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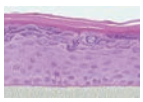

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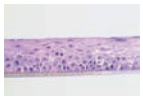

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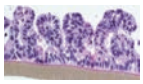

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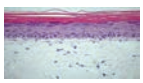

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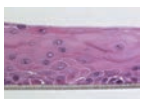

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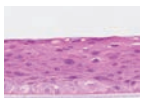

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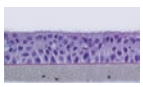

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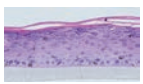

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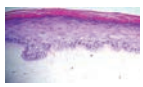

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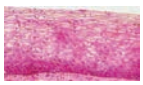
Feminine Product Irritation, Microbicide Testing, Immuno-competent, HIV-1 Infection Sexually Transmitted Infection, Inflammation, Vaginal Drug Delivery …



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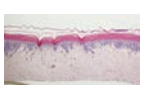

PRIMARY CELLS & MEDIA FROM USA
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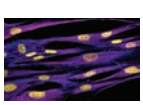

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Welcome address EUSAAT 2018

Dear friends and colleagues,

on behalf of EUSAAT, the European Society for Alternatives to Animal Testing, we welcome you to the 21st European Congress on Alternatives to Animal Testing & the 18th EUSAAT 3Rs congress in Linz, Austria on September 23-26, 2018.

During the past two decades the "Linz-Congress" has emerged in Europe as the major scientific event in the field of the 3Rs and in the year 2018 the EUSAAT 2018 3Rs Congress Linz will be the largest international 3Rs congress. We are particularly pleased that our international partner societies from China, CCARE, the Chinese Society for Alternatives to Animal Experiments, and from the USA, ASCCT, the American Society for Cellular and Computational Toxicology, are actively participating in our session on "International Progress in 3Rs Research".

EUSAAT 2018 is hosting oral and poster presentations to facilitate discussions and the exchange of ideas for the benefit of alternative methods to animal experiments. The Scientific Committee has identified the most important subjects related to the 3Rs that are currently of interest to scientists in Europe. The EUSAAT 2018 conference focuses in oral and poster sessions on Refinement and Reduction (e.g. animal welfare, education) as well as Replacement topics (e.g. disease models, non-animal tools for basic biomedical research, toxicity studies, omics techniques, advanced 3D models including recent progress in developing human-organ-chips).

After the success in 2016, this year we will host three round table discussions on topics that are of particular interest to EUSAAT members. The first on "International Progress in 3Rs Research", the second on "3R centers in Europe – National and Local Centers" and the third on "Applying human 3D models and Multi-Organ-Chips (MOCs) in Industry".

We are grateful to all speakers for contributing to the training workshop "EUSAAT - Kirkstall Quasi Vivo® Training Workshop" and in particular to the company Kirkstall for the organization of this workshop, which is open to all participants of the EUSAAT 2018 congress.

In particular, we are very proud that we are able to continue the "Young Scientists Travel Award (YSTA)" program in 2018 and want to thank the SET Foundation for their generous funding. The YSTA program provides about 20 young scientists with the opportunity to share their ideas on the implementation of the 3Rs in education and novel test systems to reduce the suffering as well as the number of animals in research.

The EUSAAT Board is pleased that the number of sponsors of the EUSAAT congresses has increased over the years, since without their continuous support we would not be able to maintain the high scientific standard and to keep the congress fee low. Both elements are equally important in order to attract young scientists from all over Europe and beyond. Therefore, the EUSAAT Board and Scientific Committee thank all of the sponsors of EUSAAT 2018 on behalf of the participants.

Moreover, we are also pleased that the abstracts of the EUSAAT 2018 conference on the 3Rs are again published in ALTEX Proceedings and we thank the editor and CEO of ALTEX Edition for helping us to pave the way for future cooperation for the benefit of implementing the 3Rs in Europe.

We finally want to thank our colleagues on the EUSAAT Board and the Scientific Committee for their continuous support in planning the EUSAAT 2018 congress.

Horst Spielmann	Winfried Neuhaus	Dagmar Jirova	Dominik Rünzler
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Young Scientists Travel Awards (YSTA)

Kevin Achberger
Laura Behm
Aurore Bourguignon
Giuliano Mario Corte
Alexandra Damerau
Carolin Drieschner
Marco Franco
Stefanie Fuchs
Justyna Gogola
Michelle Hesler
Katharina Hörst
Melanie Humpenöder
Meik Kunz
Anna Löwa
Beatriz Moreira
Roberta Nossa
Richard Paštěka
Sara Tirendi
Lena Vogt
Paul Warncke

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Towards implementation of the 3Rs in basic research and teaching in Italy: Centro 3R

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The role of universities is to provide a nurturing and liberal environment for teaching and research, as such they represent an ideal place for the definition, implementation and the promotion of the 3Rs. The first of its kind in Europe, the recently established Italian Interuniversity Center for the Promotion of the 3Rs Principles in Teach and Research (Centro 3R) was set up to meet this objective. Its mission is to promote rational and scientific thinking in experimental science through a multi-disciplinary teaching and research approach inclusive of all 3Rs as a means to accelerate the R of replacement.

Specifically, the Centro 3R will develop courses and credits on the animal care and welfare and advanced *in-vitro* methods, integrating them into bachelors and masters curricula. In the research field the Center is committed to the development of innovative methods for the implementation of the 3Rs as well as knowledge and resource sharing.

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3D epithelial culture model to study Chlamydia infection

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Chlamydia trachomatis is a Gram-negative obligate intracellular bacterial pathogen. It is the leading cause of bacterial sexually transmitted disease in the world, with more than 100 million new cases of genital tract infections with *C. trachomatis* annually. Mice are the most commonly used animals to study genital chlamydial infections. However, mice model used to study genital tract infections with Chlamydia does not perfectly mimics the anatomy and endocrinology of the human reproductive tract. Also, the pathogenesis and immune responses occurring during a human genital *C. trachomatis* infection differ from the mice counterparts.

3D epithelial cell culture has allowed the expansion of mini organ-like structures *in vitro*; this includes the 3D organoids as well as the primary cells culture; air-liquid-interface model. Today, 3D models resemble a wide range of organs and exhibit remarkable similarity to their counterparts *in vivo*. This important endeavor now provides us with new opportunities to not only explore infection processes more accurately, but also to reproduce many aspects of infection biology *in vitro*, which was not previously possible. 3D model represents an attractive tool to study the infectious diseases, including Chlamydia because current animal models do not faithfully recapitulate human pathology.

As *C. trachomatis* is the main causative agent of tubal infertility associated with salpingitis and scarring caused by complicated ascending infections. Growth conditions for long-term 3D organoids of fallopian tube epithelial cells obtained from clinical samples have been established. The system is based on maintenance of adult stem cells through supplementation with appropriate growth factors. Infection of 3D organoids with *C. trachomatis* turned out to be successful and within the organoids, Chlamydia completes its infectious cycle and induces the c-Myc oncogene via activation of PI3K. Activation of oncogenes promotes cell survival and metabolic reprogramming. These findings identify a previously unknown pathway activated by Chlamydia infection, which exhibits pro-carcinogenic features. To further improve this infection model, we plated the organoids derived cells on filters of a 2D chamber air-liquid-interface model. Under these conditions, the fallopian tube epithelial cells acquired typical polarization features, differentiated to fallopian ciliated cells and secretory cells. Continuous growth and differentiation depend on both Wnt and Notch paracrine signaling. Finally, by infecting these monolayers with *C. trachomatis* we could demonstrate that Chlamydia can replicate inside the monolayer and persist for several days. Importantly, the air-liquid-interface secretory cells produces mucus which might act as a physical barrier against the infection, which was not demonstrated using other infectious models. Ultimately, 3D model promises to be a versatile tool to study infectious diseases and pathogenesis. Further, air-liquid-interface also allows assessment of molecular communication of epithelial cells in co-culture with other cell types, such as stromal cells and immune cells.

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Modelling ischemic stroke injury at the blood-brain barrier *in vitro* using induced pluripotent stem cells

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Stroke constitutes the 5th cause of death and a leading cause of disability in the United States. Ischemic stroke constitutes the majority of stroke events. It is characterized by the occlusion of one or several cerebral arterioles by a thrombus, resulting into the onset of a severe hypoxic/ischemic injury. Such hypoxic/ischemic stress triggers the opening of the blood-brain barrier (BBB) and contributes to neuronal cell death by excitotoxicity and by brain edema. In addition to its opening during stroke injury, the BBB undergoes a second opening during the first 72 hours of reperfusion, compromising patient's recovery. In addition, sex-dependent and age-dependent factors greatly influence patients' outcome. Yet, the presence of such dimorphism at the human BBB remains unclear. Therefore, a better understanding of the cellular and molecular mechanisms by which hypoxia/ischemia induces the BBB disruption as well as its recovery during reperfusion is essential to improve the brain recovery following stroke injury.

In this study, we examined the effect of biological sex and canonical WNT signaling pathway on the blood-brain barrier response to ischemia/reperfusion injury *in vitro* using an oxygen-glucose deprivation(OGD) / reoxygenation injury using patient induced pluripotent stem cells (iPSCs). Prolonged OGD stress (24 hours) resulted in major BBB disruption in all iPSC-derived BMECs monocultures. However, acute OGD stress (6 hours) followed by reoxygenation showed differences between males and females. Females BMECs showed a worsened outcome on the barrier function compared to male BMECs, both during OGD and during reoxygenation phase. Although no differences in stabilization in the hypoxia-inducible factor-1alpha (HIF-1alpha) were noted in OGD, we noted a significant difference in VEGF secretion both during OGD and during reoxygenation, as well as a difference in responses of astrocytes/BMECs co-cultures between males and females. Furthermore, preliminary data suggest a differential effect of the activation of the canonical WNT pathway on the barrier function during OGD and reoxygenation stress.

Taken together, our data supports the hypothesis of a sex-dependent response of the BBB to ischemic stroke injury and raises the possible contribution of HIF-dependent and independent mechanisms in such dimorphism

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Introduction of the Core Facility Alternative Biomodels & Preclinical Imaging

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The goals of our Core Facility are the establishment and use of adequate human- and animal-derived cell culture models as well as the integration of preclinical imaging techniques into ongoing projects in order to develop possible alternative methods and subsequently reduce the number of animals in the trial while at the same time increasing the information gain. Various instruments and methods combine *in vivo*, *ex vivo* and *in vitro* information on pathology and physiology.

In vitro experiments in different cell systems reduce and replace experiments on animals. We provide the *CellBank Graz*, which offers a variety of human and animal-derived cell lines to researches at the Medical University of Graz. We complete our services by offering different assays, like the detection and elimination of mycoplasma and Short Tandem Repeat analysis to ensure the quality of the cell lines. To assist research questions we also perform assays for toxicity (MTS, ATP, LDH assays), proliferation and migration on request. Our liquid handler instruments like *Multidrop* and *Versette* ensure high throughput screenings. Migration and invasion, for example in wound healing processes or metastasis of cancer cells, can be monitored in real time using our *xCELLigence* system.

Our Core Facility focusses on the establishment of adequate cell culture models of rare cancers like chordoma, glioblastoma, sarcomas and their surrounding tissues. Due to the proximity to the University Hospital Graz, the cooperation between the various disciplines and the Comprehensive Cancer Center Graz a rapid isolation of the cells, a reduction of the ischemic time and comprehensive know-how is ensured. The cultivation of cells in a microstructured three-dimensional cell culture with different cell types reflects the *in vivo* situation and is optimal for the testing of chemotherapeutic agents. 3D tumor models in different matrices and scaffolds as well as embedding and staining protocols are offered. To keep cells under dynamic conditions, again reflecting the *in vivo* situation, the cells are constantly covered with medium. At the Core Facility, the *Kirkstall System* is used as dynamic system.

Furthermore, our Core Facility engages in closing the gap between biomedical research and non-invasive *in vivo* imaging. Currently we have micro-computed tomography, micro Ultrasounds and fluorescence based optical imaging available. *Micro-computed tomography* (Siemens) is a very high-

resolution, non-invasive radiographic imaging technique that can be used for both pathological and physiological analysis as well as material analysis. Thanks to well-developed contrast agents, it is now possible not only to visualize bones but also to examine soft tissues. The ultrasound system *Vevo3100* by VisualSonics is our newest purchase, made possible by a cooperation of the MUG, KF and TU Graz. Using this non-invasive *in vivo* imaging technology, the monitoring of long-term studies as well as imaging guided applications helps reducing the number of animals necessary. The *CRi Maestro Imaging System* is a cost-effective variant for fluorescence-based *in vivo* imaging. Features of this system include multispectral data acquisition, data analysis, enhanced autofluorescence sensitivity, and qualitative analysis for fluorophores.

Our team is happily available for individual questions about project planning and project implementation.

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Extracellular vesicles take part in cellular communication of Chordoma cells and can be detected using various methods

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Chordoma are slowly growing malignancies, arising from remnants of the notochord, which can be located all over the spine and belong to bone tumors. The exact molecular pathogenesis is yet to be elucidated. The cells of chordoma present variable amounts of the so-called physaliphorous cells, which contain one or several intracytoplasmic vacuoles, creating a bubbly appearance. Due to the complicated location, the usual treatment of resection and radiation is often insufficient and leads to tumour recurrence.

Extracellular vesicles (EVs) have been shown to be released by various cancer entities. These vesicles have the same topology as their parental cells and have been shown in several studies to be capable of delivering nucleic acids, such as specific miRNAs or DNA fragments, as well as proteins, like tetraspanins and growth factors, to recipient cells. Due to the delivery of bioactive molecules, EVs are thus involved into several cellular processes like oncogenic transformation, invasion, migration and angiogenesis.

