narcotics, explosives and other smuggled goods. Their workload and solitary lodging are some of the important welfare issues. Policies and regulations are to be formulated for the welfare of animals engaged in the above services also.

Stress response to blood sampling from dorsal pedal vein compared to saphenous vein in mice

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Blood sampling in rodents has historically focused on collecting sufficient blood in a simple, efficient manner. Today, with the development of laboratory tests that can be performed on ever decreasing amounts of serum, or even on a single dried blood spot, one has the opportunity to focus on the welfare aspects of blood sampling. This study used blood glucose as an indicator of stress and found no difference in stress response if the samples were acquired rapidly. However, if the animal was restrained for a prolonged time, blood glucose values increased if collected by saphenous venepuncture. Pedal venepuncture has several advantages over saphenous venepuncture with regards animal welfare and should be the method of choice when small or moderate volumes of blood are to be collected.

Health monitoring of rodents in microisolation cages. A rationale approach (with an eye to the 3Rs)

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Micro-isolation systems (mainly IVCs and FTCs) are widely used nowadays, with the aim of protecting animals and operators. Health monitoring of these units has always been problematic.

This procedure relies upon the transmission of agents in addition to other uncontrollable variables such as prevalence of disease; dose of agents that are shed by resident animals; frequency and amount of bedding transferred in addition to the susceptibility and receptivity of the sentinels.

Recent advances in technologies and platforms have enabled other approaches centered on the use of PCR to be proposed, enabling the use of immunodeficient sentinels or relying on environmental monitoring only.

In addition to the improvements in the area of molecular diagnostic, it’s now possible to perform the most advanced serological tests in rodents with the use of the dried blood spot technology. This application in conjunction with other laboratory techniques makes non terminal sampling screening of animals possible.

This can be achieved by collecting a single drop of whole blood for serology, faeces and swabs for detection of bacteria and parasites, resulting in a really easy collection procedure, enhancing the 3Rs and possibly, reducing the overall costs of health monitoring programs.
Laboratory mouse euthanasia: aversion and refinement

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The most common agents used for laboratory mouse euthanasia are isoflurane and carbon dioxide (CO$_2$). In Experiment 1, a light-aversion test was used to examine mouse aversion to a rising concentration of CO$_2$ at a 20% flow rate, 5% isoflurane administered using a vaporizer, or 5% isoflurane administered using the drop method. Mice chose to remain in the chamber longer, and were more likely to become recumbent, when exposed to the isoflurane vaporizer treatment, compared to the other treatments. These results indicate that isoflurane delivered by a vaporizer is a humane refinement for mouse euthanasia. Once mice have been rendered insensible using isoflurane, users may switch to a high flow rate of CO$_2$ to decrease time to death, but no recommendations exist for when it is safe to switch to potentially painful CO$_2$ concentrations. Experiment 2 examined three measures of insensibility (recumbency, loss of the righting reflex, loss of the pedal withdrawal reflex) in mice. The results suggest that users should wait a minimum (mean + 3 S.D.) of 79 s after the appearance of recumbency before switching to a high flow rate of CO$_2$, when using the isoflurane vaporizer method of euthanasia.

Session VIII-3: Humane principles in experimental techniques and benefits of 3Rs

Co-chairs
Jan-Bas Prins, FELASA, Leiden University, Medical Center, The Netherlands
Tim Sangster, Charles River Laboratories, UK

Session VIII-3: Oral presentations

Applying the concept of wellbeing to the advancement of Refinement

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Although, to date, strategies to identify and alleviate the experience of pain or distress have been the primary focus to achieving Refinement, it is noteworthy that Russell and Burch (Russell and Burch, 1959) contended that we should “aim at wellbeing rather than a mere absence of distress”. Further, they concluded that the “psychosomatics of experimental animals” was probably the single most important area by which Refinement would be advanced. This at a time when the mind<>body relationship was contentious, poorly understood and generally not recognised as significant in non-human animals.

The notion of wellbeing implies positive mental state, positive experiences, successful biological function and a capacity to respond to and cope with potentially adverse conditions (Anon, 2013).

Recent advances in neurobiology and ethology have provided evidence that animals experience emotional states. Whilst there is increasing evidence of the impact of negative emotional experiences on the validity and interpretation of data, the role and significance of positive experiences merits investigation.

This paper will argue that the concept of wellbeing is pivotal to achieving the goals of Refinement and that a focus on ways to enable cognitive and emotional development will support animal wellbeing, advance the goals of Refinement and potentially enhance scientific outcomes.

References


Fluorescent target array reduces mouse numbers while assessing multiple post-vaccination T-cell responses

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The high-throughput multi-parameter technique, fluorescent target array (FTA) assay, allows the simultaneous quantitation of CTL-mediated target cell killing, functional avidity and epitope variant cross-reactivity in real time in vivo. Traditionally these techniques require large numbers of mice for two point assays, peptide titration to optimise CTL avidity conditions, and epitope variant responses. Through the capacity of FTA to measure several T-cell responses simultaneously, via novel multiple cellular staining techniques, many fewer mice were required for the experimental characterisation of cellular responses post anti-viral vaccination (reduction of 6068 to 44 mice). Proof-of-concept was demonstrated via a vaccination protocol that used a recombinant (HIV-1 epitope) fowlpox/vaccinia virus prime-boost regimen in mice, followed by FTA investigations of the cytotoxic CD8$^+$ T-cells (CTL) responses to HIV-1 expression post-vaccination. This study applied the FTA assay as a screening tool to assess over 20 different HIV-1 poxvirus vaccination strategies in mice, and revealed heterologous poxvirus prime-boost vaccination regimes as the most effective for generating high quality CTL responses. The FTA assay revealed important insights into CTL function against HIV-1 infection, while reducing the required number of animals by >100-fold.
Microsampling a bioanalytical view

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The last few years has seen the interest in Microsampling dramatically increase, focused initially on the use of dried blood spots. The aim is to use smaller samples for toxicokinetic and pharmacokinetic analysis to reduce the number of animals used on studies and also to refine the data generated. The refinement in the data is to give full profiles across the company and the data from this will be presented for both microsampling in regulated studies.

We discuss the perceived and real hurdles to the implementation of animals allowing correlation of the toxicology data with the exposure and the data generated. The refinement in the data is to give full profiles to use smaller samples for toxicokinetic and pharmacokinetic analysis.

Finally, Charles River has implemented microsampling globally across the company and the data from this will be presented for both small and large molecule case studies.

Validation of pain-related grimace scales in preweaned laboratory animals

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Despite advances in pain recognition in adult animals, few methods have been validated to assess pain in preweaned laboratory animals, including mouse pups and baby pigs. It is important to have reliable and clinically useful methods for pain assessment since many potentially painful procedures are performed without analgesia on animals of this age, such as tail tipping for genotyping and ear notching for identification in mouse pups, and castration and tail docking of piglets. The objectives of this work were to develop, evaluate, and validate grimace scales and behavioural scoring as tools to assess pain in preweaned mice and pigs, and to use these tools to assess efficacy of analgesic agents for addressing potentially painful conditions in mouse pups (ear notching; carprofen) and boar piglets (castration; prilocaine/ lidocaine cream and/or meloxicam). In studies with both species, facial action units were identified and then scored by individuals blinded as to animal treatment. For each species, the change in facial action unit scores from baseline to post-procedure with changes in baseline behavioural scores with and without analgesia was used to validate grimace scale scores. Close correlation was demonstrated, suggesting that grimace scales have clinical utility for pain assessment in preweaned animals.

Decreased levels of discomfort in trained mice at experimental procedures, assessed by facial expression

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A need for more objective approaches on measurements and assessments of improved welfare has been identified. In this study, mice were trained to acclimatize to a new method for non-restrained subcutaneous injections. The test group (n=20) was trained five days per week, while the control group (n=20) was handled without training once weekly. After three weeks, both groups were injected subcutaneously.

A modified protocol for assessment of facial expressions of pain in mice (Langford et al., 2010) was used, with ear and eye scoring at a three grade scale: 0=normal, 1=slightly, and 2= totally changed/ altered. Six blinded experienced animal technicians scored the test group days 1 and 7, and both groups day 22. After one week of training, ear and eye scores significantly decreased (Ears: from 1.7 to 1.3; Eyes from 0.9 to 0.6). In addition, trained mice displayed significantly lower ear scores during subcutaneous injections than controls (0.75 versus 1.1). These data clearly show that trained mice display less discomfort than non-trained mice during subcutaneous injections, assessed by ear scoring, and that preparing animals to experimental procedures has a 3R potential. Facial expression scoring can be an important tool when assessing improvements of animal welfare in 3R projects and method development.

Reference


European refinement initiative – science-based refinement

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During the workshop “Science-based Refinement” hosted by CAAT-Europe, Novartis International, Hoffmann-LaRoche and BSL Bio-services, experts from industry, academia and animal welfare came together to discuss new approaches in the area of refinement.

Special topics were “Experimental Design” and “Animal Welfare Indicators”. As already stated by Russell and Burch (1959), it is always becoming clearer that compromises regarding animal welfare disturb the quality of science (Knight, 2001). The session “Animal Welfare Indicators” provided a new and more dynamic concept of animal welfare (Ohl and van der Staay, 2012), discussed missing pain assessment and analgesics application in labo-
Principles of animal modelling in psychiatric research with respect to 3R rules

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At the moment, with respect to the long-term development of this field, animal models are viewed as highly valuable and extensive tools in biomedical research. To mimic brain pathogenesis in human neurodevelopmental disorders, many model studies are done using rodents, with whom we can evaluate many patterns in the ethogram exhibited by the species used in specific experimental situations. It has recently become increasingly important to develop animal models that enable multiple behavioural domains to be explored in parallel and thus reduce the number of animals used as much as possible (the rule of reduction). The solution in this instance is to use more behavioural endpoints per experiment, which allows a greater number of different domains to be registered. For example, preclinical studies suggest hyperlocomotion in rodents to be equivalent to positive symptoms of schizophrenia. Moreover, exploration can be used as a marker of vulnerability to stress or anxiety. The rule of refinement is closely connected with correctly respecting the rules in experimental manipulations and standardizing testing conditions in order to attenuate interindividual variations. However, it is very difficult to fully implement replacement in animal models of neuropsychiatric disorders, due to the complexity of brain functions and brain pathogenesis.

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Gavage incidents – a urgent need for action

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Gavage is an extensively used technique for the oral dosing of substances. The technique involves the restraint of conscious animals and insertion of a rigid metal or flexible plastic tube into the oesophagus to deliver a bolus of substance directly into the stomach. Research has shown that, even when carried out properly, the procedure can induce significant stress, which could confound research results. Additionally, there is a risk of gavage-incidents, commonly reported as deaths. Gavage incidents include accidental insertion into the trachea causing suffocation, puncturing of the throat or stomach and chronic irritation leading to severe weight loss or excessive ingestion of bedding. Mortalities have ranged from 0 to 53% in single studies but to date the extent of gavage incidents across a sector is not yet known.

The aim of this study was to determine the incidence of gavage-related mortality in regulatory toxicology submissions for industrial chemicals. Over 300 independent studies were reviewed from the ECHA CHEM database for reports of gavage- attributed deaths. We observed an unacceptably high proportion of studies with gavage incidents. More humane approaches to dosing do exist but are under used- perhaps due to a failure to appreciate the extent of gavage related deaths.

The development of hormone loaded diets to promote xenograft growth

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In our facility, we use several tumours which are dependent on hormone supplementation, delivered via subcutaneous pellets, for growth. Due to supply issues, we had to develop an alternative method of DHT supplementation and decided to try delivery via a fortified diet, as this would be less invasive and stressful to the animals and more convenient for the staff. After development, the diet was tested against the LNCaP prostate tumour line and was found to be as effective in stimulating growth as pellets. We then decided to adopt the same method for estradiol delivery hoping it would eliminate associated side effects, and MCF-7 breast carcinoma growth was stimulated as required with side effects markedly reduced.

We believe delivering hormones via the diet, rather than pellets, is a major refinement in welfare terms by reducing both the need for invasive implants and in side effects, while still promoting tumour growth.
Humane principles of using animals in Russia

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Russia’s lack of documents regulating the ethical principles of the treatment of animals is what most of us are aiming for. However, replacement is a long and tedious road and it might be anticipated that we will continue per-
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Humane end-points in animal experimentation

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Biomedical research without the need to use laboratory animals is what most of us are aiming for. However, replacement is a long and tedious road and it might be anticipated that we will continue performing animal experiments for the next few decades, including experiments that are known to induce severe pain and distress, e.g., infectious disease models with lethal challenge procedures or tumour survival studies. Particularly the high severity category of experiments requires our special considerations; for animal welfare concerns, but for scientific and legal reasons as well. Our last resort in experiments with significant pain and distress is to limit the time period animals are severely suffering. This approach is generally referred to as applying humane end-points.

In this presentation I will make a distinction between unavoidable and avoidable pain and distress and the consequences for applying humane end-points. I will discuss various types of humane end-points, including opportunities to introducing non-clinical end-points. Finally, following my conclusion that implementation of humane end-points still is far from optimal, I will address existing barriers and imitations to implementing humane end-points as part of a “culture-of-care” attitude in the laboratories.

Inflammation imaging and refinement of post-surgical NSAID dose recommendations in mice

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NSAIDs treatments are common welfare interventions with the aim of reducing post-operative pain linked to tissue inflammation. However, dose recommendations derived from indirect assessments (e.g., behaviour, hyperalgesia) may be ineffectual, particularly in mice, where questionably large doses seem to be needed. We attempted a more direct determination of the anti-inflammatory effects of meloxicam (1, 5 or 20 mg/kg s/c) given 1 hour before laparotomy in groups of male BALB/c mice (n=9-12) by imaging a fluorescent COX-2-targeted probe (Uddin et al., 2010) at 7, 24 and 48 hours. Results were contrasted with efficacy/welfare estimates from body weight and automated behaviour analyses (Roughan et al., 2009) and the Mouse Grimace Scale (MGS) (Langford et al., 2010). Meloxicam dose dependently reduced inflammation relative to controls. Surgery predictably caused weight losses and abnormal behaviour changes, and increased MGS scores. Meloxicam had no detectable beneficial effects on welfare at any dose or time-point and 5 and 20 mg/kg further increased MGS scores. Imaging confirmed meloxicam’s anti-inflammatory effects, so the lack of positive outcomes suggested pain derived from factors in addition to inflammation, which may be only partially controlled by relatively large NSAID doses (Matsumiya et al., 2012; Wright-Wil-
A global initiative to refine acute inhalation studies through the use of ‘evident toxicity’ as an endpoint: towards adoption of the Fixed Concentration Procedure

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This abstract is submitted on behalf of the NC3Rs Working Group on the Fixed Concentration Procedure (FCP). Acute inhalation studies are conducted in animals as part of chemical hazard classification. Current accepted methods use death as an endpoint (OECD TG403 and TG436). The FCP (draft OECD TG433) uses fewer animals and replaces lethality as an endpoint with "evident toxicity", defined as clear signs of toxicity that predict exposure to the next highest concentration will cause severe toxicity or death in most animals. TG433 was dropped from the OECD work plan in 2007 because of a lack of evidence for comparable performance with TG403 and TG436, suspected sex differences (FCP originally only used females) and the ill-defined and subjective nature of evident toxicity. The first two issues have been resolved (Price et al., 2011; Stallard et al., 2011). A global initiative including 20 organisations, led by the NC3Rs, has addressed the last concern with the aim of making evident toxicity more objective and transferable between laboratories. The group has shared data on clinical signs recorded during acute inhalation studies for 188 substances. Preliminary results suggest signs including bodyweight loss, irregular respiration, gasping, ano-genital staining or hypoactivity are highly predictive of severe toxicity or death at the next highest dose.

References
Nutrition studies in cattle require rumen fistulation, a surgical procedure creating pain, trauma, and appalling appearance. Rumen simulation technique (RUSITEC), in vitro gas production techniques (IVGPT) and Tilly and Terry method are alternatives to rumen fistulation. In this study RUSITEC and IVGPT are compared for their efficiency to establish rumen bacteria Butyribrio fibrisolvens. RUSITEC system is a semicontinuous culture system consisting of eight vessels (capacity 800 ml) and IVGPT is a batch system with 35 glass syringes (100 ml capacity). Both the system was supplied with rumen liquor collected at the 48th hour. Colony counts in nylon bags and IVGPT syringes were supplied with 200 mg feed. Anaerobic roll tube technique was used for the culture of B. fibrisolvens in the rumen fluid collected at the 48th hour. Colony counts were 1.14 x 10^7 ±3.53 in IVGPT and 3.45 x 10^4 ±1.21 in RUSITEC. Therefore, RUSITEC is considered as the best laboratory alternative to avoid painful rumen fistulation.