We hypothesize that EVs are released by chordoma cells, play a major role in tumor progression and might be a new option for targeted therapy. Patient derived chordoma cell lines were transduced stably by red fluorescent protein and green fluorescent protein for EV visualization and cultured under normal and hypoxia conditions. Conditioned cell culture media (CCM), in which cells have been cultured, was processed and analyzed. All used media, buffers, antibodies and fluorescent dyes have undergone preparation with the purpose of excluding particles and aggregates. Analysis of CCMs using flow cytometry, nanoparticle tracking analysis, proliferation assays, microscopy and negative staining compared to plain cell culture media, in which no cells have been cultured, clearly demonstrated the existence of a range of different EVs.

To our knowledge communication via EVs and chordoma cells has not been investigated so far. The data gained to this point indicates that chordoma cells produce extracellular vesicles for cellular communication. Additional experiments aim to determine the influence of further different culturing conditions (confluency, inhibiting and stimulating supplements) on the production rate of EVs and aim to reveal the underlying conditions and mechanisms for release of EVs. Additionally the effect of stimulation/inhibition of co-cultivating chordoma cell lines together with a spontaneously occurred lymphoblastoid B-cell line from the same patient will be investigated. Moreover the characterisation of biomolecules being transported by EVs will be targeted, thus aiming to reveal a possible innovative therapy.

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Stem cell-based human blood-brain barrier co-culture models for pre-clinical development

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Introduction

In vitro models of the blood-brain barrier (BBB) are very strong tools in drug development and to study physiological as well as pathophysiological mechanisms. In order to closely mimic the BBB *in vivo* and to optimize model characteristics, we analyzed a set of different BBB co-culture models based on primary cells, human induced pluripotent stem cells (hiPSCs) and multipotent fetal neural stem cells (NSCs). The use of hiPSCs allows a very standardized, robust and reproducible model establishment. Further, they pose an effective cell source to generate functional brain cells and have the advantage to be independent of postnatal brain tissue biopsies, including their variations and limitations during *in vitro* culture.

Objectives

The aim of this study was to systematically investigate the individual impact of important BBB niche cell types (i.e. astrocytes, pericytes, NSCs) on hiPSC-derived BBB endothelial cell (EC) function and gene expression.

Materials and methods

We used standardized methods to differentiate the BBB cell types from hiPSCs as well as human multipotent stem cells. Differentiation protocols to generate brain endothelial cells as well as human astrocytes from iPSCs and NSCs mimicking the *in vivo* embryogenesis are performed as described recently. The differentiated cells were characterized by immunohistochemistry and qPCR analysis. Ten different BBB-(co)-culture setups were performed and characterized by proving the cell morphology and functionality, analyzing the gene and protein expression, ultrastructural analyses, measurement of the trans-endothelial electrical resistance (TEER) and drug permeability using reference substances.

Results

We were able to present a quadruple BBB culture model with improved BBB characteristics compared to the monoculture and most of the co-cultures. TEER up to 2500 Ω*cm^2 was achieved and at least 1.5-fold up regulation of characteristic BBB genes. Further, the important BBB related TJ proteins, CLDN1, CLDN3, CLDN4, CLDN5 could be analyzed at protein and gene level as well as by freeze fracturing technique. Moreover, the functionality of these proteins was investigated.

Conclusion

In conclusion, we were able to present a method for complex BBB quadruple model generation with *in vivo* like characteristics, which can be used in future drug development and infection studies for instance.

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Establishment of *in vitro* exposure system for safety study of theatrical fogs

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Theatrical fogs have been widely used to generate special effects and make lighting visible in the entertainment industry. Previous studies conducted in workers of entertainment industry demonstrated that exposure to theatrical fogs related to mucous membrane irritation and respiratory symptoms in workers; however, there was unclear regarding the effects on theatrical fogs on human health. In this study, we focused on investigation of the usefulness of the *in vitro* exposure system as alternative method to human study to evaluate the biological effects of theatrical fogs. To mimic the possible exposure of theatrical fogs, air liquid interface culture exposure (ALICE) of human bronchial epithelial A549 cells and reconstructed human epidermis (RhE) model were used as the *in vitro* model of lung exposure and skin exposure, respectively. Regarding the respiratory system, we generated the *in vitro* exposure system of inhalation exposure by incorporation ALICE with the fog generator to mimic real situation of human exposure in entertainment industry. The results of *in vitro* exposure system of inhalation exposure demonstrated that some theatrical fogs could induce cellular damage at 10 min exposure at flow rate of 5 mL/min and higher exposure time at 30 min caused more severity of cellular damage. In addition to respiratory system, the results of skin exposure using RhE model indicated that solutions of commercial theatrical fog used in this study did not cause skin irritation following OECD TG 439. The potential of theatrical fogs to induce skin sensitization was evaluated by h-CLAT assay following OECD TG 442E. The results showed that all solutions of theatrical fogs caused increasing of CD54 and CD86 expression higher than 200% and 150%, respectively, suggesting the potential to induce skin sensitization. The chemical compositions in commercial theatrical fogs were characterized to identify the factors related to respiratory symptoms in workers in entertainment industry. Our results indicated that most of theatrical fog solutions were mainly comprised of glycols, while another was mainly comprised of glycerin. Taken together, our results using *in vitro* exposure models indicated that theatrical fogs has possibility to induce respiratory symptoms and skin sensitization. Furthermore, our results suggested the potential uses of *in vitro* exposure system as an alternative model to human study for investigation of the safety and biological effects of theatrical fogs *in vitro*.

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Using mechanisms of toxic action to classify and predict ester ecotoxicity

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Even though esters are often used and released into the environment, little is known about their mechanisms of action in relation to their toxicity. Most of the simple esters are considered to exert a specific narcosis, while some other esters can exert toxicity related to their potential reactivity. Therefore, the critical step, before predicting the toxicity of an ester, is to determine its mechanism of toxic action (MechoA). For this purpose the classification of Bauer et al. (2018) is used in combination with an accurate modelling approach which is derived from empirical data specific to the MechoA.

The acute toxicity of esters to aquatic flora and fauna may be regressed against a hydrophobicity descriptor (i.e. log K_{OW} or water solubility), and compared with similar regressions for non-polar narcotics. The similarity between these regressions confirms non-reactive esters are simple narcotic compounds for algae but not for fish or daphnids. For the animal species, the regressions for esters are not the same as for narcotic compounds. The most likely explanation for this difference is the balance between hydrolysis rate and the toxic action of the parent and the degradation products. That is why they are considered as pronarcotics. A toxicity to algae in line with a narcotic mode of action suggests that the enzyme responsible for hydrolysis found in fish and daphnids is absent and therefore hydrolytic activity of esters by algae is negligible. Thus, di-esters appear more toxic than mono-esters for fish and daphnids because they can produce two times more metabolite than mono-esters.

The more reactive esters are usually unsaturated, like allyl/vinyl-esters and alpha,beta-unsaturated esters, whose double bond can be activated by the carbonyl group. For these compounds the substitution around the double bond plays a decisive role in effective reactivity. Thus, methacrylates which have an alkyl substituent in position alpha of the carbonyl are not more toxic than aliphatic esters for any of the three aquatic species. On the other hand, Acrylates clearly express excess toxicity and have to be considered as acting through yet another mechanism of action relating to a model dedicated to soft electrophiles.

Rather than model the toxicity only according to structural analogy, a modelling approach is used to develop QSARs for esters based on three pillars: structure, mechanism and species metabolism.

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Microfluidic engineering of vascular networks by growth factor gradient control

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Recreating vasculature for tissue engineering *in vitro* remains vital as non-perfusable tissue constructs are restricted in nutrient supply and waste removal. Microfluidic devices have emerged as an integral tool to mimic both physiologic and pathologic cellular microenvironments. But while currently employed vasculature-on-chip models are routinely used to investigate the effect of nutrient gradients [1] or interstitial flow [2] on endothelial cell sprouting, limited research elucidates the influence of these factors on physiologic co-cultures of endothelial cells and supporting mural cells. Here, we show that not only is microfluidics ideally suited to create and monitor spatiotemporal gradients in three-dimensional hydrogel cell cultures but also that growth factor supply and elution as well as type of flow regime greatly affect vascular network formation in a co-culture model of human adipose-derived stem/stromal cells and human umbilical vein endothelial cells. In a static model without flow, vascular networks showed a dependence on nutrient diffusion, as network parameters peaked at distance 2 millimeters from the nutrient supply and subsequently declined. In contrast, application of indirect flow enhanced endothelial cell sprouting due to increased delivery of nutrients into the construct, but failed to initiate the formation of mature vascular networks. By comparing vessel parameters, we found that dynamic cultivation increased sprouting activity but failed to produce vascular networks with similar vessel area coverage and average vessel length as static cultures. Assisted by finite volume CFD simulations of distribution of differently sized molecules, we found that while indirect interstitial flow in the medium channel enhances HUVEC sprouting activity, it attenuates vascular network formation due to increased growth factors elution.

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European institutions still need to close the door on animal-tested cosmetics

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The EU Cosmetics Regulation established an important precedent worldwide for assessing the human health risks of cosmetics products and ingredients with the explicit exclusion of animal-derived test data. The regulation bans not only the direct testing of cosmetics products and ingredients on animals but also the marketing of such substances that rely on the results of animal studies for toxicity assessment purposes.

The cosmetics testing and marketing bans continue to be of great political and ethical significance, as evidenced earlier this year when an overwhelming majority of MEPs voted in favour of working towards a global ban on animal testing for cosmetics [1]. Whilst the non-binding resolution voted for by MEPs is a commendable step forward, further work is required to close the loopholes that still allow animal-tested cosmetics ingredients to creep in through the back door here in Europe. Under the guise of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation, the European Commission and the European Chemicals Agency (ECHA) permit tests on animals for cosmetics ingredients under certain circumstances [2]. The position taken by the Commission and ECHA regarding the interplay between REACH and the Cosmetics Regulation is fundamentally flawed. Not only does their stance contradict both the intent and value of the two regulations, they are also at odds with the reasoning and positions set forth in recent administrative and judicial decisions.

In this presentation, we will discuss how testing decisions adopted by ECHA for ingredients used exclusively in cosmetics products may affect the ability of cosmetics manufacturers and distributors to place a product on the market in the EU, drawing on recent cases from the Court of Justice of the European Union, the ECHA Board of Appeal, and the European Ombudsman.

As consumers around the world are increasingly demanding cruelty-free products and political will is pushing forward cruelty-free industry, it is vital that European institutions ensure that cosmetics ingredients manufactured or marketed in Europe are never tested on animals under any circumstances, anywhere in the world.

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8

The Nonclinical Innovation and Patient Safety Initiative (NIPSI): Supporting human-based nonclinical approaches through advances in law, policy, education and training in the United States

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The integration of modern and state-of-the-art nonclinical approaches for assessing drug safety, efficacy and disease modeling are necessary to improve drug development.

Recent advances in the United States, such as the Food and Drug Administration's (FDA) Predictive Toxicology Roadmap and the Interagency Coordinating Committee on the Validation of Alternative Methods' (ICCVAM) Strategic Roadmap for

Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States, represent a fundamental shift in how drugs will be developed and regulated. Regulators now state the need to move away from animal testing towards methods that can be expected to be more relevant to humans because they are based on human biology and physiology, and have mapped their plans for doing so. The Nonclinical Innovation and Patient Safety Initiative (NIPSI) formed to foster stakeholder collaboration – among federal agencies, the private sector, and patient, health and research organizations – that supports innovative human-based science and addresses the factors that impede the uptake of modern, nonanimal approaches. Stakeholders initially met at a full-day NIPSI roundtable in January 2017 in Washington, D.C., then a growing group met during ancillary meetings of the Society of Toxicology Annual Meetings in March 2017 and March 2018.

This presentation outlines NIPSI recommendations for advancing the uptake of human-relevant nonclinical approaches. One project aims to change current United States Food and Drug Administration (FDA) regulations that require animal data, to reflect FDA's discretion to accept human-based nonclinical approaches in investigational new drug applications (IND) and new drug applications (NDA). This is necessary for ensuring FDA regulations at minimum keep pace with innovative science. Another project involves lobbying the United States Congress to increase funding allocated for human-based science. The presentation will also include results from a recent review of NDAs that was conducted to determine if the FDA has received data from organ chips in NDAs.

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Computational methods for the prediction of chemical toxicity

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To foresee future, the past must be consulted

Humans in the modern society can be exposed to a large number of chemicals through several different routes on a regular basis. These chemicals are derived from various sources, such as food and drink, use of pesticides in food production, prescription of pharmaceuticals, application of cosmetic and personal products, use of detergents and washing agents, clothes, and paints in addition to exposure in occupational settings. Thus, in order to control risks to mankind, the toxic effects associated with exposure to chemicals, should be assessed in advance. On the other hand, poor pharmacokinetics, side effects and compound toxicity are not only frequent causes of late-stage failures in drug development but also a source of unnecessary animal tests. In the past decade, computer-(*in silico*-)based models have matured into powerful tools for simulating and quantifying biochemical processes at a molecular level and nowadays, they are routinely used in the early stages of drug development. In the context of the REACH (Registration, Evaluation and Authorization of Chemicals) initiative of the European Union, computer-based models have received additional attention as they can predict the toxic potential of the existing and virtual compounds.

Here, we present ProTox-II which incorporates molecular similarity for acute toxicity prediction, pharmacophore-based models for 15 toxicity targets, fragment propensities and machine learning models for 17 different toxicity end points. To the best of our knowledge, ProTox-II is the freely available computational toxicity webserver enabling the prediction of the largest number of toxicity endpoints consisting of 33 models. A novelty of the updated ProTox-II webserver is that the prediction scheme is classified into different levels of toxicity such oral toxicity, organ toxicity (hepatotoxicity), toxicological endpoints (such as mutagenicity, carcinotoxicity, cytotoxicity and immunotoxicity), toxicological pathways (AOPs) and toxicity targets thereby providing insights into the possible molecular mechanism behind such toxic response.

We believe that in the process of toxicity analysis extending to drug discovery, ProTox-II *in silico* prediction platform will help to initiate focused experimental follow-up studies and to enhance hit selection and lead optimization process. Additionally, ProTox-II methods have the potential to support risk assessments for regulatory decisions such as to create novel hypotheses and get insights to the mechanisms of toxicity.

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Training and teaching in substitutive approaches to animal testing: The commitment of Italian Academia

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Today there is a pressing need to highlight the 3Rs concept to foster innovative technologies and encourage approaches, which respond more effectively to the public's demand for the replacement of animal methods. Moreover, there is also a scientific quest to identify toxicological risks for humans, animals and environment, more accurately. To accelerate the process of replacement of animals in biomedical research, education, and regulatory testing, many countries have already established institutions dedicated to the development of alternatives to animal testing. In this regard, it is necessary to establish academic degree programs in Animal Replacement Science to train the next generation of scientists, ethicists, and policy makers. The fundamental commitment of Academia is education and training, so it is in this framework that we should educate future scientists, giving them the means and impetus to develop new and appropriate experimental methods. Every researcher should know about relevant alternatives, as well as about the scientific limitations of both animal approaches and non-animal ones. In this context, it is also important to keep in mind that the lectures and training are to perform by scientists, regulators.

To address these issues, LARF, at the University of Genova, has been engaged in the organization of theoretical and training courses on non-animal methods since 2008. In 2013, the LARF team won the international Lush Prize for Training Category. The annual 2-day course is open to 24 participants, including experts in *in-vitro* methods, as undergraduate students. Thanks to over twenty years of experience in various fields of pathology and teaching, the LARF staff is able to offer their knowledge and expertise in all aspects of *in-vitro* technology. To date 8 editions of the Basic and 4 of the Advanced course are focused on a practical part and demonstrations/lessons from specialists from the academic world and leader companies in technologies to supply basic knowledge, update on innovative *in vitro* models and technologies, implementation of regulatory issues, adverse outcome pathway analytical construct, and so on. Furthermore, the experts offer hands-on experience to each participant in specific practical training modules.

Moreover, in LARF laboratory several graduating and PhD students elaborate their degree thesis on *in vitro* models and every year 20 h training modules on *in vitro* toxicology are performed for graduating students in Biology, Medicine and Surgery, as for graduates, as a voluntary stage.

Dissemination of 3R-knowledge ensures best possible practice for a predictive and reliable toxicology, by performing and optimizing 2D/3D *in vitro* models based on human cells to evaluate the health hazard.

In this context, in January 2018, the Centro3R, the first Italian Interuniversitary Center for the Promotion of the Principles of the 3Rs in Teaching and Research, was set up. Its main objectives are to promote the establishment of courses focused on the 3Rs within the core curricula of scientific degree programs; to create an open-platform to offer teaching resources on 3Rs principles; and to organize courses, seminars, and meetings within various scientific disciplines.

Establishing training programs around the world makes a huge difference to progress.

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104

Towards animal-free methods in education and training: Lessons from healthcare

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Directive 2010/63/EU mandates that animals cannot be used when animal-free methods are available for the same purpose. The use of animals for educational and training purposes in the EU is lower than for other areas of research and testing. However, they are still used under different justifications. The impact of using animals as educational and training tools has been known to be negative for students' development and their ethical attitudes towards animal lives [1,2]. Additionally, in disciplines where once animals were normally used educators have found different methods to pass on their knowledge to students and trainees.

Non-technical summaries from different EU countries of projects that use animals with the primary purpose of education and training have been analysed. Three cases were selected for their distinct relevance to qualify the type of scenarios where animals are still considered necessary in education and training. Justifications of such necessity include the belief on a higher gain to students despite alternative methods are known to be available, lack of knowledge about alternative methods, and new interventions for which educational methods may not yet have been developed. These different scenarios present distinct challenges.

Even though animals are still used for medical education, many faculties and hospitals have moved away from this practice and implemented animal-free curricula and courses from undergraduate to continuous skill training. Simulation-based education and training has facilitated a learning and training methodology that is based on a patient-centred approach. This approach evolved from a growing social concern about using patients as training subjects for medical trainees. The growing concern about the use of animals in experiments [3], urges the need to challenge our application of the 3Rs principles and to put forward concrete stepwise strategies to phase out the use of animals in science, and in particular in education and training. To that end, simulation-based and animal-free methodologies can be adapted to the fields of veterinarian and other disciplines where animals are used in a clinical or research practice. By having an animal-centred approach, the protection of animals' lives can be at the core of the educational and training methodology.

However, the growing investment in research as led to fast developments of new technologies and methods. Rapid scientific developments pose challenges for the continuous adaptation of education and training of new researchers, veterinarians, and clinicians. Fostering stable relationships between academia and industry, with strategic thinking by (clinical/veterinarian) researchers about translation into practice, can aid a prompt adaptation of curricula to new scientific developments [4,5].

Additionally, actions to support, coordinate or supplement knowledge sharing on animal-free educational and training methods is an essential pillar to improve the uptake of animal-free methods. Teaching the educators and educating the competent authorities is crucial. However, the mind-shift necessary to promote the uptake and use of alternatives may require further efforts.

Mapping the use of animals for educational and training purposes in the EU can provide a framework on which to build a coordinated strategy to move towards humane teaching methods in Europe.

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ReThink3R: Design Thinking workshops for young scientists – awareness and innovation for the 3Rs

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An advanced implementation of the 3Rs will be dependent on innovations. Their development requires an open and creative mind-set from people in life sciences. As a field with such demands and opportunities for innovation, one might suspect young scientists being most amenable for it. However, a lack of education relating to the 3Rs as well as pressures created e.g. by conventional research structures are some of the obstacles that discourage scientists from taking initiatives and working on the 3Rs. Serious possibilities to reflect the topic with others are missing. Moreover, young scientists need to be empowered to be innovators in their field through training of 21st century skills, which include critical thinking, collaboration, creativity and empathy [1].

On these grounds we developed a workshop format for PhD students and others involved in research using animal experimentation. It is based on the Design Thinking methodology and aims to create an open atmosphere and time to intensively rethink problems relating to the 3Rs, to imagine new realities and build on opportunities for their implementation. Since 2015, our interactive workshops have been realized several times in collaboration with different graduate schools in Berlin.

Design Thinking is an innovation method that combines both analytical and creative methods to find practical solutions in an iterative process. Important aspects of the process are diverse teams, a flexible work environment, visualisation, and tools for gaining empathy with users [2].

Our workshop usually begins with an assignment ("challenge"), such as "Design an institute that keeps animal suffering to a minimum" or "How can we sensitize pupils or students for the field of the 3Rs?". During the first exercises, the participants discuss and explore various issues within this challenge. By interacting with different affected people, they get to know different perspectives and necessary information to uncover concrete underlying problems. Issues were for example i) time limitations due to application writings and statistics, ii) problems in animal handling, iii) a lack of knowledge or trust in alternative methods or iv) communication problems between scientists and laypersons. All gathered information and insights are used in the solution process and shape the following generation of ideas, out of which one is developed further through quick prototyping and testing with users for validation. Finally, the participants have created validated solutions to the stated challenge based on actual problems expressed by the people they interacted with.

So far, our workshops yielded various innovative prototypes such as i) an animal handling training package with robot animals and modern glasses that guide through SOPs and acquire videos during animal handling, ii) an online program for applications for animal experiments including support in statistics, finding alternative methods, and sharing of animals or iii) a project week for schools, during which pupils deal with subtopics such as cell culture, *in silico* methods or animal behaviour. More important, our workshops empowered students with creative confidence, awareness for the variety of the 3Rs and motivation to work on their implementation, – being equipped with first hands-on experience in innovation.

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60

Microstructured thermoresponsive cell culture substrates for the controlled formation of oriented neuronal networks *in vitro*

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Today, neuroscience is predominantly based on animal research due to a lack of suitable *in vitro* models for studies of the neuronal system. Human induced neurons, and related emerging possibilities in this context, promise to be a suitable alternative to rodent cells, for example for *in vitro* disease modelling or toxicity testing [1-3]. However, neuronal cells in standard cell culture are randomly connected, which does not represent the architecture of the brain with its well-defined connections. To tackle this, various approaches to create *in vitro* neuronal networks with defined connectivity were developed. They are promising tools to investigate network properties such as pre- and postsynaptic mechanisms of neuronal cells [4-7].

Yet, most of these protocols do not provide sufficient control of the direction and timing of neuronal connections with simultaneous access for common analysis tools such as immunocytochemistry or electrophysiological recordings.

We developed thermoresponsive cell culture substrates for the spatio-temporal control of the formation of simplified neuronal circuits *in vitro*. For our approach we used microstructured thermoresponsive polymer (TRP) coatings that can be switched during cultivation from a cell-repellent to a cell-attractive state by altering the temperature of the substrate from 33°C to 37°C. Key elements of the substrates are TRP-free patterns in the shape of 50 µm wide somal adhesion spots with 200 µm distance that are interconnected by TRP-free neurite pathways of 5 µm width and 150 µm length. Since the neurite pathways are shorter than the distance between two somal adhesion spots, a 50 µm wide TRP-coated gap is created between neurite pathways and adhesion spots which allow us to control neurite outgrowth between these two structures.

Differentiated human neuronal SH-SY5Y cells were initially restricted to the TRP-free pattern of adhesion spots and neurite pathways for 4-5 days by forcing the surrounding TRP in its cell-repellent state during cultivation of the substrates at 33°C. Upon switching the TRP to the permissive state by a temperature increase to 37°C the cells extended their neurites across the gaps of TRP towards neighbouring cells. This allowed us to control the formation and connectivity of small, simplified neuronal networks in time and space. Immunocytochemistry on these artificial neuronal networks confirmed that the cells express the neuronal marker ß-tubulin III like differentiated cells on standard surfaces do. This indicates that the cells keep their differentiated neuronal phenotype. We are currently transferring our approach to induced neuronal cells. Next, we will heat up the substrate locally for an improved spatial control over the dynamic TRP surface. Such improved technologies for *in vitro* neuronal networks will help to reduce the numbers of animals used in neuroscience research.

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3D bioprinting of organ model

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3D bioprinting holds great potential to fabricate tissue constructs for research purposes and drug development. The basic idea of 3D printing, more precisely denoted as additive manufacturing, is to produce a 3D object with high spatial resolution under computer control. While the technology has matured in many fields such as rapid prototyping in the last two decades, its application to life sciences has commenced only more recently, but has already made great progress. The presentation will describe the state-of-the-art and give examples for current opportunity of application. Finally, challenges and lines of development will be discussed that will lead to the production of more realistic organ models. The final aim of 3D printed tissues is not only to replace animal models, but also to provide humanized models that represent human pathology more adequately than animal models.

In the narrow sense, bioprinting refers to 3D printing techniques that combine cells, growth factors, and biomaterials to fabricate biomedical parts that maximally imitate natural tissue characteristics. In a broader sense, printing of 3D scaffolds and their subsequent repopulation with cells can also be denoted as (indirect) bioprinting. Advanced 3D bioprinters permit positioning of biocompatible materials and/or cells with high spatial precision. The overview talk will introduce techniques for bioprinting, discuss properties of currently used materials for 3D printing and describe the state-of-the-art of 3D bioprinting of organ models. As each organ has its own special requirements, no single technology will be suitable for all envisioned applications. Dominating methods of biological printing applications are ink-jetting, extrusion printing and stereolithography. Advantages and limits of each system will be discussed.

For the bioprinting process, living cells are loaded in soft biomaterials usually denoted as bioinks. These materials must not only be biocompatible, but must also combine two essential properties: While the viscosity must be comparatively low during the printing process, the material must become stiff thereafter to maintain the structure. Currently, natural bioinks such as agarose, alginate, and gelatin as well as synthetic hydrogels such as poly-lactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), and ploy-caprolactone (PCL) are widely used.

The main focus of current research in the field is the development and characterization of organ models that reflect the biological situation better than 2D cell cultures. Major challenges are the use of primary cells that are usually more susceptible to mechanical stress during the printing process, the precise positioning of multiple parenchymal and non-parenchymal cell types, and the implication of an immune system. However, some groups already proceeded to the application of printed tissue models for research purposes, toxicology studies and drug development. For example, an advanced bioprinted liver model revealed toxicology of an approved drug that was considered safe in animal models but had to be withdrawn from the market due to adverse drug effects. Last but not least, data from our own group will be presented to use printed 3D models for infection studies with influenza and adenoviruses.

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75

3D bioprinting of lung and liver models for viral infection and transduction with viral gene vectors

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Both, lung and liver are commonly targeted by various pathogens, leading to severe infections and high mortality rates. Studying mechanisms of these infections as well as the development of antiviral strategies or efficient drug candidates is complicated by physiological shortcomings of currently used two-dimensional cell cultures and animal models. By spatially controlled depositing of biomaterials and living cells, three-dimensional (3D) bioprinting enables specific fabrication of humanized functional 3D structures, which can help to foster mechanistic understandings of infectious diseases while reducing the amount of animal usage in biomedical research.

Therefore, human lung (A549) and liver (HepaRG) cells were mixed with different types of hybrid bioinks (alginate, gelatin, Matrigel or human extracellular matrix). Subsequently, cell-laden bioinks were layer-by layer extruded using a pneumatic microextrusion bioprinter (INKREDIBLE+, Cellink) to generate three-dimensional tissue models. A suitable bioink formulation, which fulfills considerable requirements for adequate cell culturing conditions, is one of the most critical factors for effective 3D bioprinting approaches. Therefore, initially effects of the hybrid compositions concerning distribution of the cells within the 3D matrix, metabolic cell activity and cytotoxicity during cultivation within the models were analyzed. Afterwards models containing A549 cells were infected with influenza A virus, the HepaRG cell containing ones were either infected with Adenovirus or transduced with AAV vectors. Here, the antiviral immune response and viral replication as well as the silencing of an endogen target mediated by shRNA containing AAV vectors were determined.