The impact of AAALAC accreditation on compliance with animal welfare laws

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Accreditation of animal research facilities by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) is widely considered the gold standard of commitment to the well being of nonhuman animals used in research. AAALAC-accredited facilities receive preferential treatment from funding agencies and are viewed favorably by the general public. Thus, it bears investigating how well these facilities comply with animal research regulations. In this study, the incidence of noncompliance with the United States (US) Animal Welfare Act (AWA) at AAALAC-accredited institutions in the US was evaluated and compared to those at non-accredited institutions over a period of two years. Our analysis reveals that AAALAC-accredited facilities are frequently cited for AWA noncompliance items (NCIs). Further, AAALAC-accredited sites had significantly more AWA NCIs on average than non-accredited sites. This gap widens as the number of animals per facility increases. AAALAC-accredited sites also had more NCIs related to improper veterinary care, personnel qualifications, and animal husbandry. This study is the first one to demonstrate that AAALAC accreditation does not improve compliance with regulations governing the treatment of animals in laboratories.
Promoting an institutional culture of care and ethics
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AAALAC International has conducted almost 700 on-site assessments of animal care and use programs around the globe since its adoption of the eighth edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011) as one of its primary standards for accreditation. In analyzing the findings from these site visits, some patterns of challenges institutions are facing in meeting new recommendations in the Guide have been detected. Data from various regions of the world, with reference to institutional oversight, occupational health and safety, animal environment, the program of veterinary care and physical plant will be discussed to assist institutions in proactively addressing these program areas. The dataset generated from reviewing animal care and use programs around the world is unique and can serve as a valuable resource in promoting research animal welfare and high quality science. This information is described at a level of detail that will allow institutions to assess where program enhancements can be effected on the path of continuing improvement that is the foundation of an institutional culture of care and ethics.

Culture of the 3Rs: accelerating the development and the implementation of alternatives
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Biomedical research is one of the undisputed areas where progresses represent rapid and major improvements for human and animal health and well-being. Everyone working in this field is aware that the use of animals for scientific purposes is not the only way to address the challenges. All research programmes to be comprehensive include non-animal models and sometimes clinical trials. Lack of performance indicators for the 3Rs induces the false idea that the research community neglects the non-animal models.

A good culture of care within an establishment is a prerequisite for the revised EU and national legislation to deliver the anticipated improvements in welfare, use and care practices. Recital 31 of Directive 2010/63/EU requires that Animal Welfare Bodies should foster a culture of care, to ensure appropriate animal welfare care and use practices are maintained at all times. However, the responsibility and foundation for a good culture of care goes beyond that of just Animal Welfare Bodies – it rests with everyone dealing with animals bred or used for scientific purposes.

What constitutes a good culture of care?
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A good culture of care within an establishment is a prerequisite for the revised EU and national legislation to deliver the anticipated improvements in welfare, use and care practices.

Toenail trimming in mice – an alternative treatment for ulcerative dermatitis
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In 2010 technicians at our university became concerned about incidents of mice in our facility with ulcerative dermatitis (UD). The affected areas were pruritic and subsequent scratching by the mice, often in an obsessive manner, resulted in sore lesions developing.

Husbandry techniques were adapted and veterinary recommended treatments were administered but little improvement was seen. In 2011 a new approach was adopted, trimming the toenails of affected mice in order to break the itch/scratch cycle, instead of attempting to treat the UD itself. This method reduced the amount of skin damage occurring during scratching and the lesions began to heal. Daily photographs taken for ten days documented the healing process and eventual hair regrowth. Extensive data collection on all mice presented with UD enabled comparable analysis of the toenail trimming method against two other treatments, topical application of an antibiotic/anti-inflammatory gel and an IP injection of a synthetic protein thought to inhibit the itch reflex. Data analysis showed that toenail trimming resulted in much higher rates of healing than the other two treatments. As this method is also free, quick, non-invasive and simple to teach, it has now been adopted as the standard treatment of this condition at our institute.
Implementing an action plan for world class animal care and husbandry at Imperial College London

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Following publication in 2013 of an independent review by Professor Steve Brown (Brown, 2013) about how Imperial College London (2014) could deliver change to ensure it was a leader, both within the UK and internationally, in its animal care and husbandry, and in developing and applying the 3Rs, in January 2014 the College published its action plan to achieve this objective.

This presentation, offered as part of the Culture of Care session of Theme VIII, would focus on the practical challenges of improving the culture of animal welfare alongside delivering world research involving animals.

The four main commitments the College has made under its action plan are to strengthen its strategic leadership of this area, to promote fuller consideration of the 3Rs through strong links between researchers and animal faculty staff, to reform ethical review processes and to communicate more effectively both externally and internally.

To be successful in achieving these commitments, Imperial College has sought to engage widely in search of best practice for managing world leading, complex multi-site animal research facilities. A substantial reform of the governance of animal research is being implemented.

References

Session VIII-5: Poster presentation

Changing the culture of laboratory rat care

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Standard laboratory rat housing generally consists of a small cage, bedding, and a rudimentary shelter. Rats are sometimes habituated to standard laboratory procedures but are rarely socialized to humans. Because rats are inquisitive by nature and able to experience a range of positive and negative emotions, these standard laboratory conditions are likely to affect their welfare (Makowska and Weary, 2013). We housed rats in standard (six pairs) and semi-naturalistic (six groups of five) cages. When housed in the semi-naturalistic cages, Sprague-Dawley rats purchased from Charles River Canada spontaneously dug burrows, climbed, bounded, and stretched to their full capacity, all behaviours which were never seen and were not possible in the standard cages. In another study, a rat socialization protocol was developed that allowed us to raise rats that were friendly and playful even with strangers. These rats, when called, also willingly ran up to and climbed into a testing apparatus. These examples can inform changes in the way we view and care for rats. Dogs and cats used in research “should be allowed to exercise and provided with positive human interaction” (NRC, 2011); we suggest that this guideline should also be applied to laboratory rats.

References
Refinements for implant surgery: the effects of different anesthetic agents on pregnancy and pup survival

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The most critical aspect of generating genetically engineered mice is the ability to produce live animals for analysis after the appropriate injection procedure. Animals are produced by implantation of manipulated embryos into pseudo-pregnant females for gestation, parturition, and subsequent growth to the weaning stage. Animal loss can occur during any of these stages, resulting in repeated procedures and increased animal usage. One might predict that the anesthesia used during implant surgery could affect the number of pups produced. Anesthetic agents commonly used in the United States for implant surgery include Avertin (a tri-bromoethanol, tert-amyl alcohol mix) delivered by IP injection, ketamine: xylazine (100 mg/kg: 5 mg/kg) delivered by IP injection, and inhaled isoflurane (2.5% in oxygen). To determine if the type of anesthesia used during implant surgery could affect the number of pups produced, we tested each type in implant surgeries and as-sessed the numbers of pups produced. Sufficient numbers of embryos delivered by IP injection, and inhaled isoflurane (2.5% in oxygen). The results of this analysis will be presented.

Quantitation of fluorescence intensity in mice – a novel genotyping approach of fluorescently labeled transgenic mouse models

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Fluorescence proteins have been useful as genetic reporters for a wide range of applications in biomedical research. Several fluorescent markers with sufficient brightness and spectral separation are available and frequently used for the analysis of transgene activity. Here we show that transgenic mice with different coat colours ubiquitously expressing a green (eGFP) or red fluorescence protein...
(mCherry or tdTomato) can be reliably genotyped by measurement of the fluorescence intensity. We identified the tail skin of the mouse as the tissue best suited for such an in vivo genotyping approach. The fluorescence intensity not only distinguishes wild type from transgenic mice but also allows for the reliable determination of zygosity. The results obtained by quantitation of fluorescence intensity were confirmed by standard PCR analysis or test breeding. This novel approach can be used on juvenile or adult animals and allows for instant genotyping without DNA analysis. The feasibility of genotyping without tissue sampling is an important animal welfare aspect.

In summary, we demonstrate for the first time that analysis of ubiquitously expressed fluorescence proteins in transgenic mice can be reliably used to substitute DNA-based genotyping methods or test breeding.

VIII-6-620
Surplus animals in breeding: is there room for reduction?

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Recently, an international expert workshop was held in the Netherlands, entitled: “Bred but not used”. These are the animals that are purposely bred but eventually not used in procedures. In the Netherlands, the numbers of these animals have increased steadily over the past 5 years. In 2012, 579,338 animals were used in procedures while 524,735 were “bred but not used”. These include animals not suitable for experimental purposes because of their genetic make up, age and/or sex. The figures have raised concern among the public at large and the Dutch authorities. Therefore, the authorities invited twenty-two international experts on the 3Rs, animal welfare, ethics, colony management, molecular biology and laboratory animal science to discuss trends and possible actions. The following themes were identified: moral and ethical aspects; management and technology; education, training and communication; alternatives. Discussions led to an in depth analysis of these aspects of “bred but not used” and the presentation of recommendations to the authorities. The minister has included some of these recommendations in her Action plan: “Animal testing and alternatives”.

VIII-6-944
Nuclease technology reduces animals use and improves timeline for making mouse models

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Since the discovery of Zinc Finger Nuclease in gene editing in rat genome in 2009 (Geurts et al., 2009), Nuclease Technology for gene editing has transformed the landscape for creating genetically modified rodent models in facilities that have implemented this method. Additionally, this technology is not limited by the use of embryonic stem cells thus can be used to modify the genomes of any species. Recently, CRISPR/Cas9 system allows creating multiple mutations simultaneously (Wang et al., 2013). This report focuses on the impact of the technology on the number of animals used, and the timeline for generating mutant mouse models. Our data show an 85% reduction of mice used by using Nuclease Technology compared with gene targeting through ES cells. The timeline from project initiation to homozgyous mutant mouse models born for single mutation was reduced from 16 months to 2 months. For adding a mutation to a single mutation, the timeline was reduced from 18 to 7 months, and for adding a mutation to a double mutation, the timeline was reduced from 21 to 7 months. The results demonstrate that Nuclease Technology is an effective method for generating mouse models while also significantly reducing the number of animals used.

References

VIII-6-943
Generation of genetically modified mouse models: the role of the International Society for Transgenic Technologies in refinement and reduction

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After the development of technologies to efficiently manipulate the murine genome during the 1980s the generation and use of model organisms in many areas of basic and biomedical research is without alternative at present. With the proliferation of technologies, networks have formed that have provided for an exchange among each other. From these networks emerged in 2006 the International Society for Transgenic Technologies (ISTT). The ISTT sees itself as a meeting place for all those who generate and use genetically modified animals – currently still mainly mice and rats. The aim of the society is to disseminate techniques and their improvements and to facilitate the optimal use of these techniques in all laboratories employing those technologies. It has become obvious that optimization of methods leads to a reduction of the number of animals required for the generation of genetically modified animal models. Thus, the Society is a major contribution to the reduction of animal numbers. Moreover, through the dissemination of improved surgical techniques, it also contributes to Refinement. Over the last year to this day the application and optimization of new techniques of nuclease mediated gene modification is being discussed among society members. Again the driving influence of the ISTT to the dissemination of improved methods and the potential to reduce animal numbers by an increased efficiency through the implementation of state of the art technologies becomes apparent.

VIII-6-947
Reducing and verifying off-target effects when generating genetically modified animal models using genome editing technologies

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producing genetically modified animals by directly applying zygotes using novel genome editing technologies such as ZFN, TALEN and CRISPR. These technologies provide the possibility of production of more suitable animal models with more precise etiology to study human diseases and reduce the improper utilization of experimental animals. However, it is necessary to take into account that putative off-target effects of genome editing technologies might interfere with interpretations and result in ambiguous conclusions. Therefore, there is an inevitable issue about how to reduce and verify off-target effects in generating genetic modified animal models using genome editing technologies.

References
Theme IX – Global Cooperation, Regulatory Acceptance and Standardization

Coordinators
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Session IX-1: Activity updates from international scientific societies

Co-chairs
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Session IX-1: Oral presentations

IX-1-022
Activities of JSAAE (Japanese Society for Alternatives to Animal Experiments)

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The Japanese Society for Alternatives to Animal Experiments (JSAAE, http://www.asas.or.jp/jsaae/e_index.html) is a scientific organization that undertakes research, development, education, and surveillance activities for promoting international acceptance of the Three Rs as guiding principles for the proper use of animals in scientific testing. The following issues are general service of JSAAE.
1) Annual meeting of the society
2) Extraordinary symposium and workshops
3) Publication
   – J. Alternatives to Animal Experiments (AATEX)
   – Newsletters (in Japanese)
   – Home page
4) Financial support to related research
5) Validation and evaluation of new alternatives
6) Collect relevant information
7) Communication with other countries
8) Others
   – Support of International meeting
   – Collaboration with the other scientific associations Tissue culture Assoc., Mutagenicity assoc., etc
   – Communication with animal protection group

Our society was founded in 1989. There are the currently members, 389 regular members, 18 student members, three honorary members, supporting members (2 Platinum, Gold 4, Silver 23) in 29 companies.

IX-1-148
American Association for Laboratory Animal Science: past, present and future

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The American Association for Laboratory Animal Science (AALAS, http://www.AALAS.org) has a long and progressive history in the evolution of the field of biomedical research (McPhearson and Mattingly, 2000). From its beginnings in the mid-twentieth century when five veterinarians met in Chicago to the current conferences of 5000 attendees, the association has been pivotal in the advancement of responsible laboratory animal care and use, including the 3Rs, to benefit people and animals. This presentation will feature updates on traditional programs and services such as certification, e-learning, print resources, scientific information distribution, and electronic resources. In addition, attention will be paid to new products such as “Laboratory Animal Science Professional”, a magazine devoted to providing a wide range of resources and knowledge to laboratory animal science professionals and the Grants for Laboratory Animal Science program which provides funding for research about animal husbandry and welfare. The association recently established a new membership category, Global Partners, for individual member associations based outside the United States which allows members of the international association to obtain AALAS programs and services at the AALAS member prices. The presentation will conclude with predictions about the future of laboratory animal science and how AALAS will play a role in that future.

Reference
McPhearson, C. (2000). 50 Years of Laboratory Animal Science, AALAS.
North American society promotes advances in in vitro and in silico toxicology

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As efforts to replace and reduce the use of animals in regulatory testing mature, it has become clear that collaboration between diverse stakeholders is necessary to ensure successful progress. The American Society for Cellular and Computational Toxicology (ASCCCT) has, since 2010, provided a place for individuals representing non-profit, method developer, contract research, regulator, and regulated industry viewpoints to collaborate in order to speed the development and use of in vitro and in silico alternatives to animal tests.

The ASCCT offers traditional and innovative ways for members and others to collaborate, including yearly in-person meetings, an online newsletter, and an e-mail discussion list. The ASCCT’s bi-monthly webinar program provides members access to in-depth presentations by experts in their fields, covering relevant new in vitro or in silico tools, concepts, and policy efforts and simultaneously providing a venue for interested members to share their research with colleagues.

While focusing on North American events and activities, the ASCCT also endeavors to collaborate with international societies with similar missions. Finally, the ASCCT offers discounted memberships, travel awards, and free annual meeting registration to students in order to encourage the involvement of young scientists in its activities.

Cuban Group and the Latin-Ibero-American Network for Alternatives: initiatives for accelerating the implementation of 3Rs in our continent

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Latin-America has been a region with a low development of 3Rs Alternatives so far. Just few countries like Brazil can show some relevant progress in this field. In the 80s, Cuba formed its 3Rs Group with important results in the implementation of in vitro methods for Toxicology and Pharmacology research. Later, the development of the local biotechnological industry has increased the interest in 3Rs alternatives, mainly in the field of the quality control of vaccines. In spite of these advances, further implementation of alternatives is possible just in the framework of international cooperation. For that, National Groups working on alternatives in Latin America were gathered for creating the Latin-Ibero-American Network for Alternatives. Thus, it will be possible to reinforce our strengths and to overcome our weaknesses by means of the experience exchange and the scientific collaboration, courses and trainings, participation in proficiency and inter-laboratory validation studies and platform of projects fully focused on the development and implementation of Alternatives. The definition of statements, a working structure (including a Board) and an Action Plan will be the priority of this Presentation in order to give the first step toward a near future with more development and implementation of Alternatives in Latin-America.

ESTIV’s activities for the promotion of in vitro toxicology

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ESTIV is a leading European organization for in vitro toxicology that aims to strengthen networks and foster scientific exchange between organizations and professionals. It promotes the regular exchange of information by organizing the well-established ESTIV Congresses, which takes place every two years at a location in Europe. ESTIV also organizes dedicated yearly workshops, joint activities with affiliated societies and publishes regular newsletter and the ESTIV website (http://www.estiv.org). In particular, ESTIV strives to attract and integrate young scientists.

Existing for over 20 years, ESTIV cooperates closely with various European scientific organizations, governmental bodies and industrial platforms to facilitate communication and encourage research and application and use of 3R alternative methodologies. In addition, ESTIV organizes international education and training courses dedicated to in vitro toxicology and aims to encourage and extend interest in in vitro toxicology in outreach countries outside of Europe. “Toxicology in Vitro” is the official journal of ESTIV.

Activities of EUSAAT, the European Society for Alternatives to Animal Testing

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Upon initiative of local animal welfare activists, in 1991 the first European congress on Alternatives to Animal Experiments was held at the University of Linz (Austria), sponsored by Austrian, German, and Swiss government institutions, industry, and private trusts. To ensure continuity in organizing the annual Linz conferences, in 1993, the Middle European Society for Alternatives to Animal Experiments was founded. Soon colleagues from neighboring countries joined (e.g., Czech Republic, Italy, Netherlands, Slovakia, Sweden). Therefore, in 2006, English became the official congress language, and in 2009, the society’s name was changed to EUSAAT, European Society for Alternatives to Animal Testing – the European 3Rs Society (http://www.eusaat.org). During the last 20 years, the main topics of the EUSAAT conferences included ethical and legal issues of animal experimentation, in vitro pharmacology and (eco)toxicology, molecular modeling and education. EUSAAT has always maintained close cooperation with the European Commission and its services. In 2014, EUSAAT was accepted to the ECVAM Stakeholder Forum ESTAF. EUSAAT members have served internationally as co-chairs of world congresses on alternatives: Both co-chairs of WC9, Dagmar Jirova and Horst Spielmann, are EUSAAT members. EUSAAT has further established official cooperation with 3Rs societies outside Europe, such as ASCCT (USA) and JSAAE (Japan).
Alternatives to animal experiments in Korea: Past achievements and present status of the Korean Society for Alternatives to Animal Experiments (KSAAE)

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The Korean Society for Alternatives to Animal Experiments (KSAAE) initially began as an informal study group among the concerned members from academia, industries, and government. It became a formal organization in 2007 through the efforts of many members. In the same year, it held WC-6 Seoul Satellite Symposium in Korea. Since the establishment of the Korean Center for the Validation of Alternative Methods (KoCVAM) in 2009, collaboration between KSAAE and KoCVAM has made a big progress in advancing alternatives in Korea. The Cosmetic Consortium, launched in 2013, is developing new alternative test methods for cosmetics such as eye irritation assay, skin sensitization assay and photosensitization assay. KSAAE members participate in international validation studies, Validation Management Teams (VMTs), and peer review panels led by member countries of the International Cooperation of Alternative Test Methods (ICATM). Also KSAAE and JSAAE agreed to exchange information mutually. In addition to chemical testing, alternative efforts were made in the areas of biological research. For example, the Korea National Institute of Health (KNIH) started a project to develop a new animal testing method with in vivo imaging tools to trace pathogens during infection. This can reduce the number of animals for the experiment of infectious disease. KSAAE will be continuing its efforts to promote both human health and animal welfare in Korea.

The International Society for In Vitro Methods (INVITROM)

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Animal models have greatly advanced our knowledge of biological systems in many areas. Besides the ethical considerations, it is nowadays also recognized that they have major scientific limitations. Thanks to the continuous improvement and refinement of cell and tissue culture methods, the availability of (human) stem cells and in combination with advanced molecular techniques and the power of in silico analysis of large data sets, a new area for biomedical and toxicological investigations, now based on in vitro technologies, has emerged.

INVITROM (the International Society for In Vitro Methods) was founded to promote and support this new field of research and to create awareness and acceptance by the regulatory bodies.

INVITROM acts as a platform for discussion, contacts and co-operation for the enhancement and dissemination of in vitro technologies by organising annual workshops and joint symposia with related societies and by building an extensive network of experts (e-mail, website, LinkedIn).

INVITROM members believe in the strength of in vitro and in silico methodology in biomedical research and actively contribute to the improvement of these methods as new tools for expanding our knowledge in biomedicine.

Please visit the website to join and find information on INVITROM: http://www.invitrom.org
‘GUTS in the LAB’ – Award for courageous scientists doing excellent 3R research

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European Directive 2010/63/EU as being implemented into national legislation of European member states, aims for more transparency in laboratory animal research and for the public to be better informed. Its ultimate objective is to fully replace animal use in science, industry and education through the promotion of 3R-alternative approaches.

In the Netherlands, since 2007, the Dutch Society for the Protection of Animals in collaboration with the Netherlands Knowledge Centre on Alternatives to Animal Use annually awards a prize to researchers who have shown remarkable courage in their lab, in applying the 3R’s principle. Each year 4 or 5 scientists are nominated who tell about their research in a language that is comprehensible to laymen. The general public vote for their favourite candidate; the researcher with most votes is awarded the “Guts in the Lab”-award. The award is valued by both the scientific community and the public, as it bridges the gap between science and society. Transparent communication about the research is crucial, not only by the DSPA and NKCA, but also by and in close collaboration with the research institutes themselves. We hope our poster, describing the award, our strong collaboration and its past winners, will inspire many others.

Science and awareness: a journey inside the 3Rs. Didactic exhibition project

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The exhibition project by IPAM, ECOPA affiliated, is intended as a journey to get to know the scientific value of the 3Rs, aimed at outlining the theoretical and practical evolution of research methods based on the principles of Reduction, Refinement and Replacement in various fields of biomedical research. It came into being from the idea of tracing a journey through time to show the gradual replacement of animals in testing, going on to predict a future in which highly advanced science will no longer have any reason to use them.

The exhibition consists of three sections – PAST, PRESENT and FUTURE. Each section has an introductory panel and panels dealing with specific topics. All the panels were produced by 25 experts in the various fields, who wrote the texts and provided illustrations.

A total of 30 panels have been prepared, dealing with subjects ranging from pharmacology to toxicology, legislation to didactics, animal housing to neuroscience and computer models to genomics.

The exhibition was designed and set up as an itinerant exhibition and will be on display in many universities and research institutes in Italy, starting off at the Rector’s Office at La Sapienza University in Rome.

COLAMA2012 – the first Latin American congress on alternatives

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The first Latin-American Congress on Alternatives to Animal Testing in Education, Research and Industry (COLAMA2012) was held in Niterói, Brazil as a joint initiative from the Brazilian National Network for Alternative Methods (RENAMA), the Postgraduate Program in Science and Biotechnology – Fluminense Federal University (PPBI/UFF) and the Brazilian Center for Validation of Alternative Methods (BraCVAM), supported by the National Institute for Quality Control in Health (INCQS/Fiocruz). COLAMA2012 aimed to create an opportunity in Latin America for gather researchers interested in 3Rs initiatives and, most of all, encouraging the development of new research groups on this field. The congress had 47 speakers and 226 attendees, in four different themes in alternatives related on humanities, 3Rs and training and education (in collaboration with InterNICHE and Latin American conference on Humane Education and Alternatives). Industry and academy were represented by more than a hundred works by participants from 9 Brazilian and 11 international companies, and institutes (from Cuba, Argentina, France, USA, Monaco, Germany, England, and others) and 26 institutes of education. One of the most striking results of COLAMA was the creation of the Brazilian Society for Alternative Methods. The second edition is programmed to be held at CUBA, 2015.
Is international harmonization of animal care and use standards progressing as hoped?

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Several international guidance documents have been published that suggest convergence of opinion as to appropriate program infrastructure, ethical oversight, as well as practices and procedures across country borders. For example, the OIE has published a chapter in its Terrestrial Animal Health Code that specifically addresses the care and use of research animals. One hundred and seventy-eight countries/territories are members of the OIE and thus have pledged to implement those standards. In partnership with ICLAS, CIOMS has updated its international guiding principles regarding the use of animals in research. In addition, numerous countries’ regulatory frameworks and guidance documents urge implementation of the 3Rs. However, the rate of uptake of these standards at the institutional level is difficult to assess on a worldwide basis. AAALAC International is in the unique position of having an in-depth understanding of the status of animal care and use programs around the world. Thus, AAALAC has a bird’s-eye view of the progress being made globally in harmonizing animal care and use standards. The status of this international harmonization based on this extensive experience will be described.

What has changed for animal welfare in Europe?

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Animal welfare cannot be advanced by any single player. Real progress needs input and collaboration by all involved – be it animal users, animal welfare NGOs, scientists, and regulators at national, regional and international level as well as politicians.

Within the EU, the attitudes of different players in the policy scene in relation to animal welfare have shifted significantly since the late 1990s. From a typical “our side – their side of the meeting room” – approach with hardly any common ground to a collaborative productive partnership where all parties recognise the need to work together to make progress.

There are a number of contributors to this change in approach – some to do with a more general awareness of ethical issues, an increased understanding of animal needs and changes in approach to and purposes for which animals are used in the advancement of science; some linked to the economic situation and some driven by other policy changes.

By engaging actively all those parties with an interest in the use and care of animals used in scientific progress, much common ground has been found, enabling improvements in welfare without compromising scientific progress.

A scientific approach to animal replacement policy

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The legal mandate to fully apply the 3Rs, including replacement, in biosciences research has existing in the European Union since the 1986 Animal Experiments Directive, and is becoming a common fea-
ture in new and revised animal welfare legislation worldwide. Yet a disconnect remains between this policy obligation and its implementation. Factors may include an over-emphasis on decision-making at the institutional level versus at the level of research funding bodies, and a failure to overcome a perceived schism between the objectives of advancing human health and avoiding animal use.

This presentation will explore the extension of the “adverse outcome pathway” (AOP) paradigm from toxicology into biosciences research as a science-based approach to the examination of human disease processes, from molecular to individual and population levels, linking environmental and genetic causes of diseases via pathways at the cellular levels, through organ systems, to disease outcomes. The AOP paradigm would support a shift of focus away from animal models towards human-biology-based tools, such as patient-derived pluripotent stem cells, genome-wide association studies, computational systems biology, microfluidic chips, etc. Targeted research investment along these lines would, first and foremost, benefit human health sciences, while also addressing the replacement policy mandate in a meaningful way.

IX-2.846

InterNICHE activity in CIS countries and Iran: review and reflections

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InterNICHE has been active in countries of the Commonwealth of Independent States (CIS) since 1995 and in Iran since 2010. InterNICHE National Contacts and Partner organisations have worked with teachers, students, campaigners and others to introduce alternatives and replace harmful animal use. This presentation will describe the strategies employed to facilitate this curricular change within medical, veterinary medical and biological science education and training. These include organisation of outreach tours, seminars, training and exhibitions, establishment of Alternatives Loan Systems, translation and distribution of alternatives, signing formal agreements with universities for replacement, and securing media coverage. Despite significant obstacles, achievements have included replacement of the annual use of over 60,000 animals, and widespread awareness of alternatives in some countries. The positive results reflect teachers’ acceptance of the pedagogical, ethical and economic advantages of humane education and alternatives, and a growing understanding of the potential of technology to support the learning process. They also demonstrate the opportunities for collaborative action in countries with challenging socio-economic realities.

IX-2.888

The state of animal welfare implementation in the United States from the perspective of the US National Academy of Sciences

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In the United States, animal use in research is governed by two distinct but overlapping legal entities. Provisions to safeguard and advance animal welfare are present in both but philosophical and practical differences distinguish the two systems. As part of session 9.2, this talk will discuss the state of animal welfare implementation in the United States, efforts to improve it and provide some thoughts on the goal of obtaining global consistency and best practices.

Session IX-2: Poster presentations

IX-2.056

3Rs dissemination in Asia: zebrafish embryo toxicity test transferred in Sri Lanka’s universities

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Laboratory animal testing are accountable for at least 80 millions laboratory animals used each year worldwide. The ban on animal testing for cosmetics in Europe created a momentum. Since the enforcement of the ban, other countries took similar initiatives. Even by acknowledging that a lot of work still needs to be done, it is worth being mentioned that know how and confidence in validated and regulatory accepted alternatives to animal testing models for the last thirty years is robust. One should consider that those 3Rs models are ripe to be spread across the EU borders.

Under this scope, this presentation will describe the transfer of the regulatory accepted Zebrafish embryo toxicity test at Sri Lanka’s universities. Besides being regulatory relevant for 3Rs (OECD TG 236, ISO 15008), Zebrafish is a native species in Sri Lanka and a low cost toxicity model. During the talk, the audience will learn about the obstacles, the lessons learned and the required preparation to ensure success of the establishment of a Zebrafish unit facility in Sri Lanka at Uva Welassa University for toxicity testing with Zebrafish embryos. The take home message will be that similar initiative can be repeated elsewhere with any appropriate models.

IX-2.088

Animal welfare implementation in Switzerland: a successful stakeholder approach based on a joint Animal Welfare Charter

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5 years InVitroJobs – internet platform, job board and working group network for animal-free research

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The bilingual portal InVitroJobs is a project of the German Federal Association of People for Animal Rights that has the goal of promoting modern research without animal use. The development of new suitable methods is becoming increasingly complex and their understanding demands an increasing degree of scientific expertise. The platform InVitroJobs provides pertinent background reports on the path from research and development to acceptance and inclusion in test guidelines and want to illustrate how and why a particular method may not yet be adequate, what is lacking and what demands must be met to facilitate development and financial support.

Young researchers and interested parties can get a quick overview of working groups dealing with animal-free research, vacancies and thesis. We regularly publish news on research results obtained without animal testing. Under the heading “Working group – a portrait” we introduce scientific teams and companies, and discuss current developments in detail. In 2013, major topics were advantages and disadvantages of primary cultures, human-specific cell culture systems and cell lines. In the future, relevant potential developments in this area could help to avoid the killing of many animals for organs or tissue samples, particularly in pharmaceutical research and the development of vaccines.

A new guideline about euthanasia to laboratory animals in Brazil

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A new guideline about euthanasia applied to laboratory animals has last year are not permitted anymore. The focus of this work is to introduce the methods approved nowadays in Brazil in order to induce the euthanasia in rodents, amphibians, lagomorphs, birds, pigs and fish. In order to proceed these studies, we were based on the new Brazilian law to describe the methods approved to induce euthanasia in laboratory animals. For all animals previously mentioned the chemical methods with barbiturates or anesthetics are approved. Physical methods are approved only with restriction and only when we cannot use any chemical method. Carbon dioxide (CO2) can only be used for rodents and birds when a chemical method cannot be used. Carbon monoxide (CO) and ether are prohibited. These results demonstrate that Brazil has changed the law on euthanasia and recital based on the ethical principles of the 3RS. The methods that are approved and recommended are chemists and the use of physical methods are used only with restrictions and some as CO and ether are forbidden.

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Animal research is a highly emotive topic. Many people are critical of animal experiments but at the same time are demanding pharmaceutical products that meet the highest quality and safety standards. While it is common for pharmaceutical companies to have internal standards for animal research, no country has previously succeeded in aligning and extending them across a national industry. After identifying four key elements, (a) open and constructive stakeholder dialogue, (b) foster education and training, (c) promote all aspects of 3R and (d) audits and certification, the 10 articles of the Animal Welfare Charter were developed. The commitments of the charter are not only applied within the companies but are also valid for all external research and development partners – on a global level (i.e. in countries with legal requirements below the Swiss or European standard or with no animal welfare acts in place). Based on the charter, industry working groups were built and collaborations with different stakeholders such as animal rights organizations and the academia have been established. The annually published report provides an overview of company initiatives and achievements. It gives proof that the commitments are sustainable and lead to constant progress in favor of the animals.

Use of animals in military medical training by NATO nations

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Among the member nations of the North Atlantic Treaty Organization (NATO), a variety of training methodologies – including high-fidelity patient simulators, moulage scenarios, task trainers, 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.

Our survey of NATO nations shows that 23 of 28 NATO nations do not use any animals for military medical training. The United Kingdom, United States, Norway, Denmark, and Canada continue to use thousands of pigs, goats, and sheep each year for emergency medical training drills in which live animals are stabbed, shot, burned, and otherwise harmed.

These exercises persist despite the various nonanimal training methods available and these countries’ regulations requiring the use of alternatives to animals when they exist. Indeed, the few nations still using animals have acknowledged through NATO that the practice has come under “significant scrutiny” and may need to be “completely eliminated.”

This presentation discusses the survey results, attendant scientific and legal issues, and recent developments in efforts to curb animal use in Europe and North America.
Legislation and implementation of alternative methods and outcomes

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The well-known 3R (reduce, replace, refine) concept was laid down by Bill Russel and Rex Burch in 1959. Now, on the next century we have reached to the stage we possess not only the ideas and soft law derived from guidances but international hard law with the examples of EU Regulation (REACH), EU Directive 2010/63/EU and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of Council of Europe. Among the significant sanctions the mandatory stipulation to reduce, enhance and promote the alternative methods to reduce animal tests is assured. Maximising access to public legal information for all participants is part of the harmonisation and deserves common heritage of humanity.

In this paper the implementation of hard and soft law on 3 R in different aspects will be studied with the attention to regional pharmacopoeias and other relevant international institutions. Substantial case studies will be presented. In conclusion the support to intensive functional network instead of fragmented protectionist policy is expressed.

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The transposition of directive 2010/63/EU on the protection of animals used for scientific purposes – a comparison between France, Germany, the United Kingdom and Austria

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A comparison is drawn of the recent transposition of directive 2010/63/EU on the protection of animals used for scientific purposes, in the three major animal testing EU member states France, United Kingdom and Germany, as well as Austria, the author’s country of residence. First, a few flaws in the directive’s wording itself are pointed out, concerning for example the prospective evaluation of procedures, including the classification of severities. Subsequently, the different styles and emphases of the four transpositions are lined out, illustrated through the contrasting of a few particularly interesting articles of the directive with the wording of the corresponding national legal texts. Thus, the respective modalities for project evaluation, retrospective assessment, national animal protection committees as well as the differing penalties for non-compliance are described. Most notably, the UK has, for numerous rules laid out by the directive, not taken the opportunity to grant exceptions for scientific reasons, certain procedures or certain establishments. Austria has, but not in all allowed cases; whereas Germany and France have widely – emphasising clearly the facilities for research. France, for some articles, has not even transposed the directive correctly. Resuming, some possible consequences of the different wordings in transposition are lined out.