Hybrid bioinks blended with certain concentrations of Matrigel or human ECM supported the cell viability of A549 and HepaRG during standard culturing conditions up to two weeks. Meanwhile the three-dimensional distribution of the cells in the printed models during a culturing period of seven days was improved by increasing Matrigel and ECM concentrations. During this time specific liver markers such as albumin secretion and Cyp3A4 activity showed a steady increase. In addition, the 3D models could be infected with either Influenza A virus or Adenovirus, showing proper viral replication as well as an adequate cellular immune response. Moreover, transduction with AAV vectors was supported by the printed 3D models showing efficient shRNA mediated knockdown of human cyclophilin b (hCycB).

Accordingly, bioprinted lung and liver models are promising tools in the field of infection biology and viral gene vectors as basic models for the development of new antiviral strategies. Furthermore, these humanized models could overcome some of the shortcomings of current animal models that naturally do not support replication of human pathogens as well as they may help reducing the numbers of animals used in the drug development process.

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16

Meta-analysis of NTSs ameliorates identification of research areas for 3R development

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The implementation of 3R (replacement, reduction, and refinement) measures in biomedical research is not only a legal provision, but is also required to provide laboratory animals the best protection and to gain reproducible and sound data. So far, tools to support governments and third-party donors in target-oriented 3R funding are missing. To address this issue, a fine-grained overview of animal experiments, e.g. concerning methods, primary hypotheses, and statistical analyses would be most beneficial, but within the authorization process of animal experiments the publication of this detailed information is not mandatory. Therefore, an alternative approach is needed.

The Directive 2010/63/EU for the protection of laboratory animals requires that a comprehensible, non-technical summary (NTS) of each authorised research project involving animals is published by the respective Member State. The anonymised NTSs include information on the projects objectives and potential benefits, expected harm, number of animals, species, and a demonstration of compliance with the requirements of the 3R principle. The contents embedded in the NTSs offer the opportunity to generate more information for research and the public. Our approach was to analyse the duly completed NTSs submitted to the German web-based database AnimalTestInfo in 2014 to 2016 by classifying the NTSs according to the "International Classification of Diseases and Related Health Problems" (ICD) system.

Indexing NTSs with ICD codes provided a fine-grained overview of the prospective uses of experimental animals. We were able to pinpoint the envisaged benefits down to the level of the addressed disease. Thereby, we identified specific research areas with many planned *in vivo* projects, e.g. Malignant neoplasms of digestive organs, Cerebrovascular diseases, and Demyelinating diseases of the central nervous system. The development of 3R measures in these research areas may be most efficient as a large number of experimental animals would benefit from it. Hence, data drawn from NTSs can provide a basis for the development, validation, and implementation of directed 3R strategies as well as guidance for rethinking the role of animal research models.

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Statement on ethics vote: No ethics vote is required.

5

Measuring Culture of Care – a practical example

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The concept of Culture of Care is relatively new within laboratory animal science and not yet explicit legally described. Measuring or assessing your user-establishment's Culture of Care is relevant for understanding how to change this – where to start from, deciding where to go and planning how to get there. At Novo Nordisk we have developed a tool that with relatively few questions gives you valuable insight into these issues. We do not imply that the tool offers *the* descriptive definition of Culture of Care, but it represents one of several ways to describe this and should be seen as complementary to other similar initiatives.

The presentation will briefly explain the structure and elements of the survey tool and in more detail describe selected survey results. The structure and elements description includes looking at different organisational levels (the individual, the group, the management and the organisation), different professional groups (lab animal technicians, lab technicians, licence holders, lab animal vets, scientists and managers). This also includes the use of so-called surrogate markers inspired from the concept of social capital, which offers a picture of the culture, – the surrogate markers being three value based qualities (Collaboration, Trust and Integrity) and 6 operational issues (Influence, Meaning, Predictability, Social support, Rewards/recognition and Resources). The presentation will explain the surrogate markers by use of examples from the survey.

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40

EFPIA – putting animal welfare principles and 3Rs into action

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At EFPIA (European Federation of Pharmaceutical Industries and Associations) there is a foreseen importance of promoting good science and animal welfare, as well as increasing understanding of how the two are intertwined as the benefits are an essential part of promoting and enabling high quality research and 3Rs achievements. The poster describes EFPIA's commitment to laboratory animal welfare and the 3Rs and outlines areas of current focus and impact across three categories – Beyond Compliance, Leading by Example and Open Communication.
This poster will present a non-exhaustive list of examples from the pharmaceutical industry and its collaborations within the research field recognising how the sector operates to meet the requirements to effectively implement the requirements of Directive 2010/63/EU and also where researchers and technicians go beyond regulatory requirements to develop practices leading to improved animal welfare and focused 3Rs efforts in every day practice.

The pharmaceutical industry continues to work with regulators to ensure the fastest possible uptake of new approaches that balance increased effectiveness in safety and efficacy assessment and the impact on 3Rs; pharmaceutical companies work together to engage all stakeholders and disciplines in dialogue and collaborations and facilitate exchange of good practice between life science community stakeholders to improve animal welfare and scientific outcome. The collaborations with other organisations, included in the poster, support addressing emerging animal welfare issues and illustrates how mechanisms to share practices that promote good science, animal welfare and the 3Rs become operational.

Furthermore, the ways the industry is using open communication to educate, promote, and share the progress being made where animals are used in pharmaceutical research will be described.

Reference

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32

Metabolome analysis *in vitro* in NRK-52e cells might allow the prediction of nephrotoxicity and its mode of action

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During the development of pharmaceuticals, pesticides, cosmetics or other classes of chemicals nephrotoxicity is a relevant toxicological endpoint. Beyond the identification of nephrotoxicity in new compounds, mechanistical understanding and identification of the mode of action (MoA) gets more and more relevant. Reliable and robust *in vitro* methods are the preferred methods to reduce animal testing but also to identify the toxicological modes of action.

BASF has developed in the past metabolomics *in vitro* in liver cells to predict hepatoxicity of chemicals and its mode of action. Metabolomics *in vitro* in kidney cells is now a novel approach that could enable the early identification of nephrotoxicity and contribute to understanding the mode of action. Therefore, a robust system based on NRK-52e cells was established (partly within the InnoSysTox Project "Risk-IT" founded by BMBF, Germany) by cultivation of the cells on Lumox dishes (Sarstedt), treatment for 48 h and sensitive harvesting of the cells and LC-MS/MS and GC-MS/MS technology was applied to quantify > 300 endogenous metabolites. Nine different nephrotoxic compounds classified in three different mode of actions (covalent protein binding, lysosomal overload and mitochondrial DNA-interaction) and caffeine acting as negative control were applied to the system. Gentamycin-sulfate, belonging to the MoA "lysosomale overload" was tested in several concentrations and in several runs.

Principle component analysis (PCA) showed a clear dose response relationship and demonstrated the high reproducibility of the data. Caffeine, which does not share any aspects of the three different MoA of nephrotoxicity induced by the other reference compounds clustered nicely with the vehicle control (DMSO 0.5%) and the quality control (bezafibrate). For all 9 compounds a separation of the clusters in the PCA was shown compared to vehicle and negative control. Further evaluation on a specific fingerprint per mode of action is ongoing.

These already observed differences in the *in vitro* metabolome in NRK-52e cells of the 9 known nephrotoxic substances with three different modes of action suggest that the metabolome analysis could be a suitable tool for analysis of the mode of action of nephrotoxicants. This method can support on one hand side the screening process but can also contribute to identify and understand the mode of action of nephrotoxicity and has thus the potential to be applied for regulatory purposes.

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Evaluation of retinoic acid receptor alpha (RARα) interaction of chemicals by combining two techniques to a new screening approach

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Reproductive toxicity is a sensitive endpoint during research and development of new pharmaceuticals and chemicals. Therefore, early and robust identification of reproductive toxicity properties and their mode of action is crucial. It is known that inappropriate regulation of the retinoic acid receptor alpha (RARα) can lead to a range of adverse effects including teratogenicity and testicular toxicity and is a relevant mode of action (MoA) in the context of reproductive toxicity. The RARα is a nuclear receptor present in the cellular nucleus and bound to the retinoic acid response elements in front of target genes. A key step in the ligand induced transcription of those controlled genes is the recruitment of cofactors by RARα.

Two different methods (1. a cell based system in transfected HEK293, expressing RARα cells, and 2. a micro array system called MARCoNI, PamStation®) were tested to determine their strength and limitations to identify RARα-interaction (agonism, antagonism and non-response) of xenobiotics. Therefore, the co-factor binding patterns (tested by MARCoNI) for several known agonists (e.g. ATRA, AM580), antagonists (e.g. BMS195614, BMS493) and non-responder (e.g. Ketoconazole) were investigated and compared with results from reporter cell line test (HEK293), expressing RARα, to evaluate the applicability for toxicological assessment of xenobiotics.

The dose response curves (EC50) of peptides and cofactors, known as relevant for the RARα interaction, corresponded nicely to the dose response curve of the HEK293 cell line. In detail: after ATRA treatment 16 selected cofactors/45 peptide motifs revealed EC50 values between 6.6·10E-9 M and 1.0·10E-8 M and was comparable to EC50 value of 1.9·10E-9 M for ATRA in HEK293 cells without any cytotoxic effects. All results nicely show that RARα interaction of xenobiotics can be demonstrated by above mentioned methods. While the HEK293 cell line is suitable to determine the agonistic and antagonistic property of a chemical in an easy and economical way, the experiments in the MARCoNI-system revealed the molecular initiating event by influencing corepressor or coactivator-binding. Since the agonistic effects are identified properly with both methods, the MARCoNI-system allows a better understanding of mechanistical processes for diverse antagonists.

Both methods can compensate the limitations of each other and therefore the combination of both methods increases the value of each individual method. A smart testing strategy was implemented at BASF to investigate the RARα interaction including mechanistical understanding of chemicals in the screening phase of new chemicals but also for specific regulatory questions on chemicals in the registration process.

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112

It's time to rethink: Learn from negative results in animal-based research! Critical Incident Reporting System in Laboratory Animal Science CIRS-LAS.de

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Introduction

In 2016, almost 3 million animals were used for scientific experimental purposes in Germany. From today's perspective and considering the current state of medicine, the use of animals in experimental purposes cannot be completely dispensed. Every year, a large number of scientific articles based on animal experimental studies were published. But negative experiences gained from experiments get lost or are not referred in publications. The objective of the CIRS-LAS.de reporting system is the detection of negative experiences in the entire range of laboratory animal science and to avoid them in the future. In accordance to the 3R principles the portal will therefore improve animal safety and reduce the number of laboratory animals.

Materials and methods

CIRS-LAS is now supported by German Federal Ministry of Education and Research (BMBF) within its funding to support alternatives to animal experiments. The CIRS-LAS portal is based on similar databases in human medicine that allow anonymous reports of critical incidents. On CIRS-LAS portal reports can be assorted to various sub-categories applying to laboratory animal science. For example methodical mistakes, inefficacy or deviating efficacy of pharmaceutical drugs, failure in breeding lines or species-specific features can be recorded. Further explanations of the incidents, for example descriptions of experimental specifications, possible causes, upload of supporting documents and already proposed conceivable solutions, are furthermore possible. A restricted group of persons (members) is authorised to read and to comment on the database content.

Results

CIRS-LAS portal serves as an essential contribution towards the implementation of two of the 3R principles. Anonymously shared negative experiences can have an influence on the experimental setup and execution of new animal based research. Additionally, the comment section of the collected incidents serves as a basis for fundamental discussions and an exchange of ideas between scientific peers on the portal. Currently, persons from Germany, Austria and Switzerland are registered on the platform. Anonymously shared negative experiences can have an influence on the experimental setup and execution of new scientific projects. Additionally, the comment section of the collected incidents serves as a basis for fundamental discussions and an exchange of ideas between scientific peers on the portal.

Conclusion

In conclusion the realization of 3R – a reduction of the number of used animals in experiments (reduction), diminution and replacement of stressful experiments (refinement and replacement) – can be achieved by using the CIRS-LAS portal. It is furthermore conceivable that the implementation of the CIRS-LAS could serve to enhance the trust in laboratory animal science, both of the public and the scientific community. The time to rethink has been achieved – to learn from negative results in animal based research!

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Development of a cellular neurotoxicology model using genetically engineered human induced pluripotent stem cells, a 3Rs alternative to animal testing

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The generation of *in vitro* models for chemicals and nanomaterials safety assessment is a developing alternative method to animal testing. The directed differentiation of human induced pluripotent stem cells (hiPSCs) to model human organs-in-a-dish (e.g. brain, liver, kidney tissues) has become one of the most important research fields in toxicology studies. Further comparison of the *in vitro* data to existing *in vivo* results, as well its combination with *in silico* predictions, will allow the refinement of organ-specific toxicology models, and compliance with 3Rs principles of replacing animal models. Moreover, the generation of toxicity reporter cell lines, by genetically labelling toxicology biomarkers, can provide a faithful system to assess the endogenous response of cells to a compound.

We present a hiPSC-based *in vitro* toxicology assay, that can be used to test neurotoxicity at different stages of the neuronal differentiation. Human iPSCs were differentiated into neuronal cells for 21 days (TD21) and exposed to different toxicants (e.g. paraquat, valproic acid, acrylamide). Concentration-responses were investigated after acute (72 hours) and chronic (120 hours) exposure and the effect of toxicants were investigated with an ATP-based cell viability assay. The results demonstrated that chemicals affecting different biological processes might have very different toxic concentrations on human neuronal cultures.

As a next step, we develop further our system by endogenous tagging of the hiPSC line. The key point is to detect and identify the transcriptomic changes in the normal functioning of the cell which indicate a toxic effect of a compound as early as possible. The Nrf2-mediated oxidative stress response is a major pathway of toxicity. Introducing a fluorescent cassette at the endogenous locus of an oxidative stress-induced gene with the CRISPR/Cas9 technique can generate a hiPSC reporter cell line of oxidative stress. This reporter system can be then differentiated into neuronal lineage and challenged with known toxic compounds to verify its relevance in developmental neurotoxicity studies. Forward, this reporter cell line could provide a quick way to assess the toxicity of a chemical, by high-throughput screening as well. In our expectation, such reporter systems will allow to reduce the use of animal models in cellular toxicology studies.