3R work structured according to Lean Sigma results in increased refinement and reduction in toxicity testing in the pharmaceutical industry

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The aim of the project was to study effects of structured work with continuous improvements on the principles of the 3Rs in a large toxicology research unit. Would structured work according to Lean Sigma improve 3R results or would it hamper creativity? A specialized Refinement Team was set up in 2008, and a cross-departmental 3R Team was set up in 2009. Both teams worked with continuous improvements according to Lean philosophy, based on engaging all levels of staff. Implemented 3R ideas were mapped 2006-2011 and compared according to number of implemented ideas as well as cross-departmental collaborations for each R. The total number of implemented 3R ideas was 55 on Refinement and 39 on Reduction. The number of implemented Refinement ideas per year increased by almost five-fold after implementation of the Refinement team. Increases in cross-departmental projects were seen after implementation of the 3R Team; from 10% to 42% for refinement and 28% to 67% for reduction. The present study shows that Lean Sigma and continuous improvement can increase creativity and create a structured process of implementing 3Rs into toxicological research, as measured by increased number of implemented 3R ideas and increased levels of collaborations.

Alternatives in South Africa: initiatives in education, research and testing

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South Africa’s first series of workshops and seminars on alternatives was organised in 2012 following collaboration between InterNICHE and the NSPCA. Two successful workshops with international speakers were held in Pretoria. The first introduced replacement alternatives in education and training, and a multimedia exhibition provided hands-on experience. Alternative laparoscopic surgery training was demonstrated using a perfused ethically sourced animal cadaver. The second introduced the 3Rs in research and testing, with a focus on replacement. Legislation, ethics committees, the use of sentient animals in fundamental research, information retrieval, funding of R&D and validation of alternatives were addressed. Delegates from all relevant fields were present at the workshops, which were followed by seminars in universities across the country. The NSPCA/InterNICHE Alternatives Loan System, a South African library of learning tools, was also established. The events were significant for introducing the concept of alternatives in the country, and contributing to NSPCA guidance work within schools, universities and animal ethics com-
India takes a giant step forward to protect dogs from testing, with more emerging evidence that dogs add no additional value in toxicity testing

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With repeated scientific evidence emerging that dogs provide no additional value in toxicity testing, there have been series of international discussions and publications on the pertinent question “Should dogs be put through the pain of experimentation?” (Hasiwa et al., 2011; Zurlo et al., 201; Turner, 2011) Ethically, there has been a societal demand for decades, calling for a ban on the testing on dogs on the premise that dogs are companion animals, deeply sensitive and intuitive. An analysis of dog toxicity studies showed that in 92% of the studies, safety data added no more relevant information to that provided by the rat, and the other 8% did not result in the withdrawal of drugs from development, indicating that dog studies are not required for the prediction of safe doses for humans (Broadhead, 1999). Recently, a robust analysis of the value of dogs for predicting drug toxicity, based on more than 2300 drugs, showed that a negative toxicity result in dogs added no evidential weight to the probability that a drug may not be toxic in humans (Bailey et al., 2013). In light of the above, India has taken a bold step, with the CPCSEA requesting the Drug Controller General of India to look closely at the use of alternatives to dogs in testing.

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**Session IX-3: Activity updates from international validation centres**

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**Session IX-3: Oral presentations**

**IX-3-017**

JaCVAM update

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The Japanese Center for the Validation of Alternative Methods (JaCVAM, http://jacvam.jp/) was established in 2005 to promote the use of alternatives to animal testing in regulatory studies, thereby replacing, reducing, or refining the use of animals, according to the Three Rs principles. JaCVAM assesses the utility, limitations and suitability for use in regulatory studies, of test methods needed to determine the safety of chemicals and other materials. JaCVAM also organises and performs validation studies of new test methods, when necessary. In addition, JaCVAM co-operates and collaborates with similar organisations in related fields, both in Japan and internationally, which also enables JaCVAM to provide input during the establishment of guidelines for new alternative experimental methods. These activities help facilitate application and approval processes for the manufacture and sale of pharmaceuticals, chemicals, pesticides, and other products, as well as for revisions to standards for cosmetic products. In this manner, JaCVAM plays a leadership role in the introduction of new alternative experimental methods for regulatory acceptance in Japan.

**IX-3-293**

EURL ECVAM strategy to avoid and reduce animal use in acute systemic toxicity

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Assessing chemicals for acute systemic toxicity represents a standard information requirement within several pieces of EU chemicals legislation. Classification and labelling is the main purpose of conducting the test. Currently, only in vivo tests are accepted by regulatory bodies and cytotoxicity assays are recognised simply as additional tests that can be used for estimating the initial doses for tests in vivo. The development of mechanistically-based alternative methods and strategies in this area is still hampered by the limited understanding of the key toxicity pathways in humans. The EURL ECVAM strategy is based on the state-of-the-art in the area, including recent and ongoing efforts. Available data indicates that the 3T3/NRU cytotoxicity assay can be used to support the identification of non-classified substances, although results should always be used in combination with other information sources to build confidence in the decision not to classify a substance for acute oral toxicity. EURL ECVAM is therefore focus-
ing its in-house activities on the better use of in vitro and in silico methods, and on exploring the usefulness of existing data from other systemic toxicity studies. EURL ECVAM will also continue to support activities aimed at the refinement of relevant in vivo studies.

**IX-3-388**

**EURL ECVAM’s approach to the global acceptance of alternative methods**

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With the adoption of EU Directive 2010/63/EU on the protection of animals used for scientific purposes, ECVAM became the European Union Reference Laboratory on Alternatives to Animal Testing (EURL ECVAM). Its key responsibilities are to coordinate and promote the development and use of alternatives; coordinate the validation of alternative approaches at EU level; act as a focal point for the exchange of information on the development of alternative approaches; set up, maintain and manage public databases and information systems on alternative approaches; and; promote dialogue between legislators, regulators, and all relevant stakeholders in view of the development, validation, regulatory acceptance, international recognition, and application of alternative approaches.

This presentation will describe how EURL ECVAM responded to these key provisions by streamlining its validation workflow, ranging from test submission assessment and prioritization, over validation and peer review to publication of EURL ECVAM recommendations and leading projects on alternative test methods at OECD level. It will explain how regulators, stakeholders, international partners and test method users are involved in the process in a structured and systematic way. The ultimate aim is to facilitate and speed up the European and International regulatory acceptance, global recognition and use of standardised test methods and approaches.

**IX-3-560**

**A new vision and direction for ICCVAM and NICEATM**

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The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is reorienting itself to better address advances in toxicological science while reflecting the needs and priorities of its partner regulatory agencies. ICCVAM includes representatives from 15 U.S. agencies that generate or use toxicological testing information. It promotes scientifically valid test methods that better protect human health and the environment while advancing the 3Rs. In 2013, ICCVAM initiated an effort to (1) explore new paradigms for validation and utilization of alternative toxicological methods, (2) identify areas of scientific focus, and (3) improve communications with the public. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which provides support for ICCVAM, is broadening its scope to support the National Toxicology Program and the Tox21 interagency consortium. Specific programmatic changes are described herein.

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**IX-3-570**

**Alternative methods to animal experiments – ongoing activities in Germany**

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Approximately a quarter of all animals used in the EU for scientific and regulatory purposes is used in Germany. This reflects Germany’s traditionally active role in basic and applied life science research, and in the development, safety assessment and quality control of medical, industrial and agricultural products and devices. The German government has early recognized the need to support the development of alternative methods to animal experiments according to the 3R principles (replacement, reduction and refinement) by Russel and Burch (1959). Since the 1980s, research projects intending to develop alternative methods were funded with a total volume of about 150 million Euros. In 1989, the National Centre for the Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) was founded at the BfR to pursue the 3R goal by supporting the development and validation of alternative methods, advising expert panels, and providing a forum for information and education. In addition, the new legal tasks derived from the Directive 2010/63/EU have been assigned to the BfR. At the same time industry and NGOs have continuously driven forward the implementation of the 3Rs in animal experiments with scientific and regulatory purpose. An overview of ongoing activities in Germany will be given.

**IX-3-619**

**Activities of Korean Center for the Validation of Alternative Methods (KoCVAM) to promote alternative test method in Korea**


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The Korean Center for the Validation of Alternative Methods (KoCVAM) was established within the National Institute of Food and Drug Safety Evaluation (NIFDS), which is a part of the Ministry of Food and Drug Safety (MFDS), in November 2009 under the Laboratory Animal Act of 2008. The mission of KoCVAM is supporting policies on the development and approval of alternative methods, coordinating validation studies and peer reviews, and providing recommendations to regulatory authorities. Furthermore, KoCVAM promotes cooperation among domestic and international organizations, and provides information and education on alternative methods. In March 2011, KoCVAM joined the
International Cooperation on Alternative Test Methods (ICATM) and has recommended 9 alternative test guidelines on Cosmetics, including RHE test method, and MFDS has accepted all of them. From 2013, KoCVAM launched a Consortium of Alternative Methods for Safety Evaluation of Cosmetics which aims at developing and validating new alternative test methods for safety assessment of cosmetics in the areas of eye and oral mucosal irritation, skin sensitization and photosensitization. KoCVAM will continue working on alternative test methods in collaboration with different national and international organizations to improve public health and animal welfare.

The need for creating BraCVAM arose in 2008 and, immediately, members of academia, industries and validation centers engaged this idea. In 2012, cooperation between Oswaldo Cruz Foundation (FIOCRUZ) and the Brazilian National Agency of Health Surveillance (ANVISA) started the establishment of BraCVAM, created in 2013. The Brazilian validation process will follow the OECD Guideline 34 where BraCVAM will identify methods for entering the validation process and/or receive requests from test submitters. BraCVAM will inform the Brazilian National Network on Alternative Methods (RENNAMA) about promising assays, which will in turn prioritize and contribute to the validation study of the selected assays. The validation study will be supervised by a Validation Management Group, and the obtained results will be peer-reviewed by an ad-hoc Scientific Review Committee, organized under the auspices of BraCVAM. Based on the peer-review outcome, BraCVAM will prepare recommendations on the validated test method and send these final recommendations to the National Council for the Control of Animal Experimentation (CONCEA). CONCEA will in turn be in charge of the regulatory adoption of the validated test methods in Brazil following an open public consultation.

The Brazilian Centre for Validation of Alternative Methods (BraCVAM) and the establishment of the validation process in Brazil

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In vitro laboratory GLP-certified in Brazil: a successful public-private partnership to achieve international harmonization

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The boost of the in vitro methods, to meet global ethical and regulatory requirements that head nonclinical safety and toxicology stud-
Systematic reviews of test method performance: a case study using the Zebrafish Embryo Test for developmental toxicity

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Systematic reviews, with their emphasis on transparency, objectivity, and consistency, provide a framework that can be adapted to assessing the literature on the performance of alternative test methods or testing strategies, a process that can be loosely likened to retrospective validation. The Evidence-based Toxicology Collaboration (EBTC) (http://www.ebtocx.com) is pioneering this application of systematic reviews, translating the Cochrane Collaboration’s guidance for conducting systematic reviews of diagnostic test accuracy in medicine to the toxicological context (Cochrane Collaboration, 2014; Hartung, 2010; Stephens et al., 2013). Our case study assesses the performance of the Zebrafish Embryo Test (ZET) in predicting the results of prenatal developmental toxicity tests in rats and rabbits, as typified by the Organization for Economic Cooperation and Development’s Test Guideline 414 (Selderslagh et al., 2012). We have written a protocol that describes the various steps to be taken in our systematic review, such as the literature search, eligibility determination, data extraction, and risk-of-bias assessment. An overview of our approach, the protocol, and its implementation via a pilot study will be provided. While our primary aim is to assess the ZET’s performance vis-à-vis the established mammalian tests, we are also seeking to operationalize systematic review methods for the toxicology domain.

This initiative are according with most advanced programs of OECD countries to reduce uncertainty of data, increase the normalization of standard operating procedures (SOPs), quality control systems, safety procedures, records and reporting applied for in vitro studies.

The aim of Brazil, and other emerging markets, to an in vitro GLP-compliant infrastructure within international requirements and higher standards is fundamental to establish international harmonization and guarantee confidence, accuracy and integrity of data for in vitro studies produced by laboratories at different countries.

References

Evolving validation practice to meet the demands of predictive toxicology

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For validation to be a key enabling factor in the acceptance of alternative methods for regulatory use, the principles, purpose and process underlying a validation study need to be carefully considered to ensure that expectations of decision makers are properly met. The validity of a method or approach can be established in a variety of ways but ultimately the aim is to demonstrate the reliability and relevance of the data generated while describing the associated prediction uncertainty. Although reliability assessment is somewhat of a technical task, the relevance aspect has become increasingly challenging due to the fact that prediction of more complex toxicity endpoints relies on the optimal combination of multiple complementary methods. Moreover, with the emergence of mechanistic frameworks such as Adverse Outcome Pathways (AOP), the relevance of a method is often more related to its ability to capture one or more Key Events of an AOP rather than its ability to predict an apical in vivo effect. However, validation practice is evolving to meet the demands of predictive toxicology and solutions lie in the innovative use of the same tools and thinking behind the new paradigm for safety assessment.
**IX-4.799**

**The role of European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) towards internationally accepted harmonised in vitro method standards**

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In cooperation with EU Member States and in response to provisions of Directive 2010/63/EU on the protection of animals used for scientific purposes, EURL ECVM has recently established the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL, http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvm.eu-netval). Its mission is to provide support for EURL ECVM validation studies that serve to assess the reliability and relevance of alternative methods that have a potential to replace, reduce, or refine the use of animals for scientific purposes. The network aims to increase Europe’s validation capacity. The first pilot project of selected EU-NETVAL test facilities is the AR-CALUX validation study to support the development of an OECD performance-based test guideline and associated performance standards for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential. Future tasks for EU-NETVAL will be the generation of 3R testing approaches, including a process for collection of real-life data. Pathways for regulatory acceptance and a new procedure for method submission and evaluation are described.

**IX-4.925**

**Validation and qualification of new in vitro technologies for drug development**

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Over the past two years, the American Institute for Medical and Biological Engineering and the National Institutes of Health have held a series of workshops on Validation and Qualification of New in vitro Tools and Models for the Pre-clinical Drug Discovery Process. The overall goal of this series of workshops is to develop guidelines for investigators developing new technologies for the drug development process on how to validate these new technologies so that they become useful, meaningful tools. Specific emphasis has been on model systems, such as “organs on a chip”, that may augment existing models, especially animal models, in the US Food and Drug Administration drug approval process. The workshops have mainly been focused on drug toxicity evaluations with new in vitro systems but have also addressed efficacy issues, not only for pre-clinical drug development, but also for use during clinical trials and potentially in lieu of clinical trials for diseases with small populations of patients. A summary of the outcomes of the workshops will be presented.

**IX-4.854**

**Regulatory acceptance of 3R testing approaches for medicinal products**

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Regulatory testing of medicinal products is carried out to support first administration to humans or to target animal species; before carrying out clinical trials in larger populations; before marketing authorisation and to control quality during production.

In line with Directive 2010/63/EU, the 3Rs are embedded in the drafting process of regulatory guidance (European and (V)ICH). Regarding non-clinical testing for human medicinal products, new 3R methods have been accepted via multiple and flexible approaches, either as pivotal, supportive or exploratory mechanistic studies.

The recently approved ICH guidelines, ICH M3(R2) and ICH S2(R1) are good examples in this respect. Current efforts related to the revision of ICH S1 and ICH S5 illustrate ongoing work.

Although regulatory acceptance of 3Rs is possible, a formal process has been lacking and implementation of new test methods in routine regulatory testing has sometimes proven problematic. Therefore, the EMA JEG 3Rs drafted a Guideline on regulatory acceptance of 3R testing approaches (EMA/CHMP/CVMP/JEG-3Rs/450091/2012) that clearly defines regulatory acceptance and provides guidance on the scientific and technical criteria for regulatory acceptance of 3R testing approaches, including a process for collection of real-life data. Pathways for regulatory acceptance and a new procedure for method submission and evaluation are described.

**IX-4.939**

**Alternative toxicological methods for drug safety assessment in China**

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Bringing together the recent progress in alternative toxicological methods in China, this presentation introduced the development and validation of replacement, reduction, and refinement alternatives (the 3Rs) to animal testing for drug safety assessment. Current, in China, zebrafish was a model under validation for early screen of reproducive/developmental toxicity and neurotoxicity testing. In carcinogenicity, Bhas 42 cell transformation assay was validated with intra-laboratory and within-laboratory and has been used for the early toxic test of Chinese traditional medicine (TCM). Also, embryonic stem cell test, micro mass test, and whole embryo culture were validated and recommended to be used for early test for developmental toxicity. A computational toxicological model is under developing by China FDA to predict TCM toxic, especially for injection products. Also, in this presentation it addressed what has been accomplished thus far in developing acceptable alternatives to traditional animal toxicological assessment and provide potentially new initiatives in China, including the use of stem cell. Finally, the abstract discussed regulatory acceptance of alternatives in China.
Session IX-5: Regulatory acceptance of alternatives

Co-chairs
Anne Gourmelon, OECD, France
Derek Knight, ECHA, Finland

Session IX-5: Oral presentations

IX-5-107
Regulatory acceptance and use of the Extended One Generation Reproductive Toxicity Study within Europe

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Two-generation study (OECD TG 416) is the standard procedure within REACH to test reproductive toxicity effects of chemicals with production volumes >1000 tonnes and encounters ethical, economical and scientific objections. The Extended One Generation Reproductive Toxicity Study (EOGRTS) was incorporated in the OECD test guidelines in 2011 (OECD TG 443) and thereby became an internationally validated method. This protocol reduces animal use for reproductive testing by about 40% and is far more informative. However, its regulatory acceptance within Europe is a challenging process. This research describes the factors influencing the three stages of regulatory acceptance and use of the EOGRTS, using literature research and expert interviews. The stage of Formal Incorporation into the OECD was stimulated by retrospective analyses of the F2 value, strong advocates and the push of US and EU chemicals legislation. The Actual Regulatory Acceptance within REACH is withheld by several legal, economic and scientific factors. The Use by Industry lingers due to uncertainty about the regulatory acceptance, costs and manageability of the EOGRTS. The existing debate is fed by two opposing frames i.e. the frame of precautionary and the frame of innovation. The 4C’s of commitment, communication, collaboration and coordination are vital to enhance the process.

IX-5-652
Can the ADAPT principles help regulatory authorities implement the 3Rs?

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Within the regulatory sector there are a number of stages after validation that must be passed before an alternative method can be considered to have replaced a specific animal test. Failure by regulatory bodies to recognise and take responsibility for each stage has, in our opinion, been a major reason for some of the delay in the implementation of methods replacing acute toxicity, skin irritation, pyrogenicity and reproductive toxicity amongst others.

We have created the ADAPT checklist for regulatory bodies to ask themselves to ensure alternatives are not being unnecessarily delayed. Assessment -does the body have a proactive mandate to assess the suitability of new methods for their sector? Decision -who takes responsibility for deciding whether an alternative method is suitable? Acceptance -have all bureaucratic hurdles to acceptance such as the need to revise guidance and/or legislative text been identified? Policing -are there mechanisms in place to monitor the use of alternatives and will action be taken if animal tests are done unnecessarily? Transparency -does the authority inform all stakeholders of their actions at each stage?

In this presentation we provide examples of each ADAPT stage where alternatives have struggled and what action should be taken.

IX-5-425
Promote the use and outspread of alternative methods in China through standardization and administration acceptance

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The development of 3R alternatives in China is full of conflicts and hope. The lack of national 3Rs regulations meets increasing conflicts with industries’ needs. The slow popularization of 3Rs concepts and alternative technology in China makes it difficult to keep pace with the rapidly-developing modern bioscience. The absence of national level coordination mechanism also restricts the 3Rs development. Under this background, the major governmental department AQSIQ system, takes the leading position in promoting understanding and acceptance, effectively moves forward the alternative methods across the country. AQSIQ has accepted and implemented more than 20 alternative-testing standards. The National Standardization Administration also adopted some in vitro methods from OECD guidelines. The China National Accreditation Service for Conformity Assessment has accelerated the inter-laboratory transfer and laboratory network construction process of alternative methods. AQSIQ also released the validation guideline according to international standards and accomplished the first multi-center validation of the in vitro skin-irritation-test in China. Actively driven by AQSIQ, with the benefits from international communication and cooperation, through education and training, the China Food and Drug Administration panel also puts forward a five-year planning advice regarding alternative methods. China is now in a new stage of development towards 3Rs and alternative methods.
**Possible roles for non-standard methods in the REACH registration**

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The various regulatory schemes for chemical and other specific products have their own data requirements: one illustration is the EU REACH Regulation for chemicals. The registrant can “adapt” the standard information requirements under REACH, and use other information instead: non-standard or non-GLP studies, *in vitro* studies, human epidemiology data, information from structurally-related substances (i.e. “read-across” and “chemical categories”), predictions from valid (Q)SARs and use of the weight of evidence (WoE) approach. Such non-standard information has to be equivalent to the standard studies, in that the key results from the standard method should be addressed and the result must be suitable for adequate risk assessment and classification. There is an R&D need to develop rational combined approaches for integration of tests/data/predictions into ITSs & IATAs & “test batteries” for use by industry and regulators in assessing the properties of substances. For example the JRC, with steering from ECHA, are working on a flexible “framework” Integrated Assessment Strategy for skin sensitisation to apply the OECD AOP by means of “assumption blocks” for the Molecular Initiating Event and the Key Events. Each “block” will be populated with a selection of assays and *in silico* assessment tools in a flexible manner.

**Regulatory acceptance of methods intended to become OECD Test Guidelines**

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OECD countries have tasked the Environment, Health and Safety Program to develop harmonized methods for testing chemicals. The methods are intended to generate valid and high quality data to support regulations in member countries. The regulatory acceptance is an ultimate step leading to their full implementation. Other upstream factors come into play, namely a balance between different policy mechanisms that enable the development of the alternative methods. In Europe, the regulatory framework for cosmetic products aims at striking the right balance between different policy mechanisms that facilitate the regulatory acceptance of non-animal methods (e.g., joint public-private investments in research; changes in the regulation preventing use of animals setting time pressure to get valid test methods). The validation process has been applied to filter methods of sufficient relevance, reliability and predictivity. In recent examples of OECD Test Guidelines, the regulatory acceptance has only been possible when protection of human health was not jeopardized. For more complex endpoints, the task will no doubt be harder. More work is needed to understand toxicity pathways, build integrated approaches to testing and assessment, in supplement to rigorous validation, in order to provide the context under which alternatives to animal testing can be safely applied.

**Read across strategy for the assessment of dyes with the aid of a new QSAR system**

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Dyes are commonly used in a variety of applications, including textile, wood, leather and paper coloring. In spite of the large diffusion of those substances, little is known about their toxicological profile in particular for the most complex endpoints such as reproductive toxicity, carcinogenicity and the fate in the environment. Beyond the social concern, a better definition of the risk posed by the use of dyes is now requested as mandatory by REACH. About fifty dyes have been already registered under REACH, but more than 500 will require a registration dossier by the 2018 deadline. Testing all of them is too demanding in terms of cost and animal lives. In order to set up an effective testing strategy, an advanced approach on grouping and read across will be applied, based on a new QSAR system. This is a unique project because no software is available that can process such complex molecules. The achievement is possible due to the availability of a very large number of data. In fact, the authors have received the permission to use all proprietary data that were acquired in the latest 30 years and own by the main manufacturing companies.

**SLiM: a smart way from innovations to humans**


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Safety assessment of pharmaceuticals and chemicals requires significant numbers of animal experiments. Although methods that replace, reduce or refine animal experiments (3Rs) for the safety assessment became available over the past decades, the number of accepted and
fully implemented methods within industry and regulations is low. The slow implementation process delays the innovations in product development, which is undesirable for societal and economic reasons.

Within the SLiM project good practices were obtained for a smarter and faster development, acceptance and implementation of 3R methods by intensifying the collaboration between companies, research institutes and regulatory bodies at an early phase in the method development process.

Factors that drive or withhold (regulatory) acceptance and use of 3R methods were identified by two expert panels (pharmaceuticals and chemicals), consisted of three stakeholder groups: regulatory authorities and legislators, industry and academia. To accelerate the process of acceptance, developments at micro-, meso and macro levels need to be aligned and the drivers need to outweigh the barriers. The dominant factors that are perceived to influence the process at these different levels revealed largely similar in both sectors. The four Cs: Communication, Cooperation, Commitment and Coordination are considered of key importance to augment the process (Schiffelers et al., 2014).

**Reference**


IX-5-691

**Developing regulatory acceptable in vitro methods**

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Change in the safety assessment paradigm has resulted in the introduction and, increasingly, regulatory acceptance of alternative in vitro methods as replacement for, or supplementary to, existing in vivo tests. Change was often driven by scientific or animal ethical considerations resulting in regulatory legislation for chemicals (REACH), consumer products (7th Amendment to the Cosmetics Directive) and biological therapies (FIDA, ICH).

A challenge in developing alternative methods is the need to validate against known human endpoints. Frequently, there is only animal data to validate against and this can result in a false positive or negative being attributed to the human in vivo model which, in reality was due to the weakness in the animal in vivo model (e.g., skin sensitisation models against LLNA).

Assays for dermal absorption, skin and eye irritation, phototoxicity, genotoxicity, drug transporter, hepatic metabolism, functional immunosays (cytokine release, ADCC and NAb) are now used and accepted as standard tests. Screening in vitro models are generating improved drug candidates for selection into preclinical testing. These, and other tests, continue to help achieve the goals of the 3Rs. The creation and validation of future methods may now be more affected by human ethical considerations.

IX-5-800

**Good In Vitro Method Practice (GIVIMP): guidance on the implementation of in vitro methods within a GLP environment to support regulatory human safety assessment of chemicals**

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In vitro methods, often based on the use of human cells and tissues, are submitted to international validation bodies (Rispin et al., 2004; Gupta et al., 2005; Coecke et al., 2005). Well-designed, robust, reliable in vitro methods that can run in a GLP environment for generating data sets are becoming more and more instrumental for supporting regulatory decisions. Good In Vitro Method Practice (GIVIMP) is a proposal from EURL ECVAM to issue an international guidance on the implementation of in vitro methods within a GLP environment to support regulatory human safety assessment of chemicals. GIVIMP will contribute to increased standardisation and harmonisation in the generation of in vitro information on test item safety. The Guidance will further facilitate the application of the OECD Mutual Acceptance of Data agreement for data generated by in vitro methods and as such contribute to avoidance of unnecessary additional testing. GIVIMP will take into account the requirements of the existing OECD guidelines and advisory documents to ensure that the guidance is complementary and 100% in line with these issued documents (OECD, 2004, 2005). In conclusion, GIVIMP will contribute to the use of in vitro method data to support regulatory human safety assessment of chemicals by striving that such data are being generated in compliance with GLP and based on current good scientific practices.

**References**


Considerations for alternatives to non-human primates in preclinical safety assessments

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Recent, we reviewed all the toxicity studies in National center for safety evaluation of drugs (NCSED) in the last 10 years. Total 43 non-human primates (NHP) repeated dosing studies have been performed. Most of test articles were biopharmaceuticals and about 1600 NHPs were used. However, only chimeric anti-EGFR Mab and humanized anti-EGFR Mab caused obviously toxicity. We compared the data between NHPs and rodents in all studies. No more valuable information was got from these NHPs. It suggested that we may need to consider to decrease animal number or use alternative way.

By using PBMCs obtained from monkey and human, we investigated cytokines secretion, proliferation of lymphocytes, and gene expression after antibody, phytohemagglutinin, lipopolysaccharides, and Fluzone vaccination stimulating. We found that there were significant difference in function of T cell proliferation and cytokine secretion between human and cynomolgus. The gene expression profiles data confirmed that the differentially expressed genes involved in immune regulation and response in human are more complex and sensitive than that in monkey. It suggested that we should be caution when predict T cells related toxicity got from animals to human, and in vitro tests used human peripheral blood may be a useful method to evaluate immunotoxicity.

Session IX-6: Breaking down barriers and promoting international cooperation on 3Rs

Co-chairs
Rodger Curren, IVS, USA
Nick Jukes, InterNICHE, UK

Session IX-6: Oral presentations

EPAA – a key player in shaping the future of 3Rs

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Since its creation in 2005, the vision of the European Partnership for Alternative Approaches to Animal Testing (EPAA) has been to promote the 3Rs approaches in regulation through better and more predictive science. Our mission is to promote the development, validation and implementation of alternative approaches, but also enhance the acceptance, harmonization and mutual recognition of tests by regulators at national, European and international levels.

EPAA brings together 37 companies from 7 industry sectors and 5 DGs of the European Commission: this unique knowledge-sharing platform launches working groups or studies to define research gaps in the development of 3Rs as well as to improve their implementation in safety regulation.

In 2013 for instance there were 11 ongoing projects related to Science and Regulation, and 6 scientific workshops were organised on topics as varied as stem cell research, skin sensitisation or vaccines consistency. In 2013, EPAA also organised events with external partners such as the European Parliament and SEURAT-1, an illustration of the EPAA’s wide network of contacts.

The unique nature of EPAA, its broad membership, its proven results and the width of its mission make it an effective cross-sector platform to further support the development and acceptance of alternatives.

The International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) in 3Rs Leadership Group: the pharmaceutical industry’s promotion of alternatives

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In January 2012, the IQ 3Rs Leadership Group (LG) of the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) was established. The IQ 3Rs LG is made up of senior veterinarians, biomedical scientists and 3Rs specialists from IQ member pharmaceutical/biotechnology companies. The mission of the 3Rs LG is to promote sharing and integration of high quality scientific practices to advance the Replacement, Reduction, and Refinement of animals used in the discovery and development of new medicines, vaccines, medical devices and health care products for humans and animals. Our first two years were dedicated to creating a variety of Working Groups (WGs) to promote 3Rs in a range of areas and ini-
tating several 3Rs projects. One of the main goals of the IQ 3Rs LG is to gather benchmarking information about alternatives from across the industry to highlight strengths and identify gaps and to promote scientific research to further 3Rs innovation. A third goal is to facilitate communication and education about 3Rs advances in a more systematic manner across the biomedical research community via WebEx conferences, journal articles and seminars at international scientific meetings. Another important goal is to develop industry consensus on scientific positions related to alternatives to advance the science, animal welfare, and innovation on 3Rs issues with external stakeholders (legislators, regulators, NGOs, CROs, academia). This presentation will provide a more detailed overview of the IQ 3Rs LG and its various WGs and current 3Rs initiatives to further our collaborative reach and progress our goal of communicating 3Rs advances globally. We will also present our current collaborative efforts in the advancement of the 3R’s globally. Members of the IQ 3Rs LG are serving in various functions, e.g., theme coordinators, co-chairs and presenters to support the success of this World Congress.

IX-6-496

A seat at the table: advocating for replacement at the OECD

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The current multi-national and multi-sector scope of many companies requires a coordinated approach to toxicity testing and assessment guidance. The International Council on Animal Protection in OECD programmes (ICAPo) is uniquely placed to provide a united voice for the replacement of animals in toxicity testing and chemical hazard assessment guidelines and programmes. ICAPo comprises groups from Asia, Europe, and North America, and provides consensus policy and scientific interventions to expert meetings, test guidelines, guidance documents, and work plan proposals. Since 2003 ICAPo has worked to reduce and refine existing and new in vivo test guidelines, though most of its efforts aim to increase the portfolio of in vitro and in silico methods and tools available to companies and regulatory agencies. For example, ICAPo helped to draft guidance on strategically reducing fish testing and recommendations for furthering in vitro thyroid disruption test methods. Nine out of the last 11 test guidelines published by OECD are in vitro guidelines. Today OECD leads the international coordination of adverse outcome pathway development and AOP-informed testing strategies, and ICAPo is committed to making financial and scientific contributions to this process to ensure the ultimate adoption of a non-animal predictive toxicology paradigm.

IX-6-503

Lack of infrastructure can be a barrier to the acceptance of 3Rs methods; education and training can provide the solution

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Policy changes, especially those addressing regulatory requirements for the safety of new products, can be impeded for several reasons. In some cases decision makers in national regulatory bodies are unaware of the science supporting proposed new methodologies and are hesitant to adopt them solely on the insistence of other countries. This is not entirely unexpected since such decision makers may be more frequently exposed to political concerns rather than the applicable scientific ones. Another barrier exists when the technical infrastructure to properly conduct new in vitro methods has yet to be established in a country’s domestic laboratories. Both barriers exist in the area of non-animal methods for toxicity testing where significant international differences in acceptance exist. Europe and the US, for example, are quickly moving towards using human-derived cells and tissues – rather than animal based models – to assess many toxicological endpoints, while other countries may be reluctant to make a change because their scientists have not had sufficient time to develop sound data bases of information. We have found that providing specific hands-on training and education on standard methods directly to regulators and scientists in these countries has significantly improved the recognition and acceptance of new 3Rs approaches.

IX-6-585

A process for regulatory science development and application

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The Critical Path Initiative: From Stagnation to Innovation provided a strategy for strengthening the biomedical enterprise. This document outlined mechanisms by which FDA could work with the regulated industries and other stakeholders to further regulatory science. Specifically, this process was developed for genetic and genomic information to be reviewed under the Voluntary Genomics Data Submission process (VGDS). This process was extended to include other types of data including proteomics, metabonomics, and imaging. Submission of these data types to the Agency permitted the development of internal expertise, tools, and processes for the analysis and exploitation of new data types in the regulatory review process. In addition to the regulated industry, consortia such as ILSE/ESI that are umbrella consortia have contributed to developing the regulatory review process by forming tripartite relationships and public-private partnerships to enable data sharing, collimation, and use by the Agency and its stakeholders. Qualification of biomarkers through the VXDS process has led to a focused method for context dependent biomarker and regulatory tool qualification through the activity of the Biomarker Qualifi-
functioning of the 3Rs at the policy level. Alternatives to animal testing worldwide as well as the details of the talk, the author will provide an overview on the progress on initiatives e.g., North & South America and Australia. In the last part will also be the opportunity to map and present ongoing worldwide with societal demands (e.g., European citizenship initiative). This of the talk, the author will focus more on legislative proposals linked main actors on the web and where they are located. In the second part of the knowledge. It will be the opportunity to discuss who are the platforms (e.g., Wikipedia) or websites, which ensure dissemination this presentation intends to illustrate with concrete examples how to animal testing: raising awareness beyond the community. It has been observed that 25% of laboratory animal use world to use the same study types and avoid unneeded animal studies that can be address already with alternatives. 4) Technical issues, most in vitro methods are water based systems, this can potentially limit the testing and identification of the hazard potential of certain test substance classes. Consequently we observe that there still is room for improvement for effective use of 3R studies for regulatory purposes.