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Characterization of a lung/liver organ-on-a-chip model

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Until few years ago, *in vitro* models could only poorly mimic the processes observed in the human body. During drug development, their inability to mimic complex physiological processes was an important reason for drug withdrawals. The combination of 3D *in vitro* models with an engineered microenvironment, resulting in the so-called "organ-on-a-chip" technology, allows these limitations to be overcome. This technology enables the study of complex organ interactions, better simulating processes occurring *in vivo* and therefore leading to better prediction of drug-associated toxicity.

With the aim of creating a model able to assess the toxicity of aerosols accurately, Philip Morris International recently developed a new lung/liver-on-a-chip device combining a bronchial tissue at the air-liquid interface with HepaRG™ liver spheroids. A specifically designed peristaltic pump allows for a continuous medium circulation and thereby enables lung-liver crosstalk. Stability of both tissues in the chip over 28 days, alone and in combination, was first evaluated. At the end of the experimental period, key liver (albumin secretion, cytochrome P450 activity) and bronchial tissue (transepithelial electrical resistance (TEER), cilia beating frequency, morphology) characteristics were comparable to control tissues that were maintained in the incubator. Using this lung/liver-on-a-chip platform, we further demonstrated the role of the liver compartment in metabolizing and inactivating a pulmonary toxicant: aflatoxin B1. When only bronchial tissues were exposed to this compound, a severe decrease of TEER values and adenosine triphosphate content was observed, along with an increased number of apoptotic cells. Conversely, in the presence of liver spheroids, bronchial tissues were unaffected by the compound. In parallel, specific inhibitors of enzymes involved in the metabolism of this compound were used to demonstrate the toxicity of the parent compound and their metabolites.

Our results demonstrate that the lung/liver-on-a-chip platform can be extremely beneficial for future toxicological analysis.

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151

Alternative methods for testing of eye irritation caused by agrochemicals

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Draize-test is the basis of the OECD 405 guideline, which is one of the most criticized *in vivo* methods, because of the injuries of the test animals and subjective nature of the test in recording the results.

Nowadays, several *in vitro* tests are available which can be partly or totally replaced the *in vivo* eye irritation testing depending on the circumstances. The isolated chicken eye test and EpiOcular™ model are part of these alternative methods. Five different agrochemicals were examined with both methods the following way.

The Isolated chicken eye test method: The test compound was applied in a single dose onto the cornea of isolated chicken eyes in order to potentially classify the test compound as either 1: causing "serious eye damage" (category 1 of the Globally Harmonised System for the Classification and Labelling of chemicals (GHS)), or 2: not requiring classification for eye irritation or serious eye damage according to the GHS. Tested corneas were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 minutes after the post-treatment rinse. The endpoints evaluated were corneal opacity, swelling, fluorescein retention, and morphological effects. All of the endpoints, with the exception of fluorescein retention (which was determined only at pre-treatment and 30 minutes after test substance exposure) were determined at each time point mentioned above.

The EpiOcular™ Model: The test compound is applied to the surface of the cornea epithelial construct for a fixed period, removed, and the tissue allowed to express the resulting damage. The irritation potential of the test item may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EpiOcular™ tissue. The test item is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1), if the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) 60% compared to the negative control. Depending on the regulatory framework in member countries, the test item is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (>) 60%.

Concurrent positive and negative controls were included in both methods. In these *in vitro* eye irritation studies, using the Isolated Chicken Eye and EpiOcular™ model with five different agrochemicals, irritation potential were observed in three test items and no effect were seen in two test items out of five. These results correspond to the available information about the tested agrochemicals, so these studies with isolated chicken eye and EpiOcular™ are considered to be successful.

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OECD 405
OECD 438
OECD 492

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101

Human rabies vaccine glycoprotein G ELISA as an alternative to the challenge test: Selection of a candidate method and future strategies

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Immunization with the native trimeric form of the rabies glycoprotein G induces the production of neutralising antibodies and protection against lethal challenge. In human rabies vaccines manufacturing, antigen quantification is used for the formulation of the final vaccine lot. Official release control of human rabies vaccines relies upon an *in vivo* potency test in mice, the NIH test [1]. The NIH assay is a significantly variable test based on immunisation followed by an intracranial virus challenge and is using a large number of mice of which many suffer tetanus symptoms. The replacement of the NIH test is thus a high priority for the implementation of the 3R concept.

An EPAA meeting in 2012 focused on gaps in technical knowledge and validation of *in vitro* G antigen quantification methods for potency testing, with the view to propose a strategy for the replacement of the NIH test. Participants stressed out that the current *in vivo* assay should not be used for correlation with the *in vitro* methods since it is highly variable, and that an agreement strategy should therefore follow. It was also agreed that the alternative glycoprotein G ELISA method should be able to discriminate between potent and sub-potent batches.

An International Working Group including regulators, rabies science specialists and vaccine manufacturers was formed to coordinate an inter-laboratory study aiming at identifying the most suitable replacement assay. A protocol was established to compare several ELISA methods, using potent and sub potent human rabies vaccine lots. The data from this study indicated a good agreement between the ELISA and the NIH test. One of the tested ELISAs was selected for its ability to discriminate potent from sub-potent lots but also to detect the main virus strains used in vaccine manufacturing [2,3]. The results of this study were presented in 2015 at an EPAA group workshop and were published [3].

Based on these results, an international collaborative study (coded BSP148) was launched by the Biological Standardisation Programme of the Council of Europe and the EU Commission to further validate the transferability and robustness of the selected ELISA. This BSP study should support to the global replacement of the *in vivo* NIH test by an *in vitro* method for the official release potency test of human rabies vaccines.

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139

A high throughput and cost-effective method to profile compounds interactions with efflux transporters using Caco-2 cells line

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Caco-2 cells, derived from a human colorectal adenocarcinoma are widely used in the pharmaceutical industry to screen for new chemical entities in the early phases of drug discovery. These cells exhibit an enterocyte-like phenotype, spontaneously form tight monolayers and express efflux transporters such as ABCB1 (P-glycoprotein), ABCC2 (MRP2) and ABCG2 (Breast Cancer Resistance Protein, BCRP).

Since several drugs could be substrate for these efflux transporters resulting in drug-drug interactions (DDI) both the FDA and EMA recommend the systemic evaluation of whether new drug candidates are substrates (and thus competitive inhibitors) for these transporters.

This is traditionally achieved by measuring the bidirectional transport (i.e. efflux ratio) of the compounds across barrier forming cells expressing those transporters such as Caco-2. The limitations of this methodology are the long culturing period of at least 3 weeks to allow for differentiation of the Caco-2 cell monolayers and the technical difficulties (and associated costs) to accurately quantify the compounds transport across the cellular monolayer.

By cultivating Caco-2 cells for 6 days on 96-well plates and using fluorescent substrates, we set up a cost-effective methodology to discriminate whether compounds are substrate for the efflux transporters express by Caco-2. The cells are first loaded with fluorescent probes for ABCB1, ABCG2 or MRPs and their clearance out of the cell monitored in presence or absence of the test compounds measured in real time using a fluorescence plate microreader. Here we propose to use the effects of compounds of the rate of clearance from the cells (Kout) of the fluorescent probes as a first-tier approach to evaluate compounds interaction with these transporters

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An *in silico* approach to predict aromatase toxicity for azoles

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Aromatase is an enzyme member of the cytochrome P450 superfamily coded by the CYP19 gene. Its main action is the conversion of androgens into estrogens, transforming androstenedione into estrone and testosterone into estradiol. This enzyme is present in several tissues and it has a key role maintaining the balance of androgens and estrogens, and therefore in the regulation of the endocrine system. Endocrine disrupting chemicals interfere with the biosynthesis and normal functions of steroid hormones, so determining if a chemical is an aromatase inhibitor is crucial for guarantee its security. In this sense, the use of *in silico* tools to predict if one compound is an aromatase inhibitor, could optimize the process of introduction of new chemicals to the market. This study intends to develop computational models able to predict the toxicity of chemicals on the aromatase enzyme. Total 327 azoles were collected from the Tox21 library (85 monoazoles, 201 diazoles and 48 triazoles), classified as inhibitors or non-inhibitors of aromatase. Three predictive models were developed, using each of these subsets of compounds (M1, M2, and M3, respectively), based on the determination of structural alerts that were identified as related to the toxicity. The models provided a good classification performance. M1 identified 13 structural alerts (Acc 0.78); M2, 28 structural alerts (Acc 0.80) and M3, 9 structural alerts (Acc 0.91). The MCC values for M1, M2 and M3 were 0.59, 0.69 and 0.83, respectively. Additionally for subset of triazoles, to have mechanistic insight, the toxicity (AC50) for the active molecules have been regressed against the quantum-mechanical descriptors. The regressed model based on single descriptor shows that charge on nitrogen at the 2nd position have certain role for the toxicity as R2 values is 0.21. The three descriptors based model suggested that the charge on nitrogen at 1st and 2nd position and HOMO energy of the molecules gives R2 value greater than 0.44. The classifiers based on structural alerts showed a good performance and could be useful to predict the toxicity of monoazoles, diazoles and triazoles on the aromatase enzyme. The regression model did not satisfy the condition for good QSAR model. However, the regression analysis suggested that there is flow of electrons from the traizoles to the aromatase because both the nitrogen have the negative charge and HOMO was directly proportional to AC50. The results obtained from the regression analyses were also consistent with structural alerts because the fragments having the electron releasing nature are also contributing to activity. Even so, it remains to increase the number of compounds in the subsets, which would improve the classification performance and to extend the domain of application of the classifiers.

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The ROle of the University in Teaching and Education on ALTernative: ROUTE ALT

Francesca Caloni and Alessia Bertero
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In order to train new scientists and future researchers on alternative methods in toxicology, the role of Academia is fundamental, to develop a mentality and a way of thinking.
Following the activity the University of Milan, Department of Veterinary Medicine, and Integrated Teaching Strategy, through a continuous education approach, based on a previous long and validated experience, from under- to post-graduate, is suggested. The pathway of education carried out in our faculty in the recent years can be summarized as follow:

Undergraduate level:
1. Lessons in Bachelor Degree in Veterinary Medicine (2 hours) and Animal Welfare and Breeding (4 hours)
2. Courses in Master Degree in Veterinary Biotechnological Sciences with 2 modules: the first one entitled "Alternative Methods in Toxicology", 4 credits, 30 hours, inclusive of 18 hours of lectures and 12 hours of practice, the second one, "Toxicology and *in vitro* models", is a 3 credits course on *in vitro* toxicology, 30 hours, inclusive of 6 hours of lectures and 24 hours of practical
3. Practical Training of 100 hours on alternative methods of Replacement in Toxicology in Master Degree in Veterinary Biotechnological Sciences

Postgraduate level:
4. Lessons (2 hours) in 2 professionalizing courses, for post-graduate: named Law and Veterinary Legislation Course and the second one Animal Welfare and laboratory Animal Care Course
5. Lessons (8 hours) in Specialization Course in laboratory animals school (6-8 hours).

The educational route could start from the bachelor degree, with common programs in different curricula in bioscience, than at least one module in the specific master degree, and finally a continuous post-graduate education in second level masters, professionalizing courses, specialization schools. Moreover PhD specifically dedicated to alternative and 3Rs, as recently proposed, could implement the attractiveness and expertises.

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Consensus ways of alternative to animal testing in China

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With more and more countries lining up to do the same implementation of full animal testing ban follow EU since 2013. In the recent 10 years, the alternative technology in China has been developed gradually with the harmonization relevant legislations, hold on various theoretical and hands-on training, validation and accept of internationally recognized methods, propagation of alternative standards and in-depth investigation on *in vitro* bioscience. Barriers also faced that demands infrastructure constructing, technology capability building and national standard system formed through international cooperation, technical assistance transformation and self-innovation. China is now speeding up to reach consensus, accelerating the technology development and attempting to incorporate non-animal, *in vitro*, testing methods into national regulatory system.

Industries have maintained a high growth trend recent years in China, including cosmetics, biological and medicine. They have a strong desire to participate in innovation and international competition. The cosmetic industry is pushed by the scientific and business advantages that alternative tests provide and increased accessibility to markets where animal testing is prohibited. In recent 10 years, CRO institutes have focused on developing and provide alternative methods and platform of *in vitro* test system to Chinese market. For example the Chn-Alt Biotechnology, founded in 2010, is the first third-party institute specializing in non-animal testing, technical training and research & development of new *in vitro* methods for testing cosmetics and chemicals. There are 5 R&D centers in the Chn-Alt which individually focus on origin-on-chip, skin immunity, TCM and plant extract, integrated testing and big-data system. In addition to toxicology services, they also offer efficacy testing and innovative methods based on industry's needs, collaborate with institutes and universities home and abroad on basic research.

Notably, the most historic and influential Chinese alternative conference has conducted eight times up to now. The conference, which was held on in 2011 first time, has different theme and attached hands-on training, the attendance annually has grown. It was an opening platform for academic communication specializing only in "replacement" in China. The hands-on training of test endpoint includes skin and eye irritation, skin sensitization, genetic toxicity, endocrine disrupt and efficacy screening. Moreover, it contains interpretation and application of AOP guideline, *in vitro* construction of lab and quality control, certification and validation of alternative methods and so on. Academic meeting and training provide the platform for broadened consensus of regulators, research institutes and industries. The first translational toxicology courses for postgraduate has launched in Shanghai Jiao Tong University in September 2017. A cosmetic *in vitro* database project lead by Guangzhou Chn-Alt Co. has launched. An E-educational course under the portal website http://www.vitrotox.com/ set up by Chinese Center for Alternative Research and Evaluation (CCARE) has begun at 2016. It will provide training videos, Chinese versions of OECD test guideline and AOP-related document, promote the awareness of the state-of-the-art alternative technology and updated global non-animal testing policy.