**Implementing the 3R methods and hurdles for their application – a perspective from the chemical industry**

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The toxicological potential of chemicals must be determined by law and often requires animal studies. We are committed to animal protection and apply 3R principles whenever possible. Here, we report on some hurdles that BASF SE has encountered in the recent years. Hurdles for reduction of animal numbers used for regulatory purposes exist in several forms

1) Timeliness for the acceptance (e.g., validation/acceptance for sensitization may come too late)
2) Changes in the original designs to reduce animal testing in the extended one-generation reproductive toxicity study may result in the performance of an extended 2-generation study
3) Different international standards (e.g., eye irritation in vitro works for GHS classification, but not for the Brazilian system), in this context, there is a need for harmonization of regulations all over the world to use the same study types and avoid unneeded animal studies that can be address already with alternatives.

**International collaboration: case study of InterNICHE in India**

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InterNICHE is a volunteer-based organisation and network with National Contacts and Partners in over 40 countries. The opportunities and obstacles of working across national and cultural boundaries, and of negotiating for replacement across differences of opinion, have provided experience that feeds into all InterNICHE activity. With successful outreach performed, projects developed, and resources provided, this presentation will explore the nature of successful international collaboration for progressing replacement. The case study of InterNICHE work in India will be used to illustrate its potential.

**Science communication in alternatives to animal testing: raising awareness beyond the community**

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This presentation intends to illustrate with concrete examples how to spread the word as well as raise and maintain awareness beyond our 3Rs community. In the first part of the talk, the author will discuss multiple social media tools available (e.g., Twitter), participative platforms (e.g., Wikipedia) or websites, which ensure dissemination of the knowledge. It will be the opportunity to discuss who are the main actors on the web and where they are located. In the second part of the talk, the author will focus more on legislative proposals linked with societal demands (e.g., European citizenship initiative). This will also be the opportunity to map and present ongoing worldwide initiatives e.g., North & South America and Australia. In the last part of the talk, the author will provide an overview on the progress on alternatives to animal testing worldwide as well as the details the functioning of the 3Rs at the policy level.

**Regulatory acceptance and use of 3R models for pharmaceuticals and chemicals: Expert opinions on the state of affairs and the way forward**

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Pharmaceuticals and chemicals are subjected to regulatory safety testing accounting for approximately 25% of laboratory animal use in Europe. This testing meets various objections and has led to the development of a range of 3R models to Replace, Reduce or Refine the animal models. However, these models must overcome many barriers before being accepted for regulatory risk management purposes. This paper describes the barriers and drivers and options to optimize this acceptance process as identified by two expert panels, one on pharmaceuticals and one on chemicals (Schiffelers et al., 2014). To untangle the complex acceptance process, the multilevel perspective on technology transitions is applied. This perspective defines influences at the micro-, meso- and macro level which need alignment to induce regulatory acceptance of a 3R model. This paper displays that there are many similar mechanisms within both sectors that prevent 3R models from becoming accepted for regulatory risk assessment and management. Shared barriers include the uncertainty about the
value of the new 3R models (micro level), the lack of harmonization of regulatory requirements and acceptance criteria (meso level) and the high levels of risk aversion (macro level). In optimizing the process commitment, communication, cooperation and coordination are identified as critical drivers.

**Reference**

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**IX-6-195**

**A decision tree to facilitate the replacement of laboratory animals in Brazil**

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We aimed to develop a decision tree to facilitate validated alternative methods (VAM) implementation. First, does a VAM exist? If yes (Y), is the laboratory director (LD) motivated to change? If Y, is there: Branch 1 (B1) Knowledge about VAM costs?; (B2) Qualified human resources?; (B3) Resistance to change by the staff?; and (B4) Incompatibilities between specific norms and Brazilian Animal Protection Law (1)’? For B1, if Y and VAM costs less than animal use, go to B2; if no (N), costs should be studied. For B2, if Y, go to B3; if N, training should be sought. For B3, if Y, staff should be educated about law, ethics and the 3Rs (2); if N, go to B4. For B4, if Y, norms should be denounced to appropriate instances; if N, implement VAM. If LD is unmotivated, does he/she know Brazilian Animal Protection Law (http://www.planalto.gov.br/ccivil_03/Leis/L9605.htm)? If Y, law enforcement is required; if N, LD should be educated about law, ethics and the 3Rs (Russell and Burch, 1959). If LD becomes motivated, he/she is ready to move to B1. If a VAM is not available, it should be developed. This decision tree provides guidance to address the main obstacles for laboratory animal replacement (Bones et al., in press).

**References**

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**IX-6-199**

**Ban safety tests on animals**

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Every day we surround ourselves by products and substances that have been tested on animals to establish their safety. And every day, new products and substances enter the market. Many of the legally required safety tests have existed for decades and new ones are being drafted. A very important institute in safety testing is the OECD. Many safety tests rely heavily on laboratory animal use. Yet, do animal tests reliably show that substances and products are safe? Are these tests predictive for humans? More and more studies are being published that cast doubt on the value of animal tests. They plead to change the protocols and for a paradigm shift, but this takes (too much) time.

The Dutch animal protection organisation, Dierenbescherming, believes that change can be accelerated by a ban on safety testing, say as of 2025, in comparison (not equal) to the cosmetic campaign. A petition was started to ask the support of the public, to make them aware of the problem and to involve them in the discussion on this subject. Safety is regarded as self-evident, but not at the cost of animals. Ideas of how to accomplish this, we want to present at the World Congress.

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**IX-6-214**

**Animal use and cost estimates for the proposed US policy on cosmetics. Comparison with EU and other World States**

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The proposed US Safe Cosmetics and Personal Care Products Act (H.R.1385) asks for highly demanding in vivo safety evaluations of all cosmetic ingredients that are marketed in the US, including both existing and new chemicals. A detailed analysis (Knight and Rovida, 2014) demonstrated that approval of that bill would cause animal use in a ten year period to increase up to more than 11 million animals with a cost over 9 billion US dollars. It also demonstrated the impossibility that the evaluation process could keep pace with the large and ever-growing number of cosmetics ingredients.

In contrast to that proposal, a new US Bill, the Humane Cosmetics Act, was recently presented to phase out cosmetic animal testing and the sale of cosmetics tested on animals, harmonized with the EU provisions (EC 1223/2009).

The situation worldwide is now confusing as other countries, for example, Japan, are requesting animal tests to authorize the use of cosmetics ingredients.

Details of the US proposal in comparison with the provisions of other countries will be presented.

**Reference**

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**IX-6-255**

**Cost comparison between the mouse inoculation test (MIT) and the virus isolation in cell culture (VICC) for rabies diagnosis**

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Because the decision for using laboratory animals is frequently based on cost aspects (Bones et al., in press), our objective was to compare the costs to perform the Mouse Inoculation Test (MIT) and the Virus Isolation in Cell Culture (VICC) for rabies diagnosis in Brazil. Based on the observation of laboratory routine at Pasteur Institute,
Advancing the application of 3R-methods requires international cooperation. Almost every step from development to regulatory acceptance and implementation has an international dimension. Organisations, companies and social organisations that promote and advance 3R application can strengthen their impact by combining their knowledge and skills in this area. However, in the light of diversity of promising innovations and professional networks that can play a role, it is essential to create focus.

In a workshop organised by NKCA and RIVM, commissioned by the Dutch Ministry of Economic Affairs, five top priorities were identified, in which national cooperation can contribute to advancing 3R development and application in the international arena. The workshop built on the study programme, Alternatives to Animal Experiments, which identified a number of promising research areas.

A plan of approach was made to maximise the international impact of 3R activities in the Netherlands. An inventory was made of organisations that must be involved in preparing these plans. An organisation must be identified for each priority to take the lead in strengthening national cooperation. The National Committee can take a leading role bringing together the relevant parties. Social organisations are part of the international network and can add strength to the message.

**References**


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**IX-6-444**

**Combining 3R strengths in the international arena: together we are strong!**

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EU Directive 2010/63 places the 3Rs at the core of a Europe-wide programme to protect animals used in science. The UK recognised successful implementation of the Directive would require a collaborative effort across UK government as well as the scientific community and non-governmental organisations (NGOs). New initiatives were needed to promote the development and widespread adoption of 3Rs advances.

The UK Coalition Programme for Government (http://bit.ly/UbIpr6) committed to a Delivery Plan (http://bit.ly/1eNCLBh) to work to reduce the use of animals in research. The Plan has three strategic priorities: putting the 3Rs at the heart of a science-led programme; influencing their adoption internationally; and promoting understanding about the use of animals where no alternatives exist.

The Plan uses the UK’s expertise in science and innovation to support its delivery and has been well received by scientists, government officials and welfare communities, many of whom are involved in the delivery of the plan. Measures of success and key milestones have been identified and will be reviewed annually, starting in 2015.

Through this unique and coordinated national approach we are demonstrating how, whilst rigorously promoting and implementing the 3Rs, we are able to continue to deliver scientific benefits for people, animals and the environment.

**IX-6-795**

**Cooperative industry activities to support international advancement of non-animal testing methods**

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Although many cosmetic, personal care, consumer product and raw material supplier companies have been working for decades to eliminate animal testing, in some countries regulatory authorities still require animals for product safety testing. While there are differing hurdles to acceptance of non-animal methods around the world, a common difficulty is lack of technical training. IIVS, a non-profit and world leader in the validation, training and application of non-animal test methods, has organized a group of companies to form the Industry Council for the Advancement of Regulatory Acceptance of Alternatives (ICARAA). ICARAA is a working group which provides counsel and financial support of IIVS’ mission to increase the use and adoption of *in vitro* methods internationally. Led by IIVS, ICARAA activities focus on educational programs that include lectures, hands-on training and data interpretation. Many of ICARAA’s activities are currently in

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**Working to reduce the use of animals in scientific research – a national approach**

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*Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

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China where there is keen interest on the part of the regulatory authorities to understand how non-animal approaches can be used to substantiate safety. This collaboration between regulatory agencies, industry and a technical institute serves as a model example of how to promote the practical acceptance of non-animal techniques and facilitate the movement away from animal testing for regulatory purposes.

Session IX-7: Harmonising ways to capture pathway-knowledge in toxicology

**Co-chairs**
- Hristo Aladjov, OECD, France
- Clemens Wittwehr, EURL ECVAM, JRC, Italy

**Session IX-7: Oral presentations**

**IX-7-286**

**Adverse Outcome Knowledge Base (AOP-KB)**

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An Adverse Outcome Pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between the two anchor points – the Molecular Initiating Event (MIE), and an Adverse Outcome (AO), connected by a causal chain of Key Events (KE).

To give the scientific community the possibility to enter, share and discuss their AOP related knowledge at one central point of information, the OECD has launched a project to develop the “Adverse Outcome Pathway Knowledge Base” (AOP-KB – http://aopkb.org/aopwiki/index.php/Main_Page), where AOP developers can create an AOP wiki page and then build an AOP by linking related information about MIEs, KEs, AOs and Chemical Initiators. Controlled-vocabulary drop-down lists from which to select Methods, Actions, Biological Objects, Life stages, Species, etc. related to the AOP simplify the entry of ontology-based information. Information regarding KEs shared among multiple AOPs is stored on a single page to eliminate redundant entries and make the collective knowledge about those entities available in all AOPs containing them.

The presentation will give an overview of the ICT architecture of AOP-KB, its main user interface elements, the AOPs currently contained within the KB and ways to interact with the system as data provider or data user.

**IX-7-426**

**Organizing the adverse outcome pathways knowledge – the Effectopedia way**

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Adverse Outcome Pathways (AOPs) describe the causal linkages in a chemical induced cascade of biological responses, across different levels of biological organisation, leading to an adverse outcome over time. The knowledge needed for AOP development is distributed across disciplines that do not normally collaborate. Evolving a common language and understanding requires interactions of experts within a clearly defined context. Effectopedia (http://www.effectopedia.org) – an online collaborative platform – defines this context as multidimensional organizational space, which can visualize AOPs against chosen pair of dimensions (e.g., time to effect vs. level of biological organization). The pathway space helps scientists with different backgrounds determine where their knowledge belongs, and also aids them in identifying both the larger scope of their research and the individual experts who might be actively interested in it. New contributions are immediately distributed to interested parties, keeping all information current, documented and open for discussion, whilst giving credit to original authors and reviewers. Effectopedia space also helps biological responses (effects) to be defined just once and shared across pathways that include them. Shared effects become common nodes in the network of connected pathways which can be utilized for vulnerability analysis, hazard assessment or identifying the most used/needed/resource consuming test assays.

**IX-7-699**

**Using the AOP knowledgebase to record an adverse outcome pathway for respiratory sensitisation**

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The aim of the OECD Adverse Outcome Pathway (AOP) programme is to organize and harmonize available knowledge about toxicological pathways in order to facilitate the development and use of chemical grouping, QSARs, and *in vitro* methods.

There is considerable interest in developing test methods to identify chemicals that cause respiratory sensitisation, and especially methods that discriminate between respiratory and skin sensitisers. Using the already well-supported pathway for skin sensitisation as a guide, a group of experts surveyed the literature to identify evidence to confirm the AOP; this information was entered into the AOP Wiki, part of the AOP Knowledge Base (AOP-KB).

In order to avoid redundancy and divergent descriptions of the same key events (KE) and to encourage the inter-connectivity of different pathways, shared molecular initiating events and KEs should be shared between AOPs in the Wiki. For example, covalent protein binding is a shared MIE between skin and respiratory sensitisation; other KEs within the two pathways are similar. This leads to consideration of whether, and how, the two pathways should share MIE/KE descrip-
tions. AOP authors must consider not only how the AOP fits into the Wiki but how the AOP, once built, will be used.

IX-7-771
**Using DRAGON to organize data and decisions for AOPs**

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ICF International’s DRAGON is a database platform designed to store qualitative and quantitative results from scientific literature to enable the data to be searched, analyzed, synthesized, and reported in support of systematic literature reviews for risk assessments. DRAGON consists of modules for literature categorization and evaluation as well as data extraction from animal toxicology studies, human epidemiology studies, and *in vitro* studies. EPA and NTP have provided funding and content support.

To facilitate development of Adverse Outcome Pathways, DRAGON users can import literature citations from multiple sources, use DRAGON-Screen to review titles and abstracts of scientific articles to identify literature relevant to a given AOP, tag literature to specific key events along an AOP, and, in cellDRAGON, extract data from the literature to serve as evidence supporting a key event. The DRAGON evaluation module provides a place to record decisions on the quality and applicability of studies and can be customized for each assessment. Common language and ontologies used in data extraction modules facilitate synthesis of evidence streams to connect, for example, mechanistic data indicating a molecular initiating event and apical adverse outcomes reported in an animal study. Data and decisions entered in DRAGON can be exported in various formats.

**IX-7-829**

**Skin sensitization AOP proof of concept implementation in the OECD QSAR toolbox**

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Adverse Outcome Pathway (AOP) concept provides a transparent mechanistic justification and weight-of-evidence approach to reduce uncertainty in the predictions for complex toxicological endpoints. As part of OECD QSAR Toolbox development a proof-of-concept AOP for skin sensitization was implemented. The skin sensitization AOP demonstrates the new functionalities using data rich chemicals, by connecting existing *in-silico* alerts, *in-chemico*, *in-vitro* and *in-vivo* test assays as nodes of a directed graph. When a chemical is subjected to the AOP first the *in-silico* alerts profilers are executed. If a chemical has the potential to cause skin sensitization according to protein binding profiles then it can “pass” to the next level of *in-chemico* tests. If experimental data is available for the chemical it can pass or not each test and move to next downstream nodes of the graph. Alternatively, if measured data is not available a category of similar analogues can be used for read across and once again verify if the node is passed. This process continues until all nodes of the graph are marked. The Toolbox can also be used for simulating skin metabolism allowing to identify the outcomes for both the parent chemical and metabolites.

**Session IX-7: Poster presentation**

**IX-7-860**

**Quality assurance of emerging technologies in toxicology**

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Recent publications from the National Research Council, the EPA and the European legislations are among the drivers of the current landscape change in risk assessment and toxicity testing. At the center of this advance is the conviction that emerging technologies such as -omics-technologies, high throughput screening and computational toxicology could make toxicity testing more efficient in terms of time, cost and relevance to human exposures. This conceptual framework offers many opportunities; challenges, however, need to be addressed to ensure a sufficiently robust and informative outcome. The ongoing “Human Toxome” research project (Bouhifd et al., 2014; Hartung and McBride, 2011) is making use of transcriptomics and metabolomics technologies for Endocrine Disruption pathways of toxicity elucidation. The degree of maturity of these technologies and acceptance of the scientific and regulatory community is dissimilar. Although microarrays have been extensively used for more than a decade, debate is still ongoing about the reproducibility of experiments and the comparability of results at different sites and platforms. Moreover, consensus is still to be achieved concerning best practices in critical aspects including data generation, analysis and interpretation and pathway information generation (Leist et al., 2012). A major challenge is becoming obvious: How to make sure sound and relevant information (e.g., toxicity pathways) is derived from these new tools?

**References**


Session IX-8: Towards harmonisation in the application of alternative approaches within chemical regulation and management

Co-chairs
David Dix, EPA, USA
Bruno Hubesch, Cefic, Belgium

Session IX-8: Oral presentations

IX-8-633
Addressing residual uncertainties to enhance read-across: an Industry perspective

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Arguably the single largest challenge associated with the regulatory use of read-across is the perception of additional uncertainty arising from a hazard characterisation not based solely on substance specific toxicological data. Addressing such uncertainty holds the key to assure scientific confidence in the use of read-across without the need for new animal test information.

At the same time it is important to acknowledge the shift in focus in the field of toxicology towards the use of technologies such as high-throughput assays, high-content assays, and their associated predictive models all of which are anchored to adverse outcome pathways (AOPs). Read-across offers a convenient platform for implementing and evaluating these tools while benefitting from the potential reduction in uncertainty as a consequence of the additional information they bring. This presentation will therefore highlight cases where read-across has been used successfully and unsuccessfully to support regulatory submissions with an emphasis on what factors contributed to the ultimate acceptance/rejection of the approach. It will then consider future opportunities in terms of incorporating biological activity, high throughput/content assays, cheminformatics, AOPs and other emerging approaches to support read-across application, thus identifying and reducing uncertainty and therefore increasing the confidence in the predictions made.

IX-8-749
OECD revised guidance on grouping of chemicals

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The OECD is actively working on the development of tools and approaches to reduce or replace animal testing such as chemical categories and (Q)SAR models. The OECD would like expand the concept of grouping chemicals using different approaches of data gap filling as outlined in the revised OECD guidance for grouping of chemicals (OECD, 2014). New ways of grouping chemicals into toxicologically appropriate categories include for example grouping based on adverse outcome pathway (AOP), which is a framework for describing the events at the different levels of biological organization and other key dimensions and their causal relationship to the in vivo endpoint under consideration. In fact AOPs shift the emphasis from just intrinsic chemical activity to chemical activity plus the key events that occur across the different levels of biological organization. In this way, AOPs form a solid mechanistic reasoning to support the use of read-across and categories, thus reducing the need for toxicity testing of a substance. The presentation will outline the revised grouping guidance, including the role and application AOPs in forming chemical categories and read-across, and how the AOPs could be implemented into the OECD Toolbox.

Reference

IX-8-896
Assessment of read-across: an ECHA perspective

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Read-across data under certain conditions are accepted under REACH to address information requirements. The legal requirements are described in Annex XI (1.5) of the Regulation, specifically the results should be “adequate” for classification and/or risk assessment, have “adequate and reliable” coverage of the key parameters as in the standard test method, cover a comparable or longer exposure duration, and there must be “adequate and reliable” documentation. There is no explicit guidance to interpret what constitutes “adequate” and “reliable”, how these relate to the “acceptance” of read-across or how to deal with any uncertainty that is introduced. In an effort to address such
The 3Rs principles started to become known in China from the 1990s onwards. Although the practical implementation of the 3Rs in China has progressed more slowly compared to other regions such as Europe, significant advances have been made in the last decade. The Alternative Animal Test Group of GDCIQ has established and is using more than 10 in vitro test batteries for the toxicological assessment of cosmetics. The major governmental department AQSIQ has accepted and implemented more than 20 alternative-testing standards. The use of non-testing approaches such as (Q)SARs, TTC and read-across have also gained traction by the different researchers that is helpful to promote the new chemical substance notification legislation process. China is now in a more open attitude to engage in the development and application of alternative approaches.

This presentation will provide an overview of how alternative approaches are being applied and accepted for regulatory purposes through scientific pathway within China with particular focus on non-testing approaches such as read-across. Perspectives on the opportunities, challenges and difficulties in the use of alternatives will be highlighted.

Application and uptake of alternative methods in China: where does read-across fit?

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Session IX-9: Establishing criteria for an independent 3R-index: “Access to 3R’s”

Moderator
Herman Koëter, Orange House Partnership, Belgium

3Rs in Corporate Social Responsibility programs – possibilities for an independent 3R-Index

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Consumers, patients and animal welfare organisations ask for more openness and transparency from public and private organisations, which is in line with the new European Legislation (Directive 2010/63/EU). Smart companies and research organisations see the interesting possibilities of reduction, refinement and replacement of animal experiments as part of their Corporate Social Responsibility. A Dutch consortium has looked into the feasibility of setting up a 3R-Index as a benchmarking tool, in line with the “Access to Medicine Index” http://www.accesstomedicineindex.org. This 3R-Index will add insight into what is already possible in the field, identify gaps and new opportunities. Additionally it will identify frontrunners and recognizes the effort of industry and research organisations to improve the implementation of 3R methods. A brainstorm session with relevant stakeholders was initiated and themes and indicators for benchmarking are identified, such as 3R policy, stakeholder engagement, quality standards, capacity building and governance. It is clear that there is much that can be achieved with personal commitment and more openness of companies and research institutes. Not only for the animals, but also for science and innovations. The global 3R-Index could be a powerful incentive for industry and research institutes, as the Access to Medicine Index has already been demonstrated.
**Emulating the human vasculature in a multi-organ-chip platform**

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Our multi-organ-chip (MOC) platform contributes to the ongoing development of in vitro substance testing systems with the ultimate aim to replace animal models. It comprises two independent circulatory networks at the scale of a microscope glass slide. On-chip micro-pumps provide pulsatile circulation – enough to support microliter amounts of human tissue constructs in defined cultivation cavities. Each circuit contains 600 µl of volume, only, enabling autocrine and paracrine crosstalk through the enriched medium.

In a subsystemic testing structure, like our MOC, artificial vessels are vitally important. In particular an endothelial barrier within the chip potentially interacts with medium constituents and regulates their diffusion into subjacent tissue. Recreating in vivo conditions, first, the flow behaviour was analysed and optimised using particle image velocimetry (PIV). Upon seeding into the channel-structures human dermal microvascular endothelial cells (HDMECs) exhibit proper elongation and orientation within four days induced by the flow shear stress. The cells were dynamically cultivated for up to 40 days. Data of the colonisation and the viability of the endothelial cell layer will be presented as well as functional staining of CD31, von Willebrand factor, and VE-cadherin. Further developments to facilitate a real perfusion of introduced organoids (e.g., liver) will be addressed.

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**A 3D culture system for investigating inflammatory responses in human lung**

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Respiratory inflammatory diseases are amongst major causes of morbidity and mortality worldwide. Lung fibroblasts have been shown to play a key role in local immune responses during inflammation. However, the role of molecular pathways such as nuclear factor kappa B (NF-κB) activation in human have remained elusive partly due to the limited physiological relevance of animal models and lack of biomimetic in vitro models. We have developed a 3D culture of lung fibroblasts on porous electrospun fibres resembling human lung matrix. In order to investigate NF-κB activation in response to pro-inflammatory stimuli, we developed two detection systems based on nuclear localisation of p65 subunit and release of a soluble luciferase reporter construct. Using these systems, we can detect NF-κB activation in response to TNF-α in dose dependent manner with high sensitivity. Interestingly the 3D model remained responsive to TNF-α at higher concentration (20 ng/ml) whereas 2D culture controls reached a plateau at 10 fold lower concentration. The dynamic range of responses in 3D cultures reflects a more in vivo like physiologic receptor expression and cytokine profile. We therefore believe our 3D culture detection system provides a sensitive and biologically relevant tool for studying the regulation of NF-κB activation in lung fibroblasts.

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**In vitro skin corrosion as a pre-validation study of Reconstructed Human Epidermis in house developed**

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Currently there is a strong global trend towards the development of in vitro tests to replace the use of animals in safety evaluation tests. In Brazil, this practice is in progress and should be quickly implemented meeting the international humanitarian concepts. In the present study we developed a model of Reconstructed Human Epidermis (RHE), which consists of a differentiated three-dimensional epidermal tissue reconstructed from normal human keratinocytes in a chemically de-
fined medium and air liquid interface growth. To validate the model, it was tested for skin corrosion, following the principles of the Guide 431 (Organisation for Economic Cooperation and Development - OECD). Four substances from the list indicated in the Guide 431 were tested, two corrosive references (Lactic Acid and Octanoic Acid) and two non corrosive references (Benzylacetone and Lauric Acid), as well as positive and negative controls. The results show that three of four substances (Lauric Acid, Lactic Acid and Octanoic Acid) and the controls could be classified in the expected categories of the Guide 431. Therefore, we demonstrated the potential of our RHE model as a test method relevant and reliable and may be used for research and chemical risk assessment.

References

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X1-276 *
Biotransformation of testosterone and 2,4-toluenediamine by human skin and reconstructed tissues

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Reconstructed human skin (RHS) gains increasing interest in preclinical drug development, but as with human skin, knowledge about biotransformation capacity is rather poor although this can be highly relevant for genotoxicity and sensitization testing. We compared the metabolism of the standard compound testosterone and the industrial chemical 2,4-toluenediamine (2,4-TDA) in excised human skin, RHS (PhenionFT, EpidermFT) and undifferentiated keratinocytes and fibroblasts.

Biotransformation of radiolabeled testosterone was determined by HPLC coupled to a radiodetector and 2,4-TDA and its metabolites were quantified by HPLC-UV.

Testosterone and 2,4-TDA metabolism by RHS exceeded biotransformation in human skin, yet, the metabolite profile was close. The mono-N-acetylated derivative N-(3-Amino-4-methylphenyl)acetamide was the only metabolite of 2,4-TDA found in all test matrices and the formation ranked as: RHS > human skin ~ keratinocytes > fibroblasts.

Metabolism of testosterone and 2,4-TDA in human skin tissues is dominated by phase I and phase II reactions, respectively. Reconstructed tissues appear to be an adequate test matrix for the investigation of cutaneous metabolism of xenobiotics and thus can be used for non-clinical drug development as well as the investigation of biotransformation-related toxicological endpoints.

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X1-434 *
In vitro models for neuroimmunological diseases

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Activation of the immune system is a common hallmark of neurodegenerative diseases as Multiple Sclerosis and Alzheimer’s disease. This involves responses generated by the innate immune system within the central nervous system (CNS) and cross-talk of the CNS with cells of the peripheral adaptive immune system.

Various non-human primate (NHP) in vitro models are available to study neuroimmunological involvement in the pathogenesis of neurodegenerative diseases. However, these models are associated with considerable discomfort to the animals. We aim to complement these models and to finally reduce and replace their use by in vitro models. We have developed various NHP in vitro models including organotypic brain slice cultures, mixed glia cell cultures, dissociated primary glia cell cultures, and co-cultures of glioma cells with cells of the adaptive immune system. These cultures are all initiated from surplus brain tissue that becomes available from other experiments.

The advantages and disadvantages of NHP in vitro CNS models compared to other available in vitro models will be discussed as well as their relevance for human diseases. Furthermore, data will be presented on the use of these models as prescreening tool for in vitro drug testing and to mimic different aspects of neurodegenerative diseases in vitro.

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X1-445 *
Human in vitro adipogenesis assay for testing modulators of adipogenesis

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According to WHO, 1.6 billion adults are overweight, and at least 400 million obese, and numbers are increasing annually. Therefore, there is a lot of interest to study effects of chemicals on adipose tissue and to develop drugs that inhibit adipogenesis. However, animal models currently used to mimic human adipose tissue metabolism correlate poorly with human.

We have developed a novel human in vitro adipogenesis assay for testing the effects of chemicals on adipogenesis. Our model consists of human adipose stem cells (hASC) which are differentiated with adipose tissue extract (ATE) (Sarkanen et al., 2012), ATE is cytokine rich solution which contains the major adipocytokines and induces differentiation of hASC towards adipocytes.

The performance of the assay was tested with substances that effect adipogenesis. Evodiamine, 4-hydroxy-tempo, Bromelain, sodium-meta-arsenite, resveratrol, GW9662 and T0070907 showed strong dose-dependent adipogenesis inhibition in the assay. The anti-adipo-
The first phase of bone fracture healing is characterized by hypoxia and inflammation being susceptible for interfering effects of medications and environmental conditions leading to delayed or non-unions. However, to study the underlying mechanisms, mainly osteotomy models in rodents are enrolled (Histing et al., 2011). The insertion of fracture gaps is accompanied by stress and strain for the animals whereas the interpolation of results to human is challenging. Hence, we are developing a 3D-scaffold-free fracture model including a fracture gap filled with a simulated fracture hematoma. Therefore, we use primary human cells such as mesenchymal stem cells and clotted blood cells. To create a suitable in vitro fracture hematoma model, we studied intensively the naturally occurring hematoma in human patients and investigated them (Sarkanen et al., 2011).

**References**


* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

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**Validation of a mock circulation loop for the development of ventricular assist devices with physiological data**

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Cardiovascular devices, such as ventricular assist devices and artificial hearts, are a promising solution to address the burden of cardiovascular disease. These devices require extensive animal testing prior to clinical implementation. Mock circulation loops (MCL) are mechanical representations of the human circulatory system and are used to optimize cardiovascular devices prior to animal experimentation. This study aimed to use human data to simulate common patient scenarios in a MCL for improved in vitro cardiovascular device evaluation.

Haemodynamic parameters were measured continuously via non-invasive impedance cardiography in healthy subjects completing a Valsalva manoeuvre, during postural changes and in transitions from rest to exercise. This data was used to simulate and validate common patient states and transitions between states in the MCL.

The haemodynamic parameters obtained from humans during changes in patient state were successfully implemented in the MCL. This resulted in an accurate system capable of replicating common patient conditions and, therefore, an improved system for in vitro evaluation of cardiovascular devices.

The combination of human haemodynamic data and a MCL resulted in an accurate cardiovascular device evaluation system. This system promotes earlier optimisation of devices and a reduction in the number of animal trials required for device validation.

* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

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**A human iPSC-derived 3D model for the assessment of gene/environment interactions during neurodevelopment**

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Concerns about developmental neurotoxicity (DNT) have increased recently due to the evidences that exposure to different chemicals may contribute to neurodevelopmental disorders. Developmental neurotoxicity assessment of drugs and chemicals are costly ($1.4 million per substance) and consumes a large number of animals (mainly rats). These experiments impose medium to very severe stress on animals. Our aim is to develop a human-relevant, high quality in vitro 3D model applicable to micro-physiological systems to test chemicals/
genetic factors which affect neurodevelopment. Furthermore, combining human induced pluripotent stem cells (iPSC) from diverse genetic backgrounds (donors) makes it possible to incorporate idiosyncracies such as genetic polymorphisms into in vitro toxicity assays. Within this work, healthy and Down’s syndrome 3D models kept in culture for up to eight weeks, have been characterized by immunostaining and gene expression showing neural precursor cells (nestin), mature neurons (neurofilament, β-Tubulin III, Map2), synaptogenesis (synapsin) and astrocytes (GFAP, S100β). Here we present the applications of this emerging alternative technology and identify possible future directions of our developed in vitro 3D brain microphysiological system from iPSC.

* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

X-1-926 *

Use of reconstructed human epidermis as alternative models for efficiency studies against the penetration of organophosphates

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Topical skin protectants (TSPs) have been developed as complementary protections against the penetration of toxic chemicals as organophosphates (OPs, cholinesterase inhibitors). Their efficiency is measured by their abilities to prevent OPs penetration. The most used model is pig skin due to its relevance to human skin (Barbero and Frasch, 2009). Alternative models such as reconstructed human epidermis (REp) or artificial membranes (AM) have been developed, their permeability usually much higher than human skin (Schäfer-Korting et al., 2008; Millerioux et al., 2009). This study (1) assesses REp and AM permeability to OPs and (2) determines whether these models could be used to evaluate TSPs efficiency. Firstly, we study paraoxon penetration (model OP agent) through REp (SkinEthic RHE® 4 cm² and EpiSkin 1.07 cm²) in their inserts and AM mounted in Franz diffusion cells (1.13 cm²). Paraoxon permeability could be ranked as following: AM > SkinEthic > EpiSkin as found in literature for similar lipophilic molecules (Schäfer-Korting et al., 2008; Schmook et al., 2001). SkinEthic and EpiSkin appeared to respectively slightly over-estimate and underestimate paraoxon penetration in comparison to in vitro pig and human skin (Millerioux et al., 2009; Vallet et al., 2008). Secondly, we propose to study their abilities to evaluate TSPs efficiency. Different spreading methods were studied to determine which provide a homogenous deposit on skin surface (profilometry/optical microscopy). Correlation between deposit quality and efficiency of known TSP will be discussed.