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As exposure confers resilience to proteotoxicity in a *C. elegans* model of Alzheimer's disease

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Background
Alzheimer´s disease (AD) is the main cause of dementia in elderly population worldwide [1]. Environmental factors such as diet, lifestyle and pollution have been linked to AD [2]. Human populations in vulnerable areas are currently exposed to heavy metals such as arsenic (As) and lead (Pb) and long term and transgenerational consequences of such exposure are largely unknown. Previous studies suggest that early life detrimental exposures could be inherited over generations [3] *C. elegans* is an alternative *in vivo* model that offers multipleadvantages for transgenerational studies.

Objective
In this study we investigated late life and transgenerational effects of early life exposure to As or Pb in a transgenic *C. elegans* model of AD.

Methods
Nematodes of GMC101 strain engineered to constitutively express human Aβ in body wall muscle were grown on NGM (nematode growth media) agar plates supplemented with different concentrations of Pb or As or on control metal free plates during larval development. Aβ-driven muscle paralysis was induced by shifting these animals from 20° to 25°C at the L4 (pre-adult) larval stage; paralyzed worms were scored every 12 hours. In addition to paralysis assessment, associative learning was measured in As- and Pb-treated wild type nematodes using starvation- benzaldehyde conditioning method. Proteasomal activity, following metal treatment, was determined by detecting the ubiquitin-tagged GFP protein levels in a respective transgenic strain. RNAi-mediated gene inactivation was performed by feeding worms with bacteria expressing gene-specific RNAi constructs.

Results
We found that early life exposure to Pb (50-400 parts per millions, ppm) increases, while As exposure (50-80 ppm) reduces late life Aβ-induced paralysis compared to unexposed worms. Similar effects (reduced paralysis following early life exposure) were found in worms exposed to polluted water obtained from As-contaminated sites in Mexico. Moreover, we found that As (80 ppm) did not affect associative learning in wild type N2 worms, suggesting that this dose (when transiently given during early life) confers resilience to proteotoxicity without signs of cognitive damage. Oxidative stress is one of well characterized toxicity mechanisms of As, however reduced Aβ-driven paralysis upon early life As exposure was not impaired by co-supplementation of antioxidants. As seen by the GFP reporter turnover, the proteasome activity was slightly elevated upon early life treatment with both As (80 ppm) and Pb (400 ppm) compared to control. RNAi-mediated inactivation of key proteostasis-related genes (skn1, hsf-1 and aip-1) was not sufficient to counteract As-induced paralysis phenotype. Overall these results demonstrate differential effect of early Pb and As exposure on adult proteostasis despite both metals being known toxins.

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Efficacy testing of antivirals using human upper and small airway epithelia

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Respiratory viral infections cause mild to severe diseases worldwide. We report herein the use of 3D epithelia made of human primary nasal cells (MucilAir™) or small airway cells (SmallAir™) for viral drug screening.

Clinical isolates of Rhinovirus (A16, C15), Enterovirus (EV68) and Influenza A virus (H1N1, H3N2), Respiratory Syncitial Virus (RSV-B) were added to fully differentiated airway epithelia. Multi-endpoints such as viral genome copy number, apical mucin secretion, cilia beating frequency, mucociliary clearance (MCC) and tissue integrity (TEER) were assessed daily during 4 days.

All tested viruses, including the difficult-to-culture HRV-C15, replicated efficiently in MucilAir™ and SmallAir™. Furthermore, Rupintrivir efficiently inhibited the replication of HRV-A16 and HRV-C15 in a dose and time dependent manner and restored MCC impaired by EV68. Oseltamivir reduced the replication of H1N1 and H3N2 and restored the impaired barrier function (TEER). Ribavirin, as reference antiviral against RSV, applied either apically or basally, inhibited viral replication in a dose-dependent manner and partially prevented the decrease of the mucociliary clearance.

These results demonstrate that MucilAir™ and SmallAir™ is a robust, reliable and relevant tool for antiviral drug development

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Modeling respiratory bacterial infections using *in vitro* human airway epithelia

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Pathogens colonizing the respiratory tract might co-operate or compete with each other. *Pseudomonas aeruginosa* (PA) infection are increasingly associated with acute exacerbations in chronic obstructive pulmonary disease. *Streptococcus pneumoniae* (SP), meanwhile is a main cause of pneumonia, meningitis, it can also lead to other respiratory diseases such as bronchitis.

We report herein the use of 3D human airway epithelia (MucilAir™) reconstituted *in vitro* to study interactions of PA and SP on nasal mucosa cultivated separately or together over 24 hours. Results showed that PA infection induces a loss of TEER, 20% cytotoxicity and a significant increase of Il-8 release (+100 ng/ml). On the other hand, SP strongly increases the mucus production. When inoculated together, a lower apical PA growth is observed (- 3E+3 CFU/cm^2) suggesting a growth inhibition probably due to the presence of SP producing H_2O_2 radicals.

Co-cultures between Cystic Fibrosis epithelia (MucilAir™-CF) and M0, M1 and M2 macrophages will be described. Growth of PA is more efficiently inhibited in presence of M0 and M1 than with M2.

These results suggest that *in vitro* human airway epithelium is a useful model to study bacterial infection and interaction on the human nasal mucosa.

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Genetically modified 3D human *in vitro* lung models

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Genome editing strategies have been widely used to generate cells and transgenic animals for therapeutic purposes. Today, the application of these approaches to 3D microtissues provides the opportunity to model faithfully *in vitro* human diseases and gain invaluable information on human physiology. Here we took advantage of the ability of lentivectors to deliver large transgenes into primary airway epithelial cells and of the precision of the CRISPR/CAS9 to modify genomic DNA sequences to produce *in vitro* models for respiratory diseases. First, by targeting the exon 11 of the CFTR gene we were able to establish bronchial ALI cultures reproducing the deltaF508 phenotype observed in cystic fibrosis patients. Characterization of the primary epithelia with their isogenic controls will be presented. Secondly, by targeting key proteins involved in DNA damage response we produced lung cancer models. Oncogene transformed tissues showed significant dysplasia with cribriform growth and marked areas of invasion. Enlarged regions displayed increased KI67 proliferation rate and immunostaining revealed an increase in P63 expression, a marker of early stage lung carcinoma. Of note, double mutants expressing in addition the KRASG12S mutation showed a stronger phenotype, with higher dysplasia index, compared to the single mutants. This observation fits into the current hypothesis that an initial mutation in growth-controlling oncogene followed by evasion from apoptosis/senescence due to tumor suppressor loss triggers the first steps of human lung carcinogenesis. Altogether, our data demonstrate the possibility to produce customized models of human respiratory diseases in multiple genetic backgrounds to accelerate the development of human therapies.

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SimulRator – manufacturing a 3D-printed simulator of the rat for laboratory animal training courses

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Introduction

In order to receive an individual permission to perform animal testing in the EU, it is mandatory to have appropriate competences and skills. These are often mediated through laboratory animal training courses in order to learn different techniques including handling, blood sampling and oral administration. The animals involved are mainly living rats and mice and therefore these courses are classified as animal experiments according to the Directive 63/2010/EU. To minimize pain, suffering and distress for the animals, simulators of rats and mice have been developed to represent an initial training device for technical procedures before live animals have to be deployed. Due to different reasons, it seems that these simulators are not used frequently. Therefore, a team consisting of anatomists, laboratory animal scientists, epidemiologists and an engineer with medical background are evaluating all current available rat and mouse simulators with the objectives of identifying their strength and weaknesses in order to create a specification analysis. This analysis will serve as basis for the construction of a new, anatomically and haptically realistic and cost-effective 3D-printed simulator of the rat, which combines many features for the different technical procedures required. Ultimately, using the same technology, also a simulator of the mouse will be manufactured.

Materials and methods

Already existing scans of a 12-months-old Wistar rat, taken on a NanoSPECT/CTplus scanner (Mediso, Hungary / Bioscan, France) at the Berlin Experimental Radionuclide Imaging Center (BERIC), Charité – Universitätsmedizin Berlin, Germany, serve as template for the manufacturing process. To create a printable virtual 3D-model, the softwares Slicer, Blender and CATIA V5 are used. The multi-material printers J750 and Connex 3 Object 350 (Stratasys Ltd. Eden Prairie, MN, USA) can be loaded with up to six materials at once, including any combination of rigid, flexible and even transparent materials. For soft tissues, the UV-curing photopolymers Agilus and Tango+, for hard tissues like e.g. bone the materials of the Vero-family can be used.

Results

The manufacturing of the rat simulator is currently in progress, and preliminary results show that the 3D multi-material printing technology is capable of printing anatomically and haptically realistic simulators.

Conclusion

The authors assume that simulators have to mimic the realistic anatomy regarding anatomical details, dimensions and haptics in order to be a suitable training device for laboratory animal training courses and to represent a method of refinement according to the Directive 63/2010/EU. The 3D multi-material printing technology used for the manufacturing of the present rat simulator, can be adapted to produce any simulator of any desired species.

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Newly validated 3D skin genotoxicity assays improve safety assessments of cosmetic ingredients

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In recent years, 3D test systems have been introduced as an addition to the standard *in vitro* genotoxicity testing battery. These more complex assays consider the relevant route of exposure without the need for animal experiments. Here, we report on human reconstructed 3D skin tissues which were combined with classical genotoxicity readout-parameters to mimic the dermal exposure route while their intrinsic metabolic capacity abrogates the need to add an external source of xenobiotic metabolism like that typically provided by rat liver S9 mix.

We report on the validation study of the 3D Skin Comet in which > 30 compounds were tested blind in six laboratories. Data analysis revealed a good specificity and sensitivity.

In parallel, the 3D skin micronucleus, i.e., the reconstructed skin micronucleus test (RSMN), has been validated with > 40 compounds. Use of the 2 skin-based assays enables addressing the full range of genotoxic damage leading to mutagenicity, clastogenicity, and aneugenicity. We show that the predictivity of a test battery comprising both assays is greater than the use of either assay alone.

Our findings indicate that for dermally applied compounds, the 3D Skin Comet assay and the RSMN can be used as direct replacement of animal studies when following-up on positive results from the standard *in vitro* genotoxicity test battery. Meanwhile, case studies have been submitted to European regulators of which one will be described in detail, i.e. Basic Brown 17, a hair dye ingredient. The regulators accepted the negative 3D Skin Comet assay data on Basic Brown 17 in a weight-of-evidence approach with respect to its safe use with regard to its genotoxicity potential.

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MechoAs (Mechanism of Action) QSAR model and skin sensitization screening: 3-methoxyphenols, 4-methoxyphenol and 1,4-dimethoxybenzene case study

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What is MechoA?

MechoAs use the molecular structure of a substance (SMILES code) to determine the toxicity mechanisms of the parent substance and its major metabolites. The model classifies the substances into 6 major classes (membrane destabilization, enzymatic hydrolysis, reactivity, pro-activity, indirect enzyme disruption, direct docking disruption) and a total of 23 subclasses [1]. The model has been trained with fish, daphnids, algae and rodent *in vivo* toxicity data, therefore, the MechoA model can discern the differences found in toxicity among these species uniting toxicology and eco-toxicology under one and only classification method: MechoAs. Here-in, we have chosen to illustrate the application of MechoAs for skin sensitization (SS), a human health endpoint required for all chemical Regulations involving Human health assessment.

Where is MechoA located in the AOP? & How does it relate to skin sensitization?

MechoAs predict the Molecular Initiating Event (MIE) phase by scanning for electrophilic reactivity and the major metabolites of the parent substance. Therefore, identifying if these substances are capable of binding covalently to proteins, which is the MIE of the skin sensitization Adverse Outcome Pathway (AOP).

Case study methoxyphenol analogs: how do MechoAs help to predict skin sensitization (SS)?

4-methoxyphenol and 3-methoxyphenol are positional isomers, while 1,4-dimethoxybenzene is the methylated form of 4-methoxyphenol. Eventhough these substances are structurally very similar they show different sensitization profiles:

4-methoxyphenol and 3-methoxyphenol (SS): The MechoA model assists in predicting these substances as skin sensitizing, by showing that these compounds can form 2 major metabolites of the type: catechol and a o-quinone, which are capable of covalently binding to proteins, the MIE of the skin sensitization AOP. This prediction is backed up by an LLNA study for 4-methoxyphenol [2] which classifies this substance as being sensitizing to skin. And, an *in vitro* study showing that 3-methoxyphenol can be metabolized to catechol by CYP2E1 and subsequently to o-quinone by superoxide dismutase (SOD) [3].

1,4-dimethoxybenzene (non-SS): The MechoA model assists in predicting these substances as non-skin sensitizing, by showing that the substance in a non-reactive substance and not significantly. This prediction is backed up by an *in vivo* guinea pig study showing that this substance is not sensitizing to skin [4].

Why MechoA and not MOA (Mode of action)? & Comparison to other predictive tools for SS

These conclusions reached using the MechoA model were compared to other models, such as, the classical Verhaar MOA model [5-7], which classifies all 3 substances as class 1: non-polar narcotics; therefore, non-reactive. Toxtree's skin sensitization reactivity domains [8]: which un-correctly predicts 4-methoxyphenol, and Toxtree's protein binding alerts module [9]: which un-correctly predicts 1,4-dimethoxybenzene.

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Minimising suffering in energy deprived rats

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In alignment with the 3R requirements mentioned in the Danish legislation and in the EU Directive [1] on the protection of animals used for scientific purposes, we sought to implement effective interventions to reduce suffering in an animal study using the RSPCA "Road Map" [2] as inspiration.

The purpose of this animal study was to investigate, if an obese rodent model could be a valuable adjunct to conventional healthy animals in the safety evaluation of novel anti-obesity drug candidates as suggested per guideline [3,4]. The study comprised normal weight and diet-induced obese (DIO) male and female Sprague-Dawley rats, undergoing a weight-loss of 20% induced by either food restriction or by daily subcutaneous dosing with an anti-obesity drug candidate for a period of 4 weeks. Pair-housed rats were single housed for 4 weeks prior to and throughout the study period, and underwent daily procedures related to assessment of body weight, food intake, clinical signs as well as body composition analysis, blood sampling and estrus cycle measurements.

The expected level of suffering was classified as "approaching severe" in animals treated with the anti-obesity drug candidate and 'severe' in food restricted animals. This classification was based on a) the intensive and stressful study conditions related to the numerous procedures and b) potential secondary effects related to the moderate body weight loss induced by either the anti-obesity drug candidate or food restriction and 3) experience of hunger in food restricted animals.

A pilot study was conducted to set dose levels for the main study and to investigate the effect of food restriction, monitor the associated suffering and test the utility of our pre-defined mitigating strategies for minimising suffering: intensify surveillance and initiate treatment if animals experienced clinical signs related to body weight reduction such as dehydration, piloerection, porphyrin excretion, hunched posture and abnormal posture/gait. If an animal developed severe clinical signs or approached a 20% loss in body weight, dosing/food restriction should be discontinued or the animal should be euthanized.

Our experience from the pilot study was discussed among animal unit veterinarians, study director, technicians, scientists and the Danish Animal Experiments Inspectorate which led to significant changes in the design of the main study as well as inclusion of additional refinement-initiatives to mitigate procedure-related stress. Furthermore, the step-wise approach to prevent body weight loss related clinical signs in animals were refined to include: close monitoring when body weight reductions approached minus 17%, supplementation of a palatable and energy-rich gel or alternatively a drug-holiday, if body weights continued to decrease. Additionally, a small wooden block was placed between the cage-bars to prevent the stereotypic behaviour described as 'cage biting' observed during the pilot study in the food-restricted animals.

Implementing intervention strategies, inspired by the RSPCA 'Road Map'[2], led to a successful attenuation of clinical signs in the majority of animals. In conclusion, a combination of pre-emptive collaborative, communicative and procedural improvements during the planning and execution of this animal study was an effective 3R refinement initiative.

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Development of an *in vitro* human-based multicomponent 3D joint model as basis for the simulation of an arthritic joint model

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Arthritis or joint inflammation is very common but not well understood. Arthritis is related to more than 100 different types of joint diseases and leading cause of disabilities world-wide. Although arthritis is a matter of research since more than 140 years, there is currently no valid 3D model available which is able to mimic an inflamed joint. Thus, our ultimate goal is to develop a valid human *in vitro* 3D joint model in order to simulate arthritis. The model will contain all involved tissue components and cell types enabling the interactions between cells by cell contacts, signaling molecules and metabolites. As an alternative experimental approach for traditional animal models, our *in vitro* 3D joint model will enable us to finally study the influence and efficacy of drug treatment.

To this end, we firstly developed all single components of the joint, namely the (1) osteogenic and (2) chondrogenic part, (3) the joint space with synovial fluid and (4) the synovial membrane, separately.

The osteogenic component was synthesized by seeding osteogenic differentiated human bone marrow-derived mesenchymal stromal cells (hMSC) on β-tricalcium phosphate (TCP) – mimicking the mineral bony part – coated with an additional hMSC monolayer cell-sheet to get a compact bony component. Survival, adhesion and structural integrity of the cells for up to 6 weeks could be confirmed by cellular release of LDH, LIVE/DEAD staining, and scanning electron microscopy, respectively. Osteogenic differentiation was verified demonstrating an increase in mineralized bone volume and the induction of bone-related gene expression (*RUNX2, SPP1 and COL1A1*) as compared to the corresponding control using µCT and quantitative PCR. Mimicking the chondrogenic part, a scaffold-free 3D cartilage construct was generated by chondrogenic differentiation of hMSC with intermittent mechanical stimulation. Chondrogenic phenotype was verified by HE and Alcian Blue staining as well as by the reduced expression of *COL1A1* and an abundant expression of *COL2A1*. Interestingly, co-cultivation of the osteogenic and the chondrogenic part for up to 3 weeks demonstrated colonization, connectivity and initial calcification implying a functional transitional bridging area. Non-animal stabilized hyaluronic acid was used to simulate the synovial fluid component. To model the synovial membrane, hMSC were differentiated towards the fibroblast lineage and then a confluent layer was formed on a polycarbonate membrane, which was visualized by histology. This confluent monolayer of hMSC, is easily transferable to the other components.

In summary, we confirmed and validated in a standardized manner phenotypic integrity and stability of each single component of our anticipated multicomponent *in vitro* simulation of an inflamed joint. To finalize the development of healthy joint model we will combine the established parts to provide suitable 3D multi-component joint model that enables us to study the efficacy of drug treatment *in vitro*.

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A combined *in silico*-3D lung tumor model to understand network effects of driver mutations for targeted therapy predictions

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Lung cancer shows among cancerous diseases one of the highest mortality rates due to late diagnosis and missing therapies for advanced tumor stages. Remarkably high response rates can be achieved in this tumor entity by targeted therapies as many driver mutations could be detected by large-scale sequencing approaches. Nevertheless, resistance evolvement renders these initial successes often futile. Thus, we developed a tumor model that enables to investigate interdependencies between drivers and other proteins in the complex signaling network of lung cancer [1,2].

At the department of Tissue Engineering and Regenerative Medicine we have established human tumor models on a decellularized porcine gut matrix, that exhibit several tissue characteristics to approximate *in vitro* to *in vivo* conditions. Advantageously, we observe a lower proliferation rate compared to 2D conditions and a better biomarker specific drug response regarding proliferation and apoptosis. The tumor cells are connected to the preserved basement membrane structure. Additionally, they can be cultured with components from the tumor stroma such as fibroblasts and show enhanced growth in bioreactors [3].

In order to understand molecular differences between the 2D and 3D system, we performed a microarray of 2D and 3D *in vitro* culture with two human cancer cell lines that harbor the EGFR activating mutation or not and compared gefitinib (EGFR inhibitor) -treated versus untreated expression patterns. These were integrated into a computational Boolean *in silico* model [4,5] of the lung cancer signaling network that was established according to literature and database information. Applying this model, we calculated system responses upon targeted treatments in specific mutational backgrounds. Furthermore, we sequenced newly generated gefitinib resistant subpopulations to unravel resistance mechanisms. We compared the *in silico* target predictions to results of the *in vitro* and *in vivo* models with the aim to reduce or even replace animal experiments. For efficient optimization of the model we use directly clinical data and implemented our approach into the local molecular tumor board to support therapeutic patient-tailored decisions.

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Vaccine batch to vaccine batch comparison by consistency testing (VAC2VAC)

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VAC2VAC brings together a unique One Health consortium of human and animal health pharmaceutical companies, academia, translational research organisations, Official Medicines Control Laboratories and regulatory bodies with the overall objective to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines. This means that animal-free assays – instead of animal tests – can be used to ensure that each vaccine batch produced is consistent with a batch already proven to be safe and efficacious in registration studies or in clinical use. Hence the name "consistency approach". It covers vaccine potency, safety and animal welfare. The project aims to promote global understanding and acceptance of these new non-animal methods to facilitate international harmonisation and improved vaccine availability globally.

The three main steps to reach these objectives are:

1) Development of new or optimisation of existing non-animal methods for consistency testing
This is the core activity of the project, with a focus on development and optimisation of physicochemical methods, immunochemical methods, cell-based assays, and multi-parametric assays & bioinformatics.

2) Pre-validation of selected methods
For selected methods developed in VAC2VAC, small-scale multi-centre studies will be set up to assess the transferability and inter-laboratory reproducibility of the methods. Methods that are successful in these pre-validation studies and that are proposed for inclusion in regulatory monographs, will be submitted to the EDQM Biological Standardisation Programme to be considered for further validation studies.

3) Regulatory acceptance of the consistency approach
To maximise the chances of regulatory acceptance and implementation of the consistency approach for batch release, the development of methods in VAC2VAC will involve close cooperation between public partners and industry partners in consultation with the regulatory bodies.

The presentation will outline the project in detail and discuss some early results.

Scientific Management Team and Work package leaders: Catrina Stirling[1], Denis Lambrigts[2], Sylvie Uhlrich[3], Coenraad Hendriksen[4], Odile Leroy[5], Paul Sticking[6], Elisabeth Balks[7], Anke Huckriede[8], Arjen Sloots[4], Marlies Halder[9], Joris Vandeputte[10], Hilde Depraetere[5]
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The Laboratory Animal Science and Welfare course at University of Debrecen

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The Laboratory Animal Science and Welfare course at University of Debrecen from the beginning has been organized and coordinated by University of Debrecen Committee of Animal Welfare (UDCAW). The course was accredited by National Scientific Ethical Committee on Animal Experimentation.

The topics of lectures, seminars and practices are based on the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the guidelines of the Scientific Committee of Animal Experimentation of the Hungarian Academy of Sciences and followed the educational requirements of the updated Law and Regulations on Animal Protection, (40/2013. (II.14.) Order of Government of Hungary). The course is based on a modular structure as follows: core modules, function specific modules and additional (task specific) modules.

To improve 3Rs and the quality of surgical interventions applied in experiments we introduced in the framework of task specific modules the advanced anaesthesiology. This topic including: general rules for anaesthesia and analgesia, drugs, species specific dosage of anaesthetics and side-effects of drugs. From similar consideration we are teaching about minimal invasive surgical techniques as basics of microsurgery and laparoscopy, and common surgical procedures on rodents. The practice of preparations for operation and monitoring on large laboratory animals is a new topic also. We would like to emphasise that during the above mentioned seminars and practices experimental animals were replaced with video presentation, surgical phantom models and biomodels. In case of large laboratory animals the participants are joined to a running experiment, reducing the number of experimental animals used.

The number of participants (graduated and postgraduated members of University of Debrecen) who successfully completed the 80-hours (60-hours theoretical and 20-hours practical training) course has been increased significantly after 2013. The range of faculties and institutes participating in the training has also expanded.

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A novel *in vitro* model of neuronal cultures based on 3D chitosan scaffold

Donatella Di Lisa [1], Mariateresa Tedesco [1], Paolo Massobrio [1], Nicolò Colistra [1], Mattia Pesce [1], Tiziano Catelani [1], Elena Dellacasa [2], Roberto Raiteri [1], Sergio Martinoia [1] and Laura Pastorino [1]

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The design of a 3D *in vitro* model of neuronal cultures better recapitulates the natural cell environment and cell-cell interaction, gene expression, synaptogenesis and neurophysiological circuits by studying the dynamics and connectivity of the brain tissue. In order to mimic the ECM environment, a porous and soft structure is preferred in the design of an artificial neural network [1,2].
In this work, chitosan was used for the development of a 3D scaffold. Chitosan is well known for its biocompatibility, biodegradability, muco-adhesiveness as well as its antibacterial activity [3].

In the present study, chitosan microspheres were obtained by phase-inversion process in an ethanol/sodium hydroxide solution, using an aerodynamic encapsulator. The obtained microspheres were characterized by optical, atomic forces (AFM) and scanning electron (SEM) microscopies: the average diameter was found to be around 160 ± 20 µm, spherical shape and a porous structure. The elasticity of the obtained samples was evaluated by AFM. Interestingly, the value stiffness falls in the same range of reported elasticity values for brain tissue (0.7-1 kPa) [4].

For a preliminary evaluation, the microspheres were used as support for the *in vitro* growth of primary neuronal cells (hippocampal and cortical neurons), more representative of the *in vivo* tissue and considered the main model to carry out morphological and electrophysiological characterization comparable with many models present in literature [5].

The scaffold topography, characterized by transmission electron (TEM) and confocal microscopies shows that CHI microbeads are enveloped and penetrated by a dense network of neuritic extensions which is responsible for the assembly and the stabilization of the 3D chitosan based structure. The immunocytochemistry characterization supports this information, showing an adequate adhesion and neuronal proliferation as well as neurite extension. Furthermore, it is noted that 3D cultured neurons, when compared to 2D environments, show distinct morphologies that are more representative of the *in vivo* environment. Finally, a preliminary electrophysiological characterization of spontaneous activity was conducted by Micro-Electrode Arrays. The 3D neuronal networks presented electrophysiological pattern different from those observed in 2D cultures: quasi-synchronous network bursts are mixed with high random spiking activity and the 3D networks is characterized by a longer burst than 2D ones. This behavior indicates the formation of a very dense network with a high degree of anatomical connectivity [1].

This study was an important step to evaluate the bioaffinity of neurons on chitosan scaffold; a forthcoming study will be the testing of the scaffold with induced pluripotent stem cells and the functionalization of chitosan microbeads, using brain-derived neurotrophic factor (BNDF), nerve growth factor (NGF) or other growth factors which play a critical role in proliferation, differentiation and survival of developing peripheral and central nervous system neurons.

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144

Modelling sporadic Alzheimer's disease using patient derived iPSCs

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Alzheimer's disease (AD) is the most common type of dementia affecting one in eight adults over 65. Most AD cases are sporadic, with unknown etiology, only 5% of all AD patients present familial monogenic form of the disease. In the current study our aim was to establish an *in vitro* cell model based on patient specific human neurons to study the pathomechanism of sporadic AD.

We compared neurons derived from iPSC lines of early-onset familial AD (fAD) patients, all caused by mutations in PSEN1 gene, with late-onset sporadic AD (sAD), and three non-demented control individuals. The iPSC lines were differentiated towards mature cortical neurons and AD pathological hallmarks were analysed by RTqPCR, ELISA and Western blot methods over a 10-weeks-long differentiation period.

Neurons from fAD and sAD patients show increased phosphorylation of TAU protein at all investigated phosphorylation sites. Relative to the controls, neurons derived from fAD and sAD patients exhibited higher levels of extracellular amyloid β1- 40 (Aβ1 40) and amyloid β1 42 (Aβ1 42). However, significantly increased Aβ1 42/Aβ1 40 ratios were observed only with fAD patient's samples, which is one of the pathological markers of the disease. Additionally, we detected increased level of active glycogen synthase kinase 3 beta (GSK3B), a physiological kinase of TAU, in neurons derived from AD iPSCs, as well as significant upregulation of amyloid precursor protein (APP) synthesis and APP carboxy-terminal fragment (APP CTF) cleavage. Moreover, elevated sensitivity to oxidative stress, as induced by amyloid oligomers or peroxide, was detected both in fAD and sAD-derived neurons.