Acknowledgments: The authors would like to acknowledge SkinEthic® for their technical advices and DGA for their support.

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* Supported by Young Scientists Travel Awards provided by ACT Germany and the German Foundation SET.

Session X-2: Cosmetics around the world

Co-chairs
Troy Seidle, Humane Society International, Canada
Yu Zhang, Humane Society International, China

Session X-2: Oral presentations

X-2-679

Ending cosmetics animal testing in the EU and beyond

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The campaign to end animal testing for cosmetics gained strength in the late 1970s. In Europe leaflets demanding an end to the Draize eye test and naming companies that test on animals were distributed to shoppers, and newspaper stories inspired the “cruelty-free” movement. The momentum was unstoppable; by 1991, when the sixth amendment to the cosmetics directive was proposed, consumers and politicians wanted action. The dossier was taken up by German MEP Dagmar Roth-Behrendt, and in 1993 the first EU deadline was set, banning the sale of animal-tested cosmetics from 1st January 1998. The ban was finally achieved on 11th March 2013, and further international regulatory changes followed. Now, in 2014, consumers worldwide are seeking change, and their voices are being heard in countries where animal protection is an emerging political concern. While the cosmetics market has expanded, compassion for animals has also grown and this is driving new partnerships, fostering regulatory and scientific advances. Humane Society International is proud to be working with scientists, regulators and companies to create a future in which animals no longer suffer to create new beauty products; we welcome the contribution made by the EU ban to the wider objective of promoting humane science.
Commitment of Government of India to make available only cruelty free cosmetics

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The Constitution of India provides that it is the fundamental duty to protect and improve the natural environment including forests, lakes, rivers and wildlife, and to have compassion for living creatures. Keeping this in view and the development of non-animal testing methods for testing safety of cosmetics, the Central Drug Standards Control Organization, in the Government of India took a landmark decision to prohibit animal testing for cosmetic ingredients, formulations and finished products in the country. Apart from this it is also in the process of making suitable legislation for stopping the import of cosmetic into India which has been animal tested in other parts of the world. India would be thus first BRICS nation where cruelty free cosmetics would only be available. In doing so, India will maintain its commitment to safeguard our consumers, and continues to support to domestic cosmetics market worth $950 million per year, and growing at 15-20% p.a. After making India cruelty free cosmetics marketplace, CDSCO would take the next commitment of minimizing the use of animals in testing of drugs, vaccines and medical devices. To this end, CDSCO has declared 2014 to be an “animal safety” year, in addition to being a human safety one.

Advancing legislation and policy in the United States to end animal use in cosmetics safety testing

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In 2014, for the first time, the United States Congress introduced federal legislation to end the use of animals for safety substantiation testing for cosmetics manufactured or sold in the country. Representing a significant portion of the overall world market, passage of such legislation would positively impact decisions being made in emerging markets and make it easier for existing markets to end animal testing.

This presentation seeks to provide an historical analysis of regulatory, corporate and other stakeholder’s work to promote the development and validation of modern testing alternatives with other industry leaders and federal regulators. It will provide rationale for federal legislation versus an administrative petition or other regulatory route. And, it will analyze why the United States has lagged behind other legislation versus an administrative petition or other regulatory route.

This presentation will articulate a pathway for world markets in officially ending the use of animals in cosmetics safety testing. Finally, the presentation will articulate a pathway for world markets in officially ending the use of animals in cosmetics safety testing. Ultimately, the presentation will articulate a pathway for world markets in officially ending the use of animals in cosmetics safety testing.
metric safety assessment. The Ministry of Science, Technology, and Innovation created in 2012 the National Network of Alternative Methods (Renama)\(^2\) aiming the implementation and validation of alternative methods, and development of laboratorial competence. The initial strategy focuses in the adoption of OECD guidelines, good laboratory practice (GLP), inter laboratory comparisons and training.

\(^2\)http://www.renama.org.br

**X-2-756**

**Introduction to the evolution in global cosmetics regulation**

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The European Union’s cosmetic animal testing and sales bans have set a world-wide precedent for cruelty-free personal care products. The regulation has created an opening to inform and engage the public and policymakers on issues surrounding alternatives to animal testing in emerging economies, or countries where animal testing has not been a topic of substantial public policy focus. Additionally, the bans have sparked significant investment in alternatives research within the EU as well as in countries such as South Korea which recently committed to investing US$145 million in developing its first Centre for Alternatives to Animal Testing.

This presentation will provide an introductory look at Humane Society International’s Be Cruelty-Free campaign – the largest campaign in the world working to extend the European precedent – and its leading role in advancing subsequent bans in India, regulatory proposals in Brazil, Australia, New Zealand and beyond, and China’s upcoming removal of its mandatory animal test requirements for domestically manufactured cosmetic products. The presentation will establish a basis of knowledge for further presentations in this workshop that will provide a variety of regional governmental, industry, and NGO stakeholder perspectives on the growing movement to end cosmetics animal testing worldwide.

**X-2-770**

**On cosmetics animal testing and be cruelty free campaign in China**

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China requires pre-market animal testing and does post market testing of cosmetics. The new regulation valid from June 30, 2014 removes the mandatory request of animal testing and allows domestic manufacturers to use alternative testing data in the pre-market registration.

Chinese scientists began the study of alternatives in 1997 but the development was quite slow. Only one alternative testing, 3T3 neutral red uptake to test phototoxicity, was validated in early 2012. The 12th National Five-year Plan included a brand new plan for the construction of cosmetics toxical alternative testing system, which aimed to validate another ten alternatives as well as the establishment of a domestic alternative validation center.

Be Cruelty Free China is working with the government regulatory agencies on the policy reform, the scientists on the promotion of alternative study and validation, and, the animal activists to educate the public and gain their support for the ban of cosmetic animal testing in China.

**X-2-772**

**South Korea’s evolving cosmetics regulations and investment in animal testing alternatives**

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The cosmetics regulatory framework in South Korea establishes different requirements “ordinary” products such as shampoos and lip-stick versus “functional” products such as sunscreens, anti-wrinkle and skin-whitening creams. Ordinary cosmetics are not subject to animal testing requirements for domestic sales, and the Ministry of Food and Drug Safety (MFDS) has announced that it will accept data from validated non-animal tests for the safety substantiation of functional cosmetics. According to the MFDS, only two new functional cosmetic ingredients have been registered in Korea in the past three years, and no notifications have been received for new ingredients for ordinary products. This suggests that there is little chemistry-based innovation, or animal testing, taking place in the Korean cosmetics market. In parallel, South Korea has dramatically enhanced its investment in in vitro testing capacity, ring-fencing 166 billion in national currency (~US$160 million) to establish Korea’s first national centre of excellence for the development and validation of alternatives to animal testing. These conditions favour policy alignment with the growing number of countries that have prohibited animal testing for cosmetics, or are moving to do so, in the interests of unobstructed trade and responsiveness to public opinion and consumer demand for cruelty-free.

**Session X-2: Poster presentation**

**X-2-758**

**The LUSH non-animal testing policy**

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Lush Cosmetics started 18 years ago with a founding principle that none of our finished products would be tested on animals and that we would not purchase from any ingredients suppliers that tested on animals. We believe animal models do not help us evaluate our products and that animal testing is cruel and unethical. For those 18 years, we have had to invent and bring products to market without any involvement with animal testing; despite this “hurdle”, we have a vibrant...
range of products which make our shops interesting and keep customers coming back for more. Along the years we have wondered why, if we can do this, are other companies are not taking the same stance. Our conclusions have been that legislation is needed to push companies to change their long engrained working practices and that science needs to be encouraged and rewarded for making the leaps forward needed to aid industry to remove animals from toxicity testing. For these reasons, Lush campaigns worldwide for governments to tighten animal testing legislation and two years ago we launched the Lush Prize – a quarter million pound annual award that recognises good animal-free methodologies and rewards the scientists developing them.

Reference

Session X-3: Special lectures

Co-chairs
Dagmar Jírová, National Institute of Public Health, Prague, Czech Republic
Horst Spielmann, FU Berlin, Germany

Session X-3: Oral presentations

X-3-572
BEMF award lecture 2014: Better science with human cell-based organ and tissue models

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The pioneer work of Dr Björn Ekwall in the field of in vitro toxicology indicated that cell cultures could be used to mimic toxic effects of chemicals in man. He formulated (1983) the "basal cytotoxicity concept" which is in good agreement with the present global vision in which the toxicity testing should be based on assessing the effects on key molecular events in critical cellular pathways (Adverse Outcome Pathways) tested with human cell-based tissue/organ models.

Today, we know that animal experiments may, in many cases, produce results with no or only limited relevance to man. The prediction ability of animal toxicity tests has shown to be around 50% (Basketter et al., 2012; Knight, 2007) or even around 20% (van Meer et al., 2012). A recent large prospective study showed that results from humanized mouse disease models may not be relevant to man at all (Junnhe et al., 2013). These are the main reasons for the poor probability of success of drug development accounting only 8% today (Arrowsmith and Miller, 2013). Therefore, one may also conclude that basic and mechanistic research performed with animals or animal cells has poor applicability to the human situation. In this presentation, some recent developments of alternative methods, testing strategies, and their challenges are highlighted.

References

X-3-640
A history of the 3Rs in toxicity testing: From Russell and Burch to 21st century toxicology

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The 3Rs – replace, reduce, refine – have become the internationally accepted framework guiding the development of alternatives to animal experimentation. In this presentation, we examine the history of the 3Rs in toxicology, the field in which this approach has arguably had the biggest impact (Stephens and Mak, 2014). Two developments help to frame our analysis, which concentrates largely on replacement. The first was the 1959 publication of Russell and Burch’s Principles of Humane Experimental Technique, which proposed the 3Rs framework. The second was the 2007 publication of the National Research Council report on Toxicity Testing in the 21st Century, following which prominent scientists began predicting the near elimination of animal use in toxicity testing through the development of “21st Century Toxicology.” We present the results of comprehensive citation and literature searches that track the influence of the 3Rs on toxicology over time. We also draw on timelines of various 3Rs activities. We gauge the impact of more than 50 years of 3Rs activity by focusing on the validation and regulatory acceptance of alternative methods and trends in animal use statistics (Stephens, 2010; Stephens et al., 2001). We conclude with a discussion of remaining challenges to the evolution of alternative methods.

References


Session X-3: Poster presentation

**X-3-175**

**Björn Ekwall memorial foundation**

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Björn Ekwall Memorial Foundation (BEMF, http://www.bemf.eu) was established 2001 by the Scandinavian Society for Cell Toxicology (SSCT) for the memory of Dr Björn Ekwall (1940-2000), an outstanding Swedish cell toxicologist and founder of SSCT. Dr Ekwall was a pioneer in the field of *in vitro* toxicology. Already in the 1970s he performed extensive studies showing that cell cultures could be used to evaluate the toxic effects of chemicals. In 1983 Dr Ekwall formulated “the basal cytotoxicity concept”, and some years later initiated an international project “Multicenter Evaluation of *in Vitro* Cytotoxicity” (MEIC, 1989-2000) with the aim of evaluating *in vitro* tests for the prediction of human acute systemic toxicity.

The main goal of the BEMF is to honour the memory of Björn Ekwall by rewarding scientists who have substantially contributed to the field of *in vitro* toxicology. During the past 13 years the Björn Ekwall Memorial Award has been given every year in connection with relevant scientific meetings. So far, 13 scientists have received the Award, among them excellent *in vitro* toxicologists from England, Finland, Germany, Spain, Sweden, and USA.

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ADDITIONAL SPEAKERS:

Irene Zhang, China
Borama Seo, South Korea
Emily McIvor, EU
Sara Amundson, United States
Helen Marston, Australia

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AltTox.org is an innovative clearinghouse for the latest technical and policy information relating to the development, validation, acceptance, and implementation of non-animal approaches to toxicity testing.

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To join the Human Toxicology Project Consortium, contact:
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Oral Presentations

Session II-5 “Discussion session: Application in decision making and testing strategies”
• “Evidence analysis in a Bayesian ITS” J Jaworska.

Session II-8 “Meeting new regulatory challenges following the cosmetic ingredients ban – Cosmetics Europe’s research programme on alternatives”
• “Systemic Toxicity Alternatives (SEURAT-1 and beyond)” C Mahony, A White, C Seaman, D Duché, D Keller, G Ouédraogo, M Dent, S Loisel-Joubert, Y Adeleye.
• “Continued developments in the Cosmetics Europe Eye Irritation Task Force strategy and programme for prediction and assessment of ocular irritancy” P McNamee, N Alépée, J Hibatallah, M Klaric, KR Mewes, U Pfannenbecker.

Session II-9 “Exposure”
• “Computational PBTK workflow to assess toxicity from in vivo dermal exposure” J Jaworska, J Troutman.
• “Extrapolation of systemic availability assessing skin absorption and epidermal and hepatic metabolism of aromatic amine hair dyes in vitro” C Goebel, J Manwaring, H Rothe, C Obringer, D Foltz, T Baker.

Session II-10a “Topical Toxicity – Skin”
• “Use of HPLC/UPLC-photometry for detection of formazan in in vitro RhT-based test methods to expand their applicability to strongly coloured test substances: A Cosmetics Europe Study” N Alépée, J Barroso, A De Smedt, B De Wever, J Hibatallah, M Klaric, KR Mewes, M Millet, U Pfannenbecker, M Tailhardat, M Templier, P McNamee.

Session II-10b “Topical Toxicity – Eye”
• “EURL ECVAM - Cosmetics Europe prospective validation study of Reconstructed human Tissue-based test methods for serious eye damage/eye irritation testing” J Barroso, N Alépée, T Cole, C Eskes, SJ Freeman, R Liška, P McNamee, U Pfannenbecker, AA Reus, CM Rubingh, MW Schaeffer and V Zuang.

Session II-11 “Repeated dose toxicity”
• “Cheminformatic Approaches for Analog-based Toxicity Assessments” C Mahony, S Wu, K Blackburn, G Daston.

Session II-12a “Skin sensitization”

Session II-15 “Genotoxicity/Carcinogenicity”
• “Impact of OECD guideline revisions and availability of reconstructed human skin-based methods on animal use in genotoxicity testing” S Pfuhler.

Session IV-5 “Intellectual property, data sharing and data ownership”
• “P&G’s approach to data sharing for the Seurat-1 COSMOS project” C Mahony, P Gallagher.

P&G as Presenters and/or Co-Authors underlined
Poster Presentations

Session I-4 “Novel 3D models”

Session II-5 “Discussion session: Application in decision making and testing strategies”
- “Use of the in vitro Caco-2 assay to predict the oral absorption of aromatic amine permanent hair dyes” H Rothe, C Obringer, C Goebel, J Manwaring.

Session II-9 “Exposure”

Session II-10 “Topical Toxicity”
- “The importance of understanding drivers of irritation in vivo for selection of chemicals used in the development and evaluation of in vitro serious eye damage/eye irritation assays: Cosmetics Europe analysis” P McNamee, N Alépée, J Barroso, A De Smedt, B De WEver, J Hibatallah, M Klaric, KR Mewes, M Millet, U Pfannenbecker, M Tailhardat, M Templier.
- “Tiered approach to the use of alternatives to animal testing for evaluation of eye irritation potential of cosmetic ingredients: hair dye case study” P McNamee, G vonBoelcshazy, C Goebel.
- “Suitability of histopathology as an additional endpoint to the isolated Chicken Eye test for classification of non-extreme pH detergent and cleaning products” E Cazelle, C Eskes, M Hermann, P Jones, P McNamee, M Prinsen, H Taylor, M Wijnands.

Session II-12 “Skin sensitization”
- “Refinement of the peroxidase peptide reactivity assay (PPRA) and prediction model” C Goebel, JA Troutman, HJ Dai, RLM Dobson, M Quijano, GF Gerberick.
- “Estimation of the skin sensitizing potency based on peptide reactivity, dendritic cell activation and read-across using the hair dye 2-methoxymethyl- paraphenylenediamine as example” C Goebel, J Troutman, J Hennen, H Rothe, H Schlatter, GF Gerberick, B Blomeke.

Session II-15 “Genotoxicity/Carcinogenicity”
- “RSMN (Reconstructed Skin Micronucleus Assay): Update on the ongoing validation” S Pfuhler.
- “Reduction of misleading (“False”) positive results in mammalian cell genotoxicity assays and its favourable impact on animal use” P Fowler, K Williams, L Jeffrey, J Young, NJ Hewitt, R Fautz, D Kirkland, P Carmichael, S Pfuhler.

Session VI-4 “Absorption, distribution, metabolism and excretion (ADME)”

P&G Congress Attendees
Carsten Goebel, Joanna Jaworska, Catherine Mahony, Pauline McNamee, Stefan Pfuhler, Kathy Rogerson, Helga Rothe, Harald Schlatter.

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JSAAE is a scientific organization that supports research, development of alternative methods, and education of animal welfare. The society also promote an acceptance of the Three Rs as guiding principles for the humane use of animals in scientific testing. Since its establishment in 1989, JSAAE has been collaborating with local/regional/global key stakeholders. The society hosted WC6 at Tokyo, Japan in 2007 and leaded the successful meeting. We will continue our contribution to drive “Humane Science”.

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