Based on the performed experiment we can conclude there is no evident difference except secreted Aβ1-40 levels in phenotype between fAD and sAD samples. To our knowledge this is the first study which compares the hyperphosphorylation of TAU protein in fAD and sAD iPSC-derived neurons. Our findings demonstrate that the iPSC technology is suitable to model both fAD and sAD and may provide a platform for developing new treatment strategies for these conditions.

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FISH on CHIPS: Development of a novel *in vitro* system of the fish intestine

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Piscine intestinal barrier function is maintained by specialized epithelial cells that are organized in a complex and dynamic environment. Our *in vitro* approach focuses on the creation of a realistic model of the fish intestine by culturing rainbow trout cell lines on self-fabricated microchips in the absence and presence of flow. This novel approach allows for realistic exposure and transport scenarios in order to evaluate, for example, the potential uptake of environmental pollutants in fish.

The development of the first "fish-gut-on-chip" was achieved in three steps: 1) Creation of ultrathin and porous membranes as analogue to the delicate and highly permeable basement membrane that underlines the epithelial cells *in vivo*; 2) Combination of epithelial cells and cells of a supporting lamina, namely fibroblasts, on the newly developed membranes to support cellular crosstalk; 3) Application of mechanical forces by fluid flow on the epithelial interface within a microfluidic bioreactor to mimic the passage of intestinal liquids.

Using standard microfabrication technology, we were able to engineer superior cell culture support membranes composed of either aluminum oxide [1] or silicon nitride with a thickness of only 1 µm or less and with pore sizes in the nano- and micrometer range. These membranes perfectly support the co-culture of the intestinal epithelial cell line, RTgutGC, and the newly established intestinal fibroblast cell line, RTgutF, judged based on transepithelial electrical resistance (TEER) and confocal microscopy. Indeed, adding fibroblasts to the classical epithelial barrier model [2] had a positive effect on barrier tightness, suggesting that fibroblasts play an active role in the modulation of epithelial barrier functionality. Low and moderate shear stress from fluid flow, as occurring in the intestine, was subsequently mimicked in a unique, multi-well plate based flow reactor. Epithelial cell resistance was found to functionally adapt to the distinct flow rates resulting in improved mimicry of the intestinal epithelium *in vivo* compared to cells cultured under static conditions.

In conclusion, our "fish-gut-on-chip" system recapitulates basic intestinal architecture and physiology of the piscine intestine and hence has the potential to bridge traditional 2D cell culture models and *in vivo* animal experiments.

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Off-target effects of novel purine-analog phosphonates for anti-cancer therapy

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Novel guanosine purine-analog phosphonates have been developed by molecular modelling for improved treatment of non-melanoma skin cancer [1]. The two lead structures, OxBu and OxHex, outperformed 5-fluorouracil not only in cytotoxicity tests with skin cancer cells, but appeared also effective against head-and-neck, colorectal, and adenocervical cancer cells [2]. Following initial studies on efficacy in organotypic skin cancer models [3], we herein investigate the off-target effects of these two investigational new drugs in comparison to a series of reference compounds.

While *in silico* models for skin permeation predict OxBu and OxHex to be absorbed comparably to 5-fluorouracil, both intestinal absorption and blood brain barrier permeation are predicted to be less for both test compounds [4,5]. Phosphorylated purine-analog phosphonates exhibit liver toxicity (OxHex-DP < OxBu-DP). However, liver toxicity of clinically-used adefovir-DP or tenofovir-DP surmounts the hepatic toxicity in the *in silico* model [4]. Neither OxBu nor OxHex are mutagenic substances in the Ames II Assay (*Salmonella typhimurium* reverse mutation assay) in the absence and the presence of metabolic activation.

Focusing on the intended topical application, we next study cutaneous effects and ocular irritation as this is a foreseeable misuse of dermatics. Skin irritation is neither predicted for OxBu and OxHex, nor for the phosphorylated metabolites. These *in silico* predictions were confirmed by testing OxBu and OxHex *in vitro* (OECD test guidelines no.439). Following topical administration, reconstructed human epidermis does not decline in viability following OxBu and OxHex, while sodium dodecyl sulfate reduces tissue viability to 1% compared to the negative control phosphate buffered saline. The *in silico* predictions of OxBu and OxHex as strong skin sensitizers [6,7] are confirmed by *in vitro* tests (OECD test guidelines no. 442C, D, and E). OxBu is peptide reactive and activates dendritic cells, but does not activate keratinocytes. The skin sensitization potential of OxHex could not be determined, since it was not applicable to the peptide reactivity assay due to OxHex's limited solubility. Predictions from TOPKAT's ocular irritancy module for acute eye irritation indicated mild or severe ocular irritancy of OxBu and OxHex, respectively. In contrast, neither the bovine corneal opacity and permeability test (OECD test guidelines no. 437) nor the EpiOcular eye irritation test (OECD test guidelines no. 492) showed any ocular irritation. This difference might be related to the limited predictive power of the Draize test, which has been used for the training data set of this QTSR-based *in silico* model.

In conclusion, hepatotoxicity (*in silico*) and skin sensitization (*in silico* and *in vitro*) stand out as off-target effects of the novel purine-analog phosphonates. Most of the *in silico* predictions were confirmed by *in vitro* assays, but strongly depend on the training data set. Thus, *in silico* models should include data from validated human cell-based *in vitro* tests and from clinical observations.

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Collaboration and complementarity within Three Rs centres

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Several Three Rs centres have recently been established within and outside Europe. These centres focus on a variety of innovative ways for advancing and achieving impact in the Three Rs – the Replacement, Reduction and Refinement of the use of animals used for scientific purposes.

Since 2015, the JRC's EU Reference Laboratory for Alternative to Animal Testing (EURL ECVAM) has hosted two meetings with Three Rs centres from across the EU with the purpose to bring them together to discuss and exchange ideas on a variety of topics of common interest.

Whilst the expertise within the centres may vary, during the meetings shared priorities have been identified and explored as a means of achieving impact in the 3Rs. These priorities include:

- Efforts to reduce animal use in biomedical research
- Communication and dissemination
- Promoting the use of alternative methods/models as biotechnological resources
- Education and training
- Validation towards regulatory acceptance
- Research initiatives supported by 3Rs centres

Furthermore, at the meetings the opportunity for interacting and sharing expertise and activities has served as a basis to further explore closer collaborations.

Networking the wealth of expertise available within the Three Rs centres has the potential to amplify Three Rs impact in many key areas, while allowing identifying specific strengths and priorities and exploring ways in which they could complement each other's competences in each of the three Rs.

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Evaluating tramadol and buprenorphine applied via the drinking water as pain medication in a mouse osteotomy model

Mattea Durst [1,2], Paulin Jirkof [1], Katharina Schmidt-Bleek [3], Frank Buttgereit [2] and Annemarie Lang [2]

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Managing pain in rodents is crucial for animal welfare and experimental quality. Some injectable analgesics need to be administered within short intervals; this comes with increased stress for the animals due to handling and restraining. Administering medication with the drinking water can be a method for refining the application of analgesia. However, recommendations on dosages vary strongly, and reliable voluntary drug intake after painful procedures is controversially discussed. The outline of this project was presented at the EUSAAT meeting 2016 and we now want to present the complete results.

We combined a refinement project with a basic research study on bone healing. Two commonly used analgesia protocols in mouse osteotomy models were used. Tramadol (0.1 mg/ml vs. 1 mg/ml as recommended by the GV-SOLAS) and buprenorphine/ Temgesic (0.009 mg/ml) were administered via drinking water. We used clinical and behavioural parameters e.g. Mouse Grimace Scale, clinical scoring, body weight, food and water intake and nest complexity scoring to asses reduced wellbeing and pain. Additionally, model specific pain parameters like weight bearing and locomotor activity were assessed. To assess stress burden faecal corticosterone metabolites (FCM) were examined. Additionally, bone healing was evaluated with *in vitro* µCT and histology to grade the impact of the pain management on bone healing and the experimental readout.

In all groups, animals treated with the high tramadol dosage lose the most body weight. Operated and Sham treated animals show high reduction in food intake. The water intake varies but is within a normal range. Animals show a higher intake frequency in the night than during the day and animals treated with the high tramadol dosage show less drinking events in the second night and day. FCM values peak on day one after the procedure and drop immediately on day two in all treatment groups. No differences in the PainScore between the treatment groups are detected. The scores are increased in operated and Sham groups. The nest complexity is decreased in all groups and tramadol high treated animals recover slowest. In the model-specific pain parameters animals treated with the high tramadol dosage show higher scores. In conclusion, results show a high impact of anaesthesia itself on wellbeing parameters. The high dosage of Tramadol doesn't provide better pain relief compared to the lower dose of tramadol and buprenorphine, but decreases the general wellbeing of the animals. No side effects on bone healing readouts are seen in the *in vitro* µCT and histology.

By integrating a refinement project in a basic research study, strongly needed evidence based studies on pain management and assessment can be realised under the 3R principles. Animal numbers can be reduced and refinement strategies could be implemented directly in specific animal models. With assessing influences and side effects on experimental readout, potential risks of implementing a new pain management strategy can be estimated. Therefore, researchers may be more willing to use refined methods in their animal model.

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Does lidocaine-bupivacaine as a local anesthetic reduce postoperative pain perception and improve overall wellbeing?

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Introduction

Local anesthesia (LA) as a preemptive analgesia has the potential to decrease overall post-surgical pain perception and hypersensitivity around a surgical wound site. In conclusion, it could reduce the need for post-surgical pain treatment. LA is commonly used in humans and veterinary medicine but has not yet been implemented in the routine pain management in laboratory rodents. In a laparotomy model, well-established in our facility, we test for possible benefits of a Lidocaine-Bupivacaine infiltration in combination with the systemic analgesia Paracetamol applied postoperative via the drinking water.

Methods

In female C57Bl/6 a sham embryo transfer, in male C57Bl/6 mice a sham vasectomy is conducted. Four different treatment groups are compared. The surgery is conducted with either LA in combination with systemic analgesia, with LA or systemic analgesia only. Additionally, animals without surgery receiving anesthesia and systemic analgesia only are used. Lidocaine-Bupivacaine is applied subcutaneous around the surgical site two minutes prior to the incision in the respective groups. The possible benefits of a multimodal pain management with LA over treatment with systemic analgesia only are evaluated by several parameters indicating pain and reduced wellbeing in two setups. In the first setup, we measure the body weight, food and water intake. The intake of the systemic analgesia is assessed and the animal's activity is analyzed. We investigate the nest complexity to depict changes in the animals' wellbeing and possible painful states.

In the second setup, potentially perceived pain is detected with the burrowing test and the Mouse Grimace Scale. We measure the fecal corticosterone metabolites to depict short term stress and apply the von Frey test directly on the surgical site to test reduced hypersensitivity occurring with LA.

Results

In the first setup, female mice showed no differences of the recorded parameters. In both sexes, animals drank a sufficient amount of Paracetamol according to our calculations. No wound healing disorders were observed. The results gathered on the second setup in female as well as the results on both setups in male mice will be presented at the conference.

Conclusion

With the parameters applied in the first setup in females, no differences between the treatment groups were seen so far. We think that with the parameters applied in the second setup, more precise information on the possible benefits of local anesthesia can be gathered.

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In vitro methods for identifying substances with endocrine disruption potential

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Endocrine disruptors (EDs) are exogenous ligands capable to bind to specific cellular receptors or serum transport proteins, potentially contributing to endocrine system disorders. Exposure sources to EDs may come from industry or agriculture, including consumer products such as food packaging materials, thermal paper, plastics, household products, or cosmetics. Interaction of endocrine disruptors with cellular receptors is a molecular initiation event that leads to complex effects. EDs may act in concentrations of 10-5 mol/l and mimic the regulation of endogenous endocrine and paracrine hormones and autocrine signaling molecules, thereby modulating enzyme activities, transport processes, cell proliferation, apoptosis, secretion and coordination of specific cell types. Human receptors may share ligands including endocrine disruptors with varying affinity and efficacy, of which certain may be persistent, leading to bioaccumulation, while others may be rapidly metabolised and act for a limited time. Most endogenous ligands are hydrophilic molecules unable to pass through the plasma membrane such as glycoproteins (e.g., thyroid stimulating hormone, follicle-stimulating hormone, luteinizing hormone), catecholamines (dopamine, adrenaline) and peptide hormones (prolactin, somatotropin, adrenocorticotropic, antidiuretic, parathyroid hormone, calcitonin, oxytocin, insulin, glucagon) with transmembrane receptors associated with ion channels, G-proteins or enzymes. Receptors for lipophilic endogenous ligands able to enter the cell via the plasma membrane such as steroid, thyroid (T4, T3) and corticoid hormones are located in the cytoplasm, functioning as transcription factors. The organism is exposed to a mixture of chemically diverse potential exogenous ligands with variable affinity, efficacy and resistence time, e.g., bisphenols, phthalates, parabens, alkylphenols, polyaromic hydrocarbons, polychlorinated and polybrominated biphenyls, perfluoralkyls, pesticides, synthetic hormones etc. as well as natural compounds such as mycotoxins or phytoestrogens. Therefore, it is difficult to evaluate such multisource exposure *in vivo*. Moreover, human exposure may be influenced by internal environment of the organism, medical, social and ecological factors. Various types of confounders and interactions complicate the evaluation of human and *in vivo* data. *In silico* screening tools and *in vitro* methods detecting the mode of action are therefore effective for hazard identification and should be used more intensively. In pilot studies, selected bisphenols and phthalates, antimicrobial agents based on nanosilver and phthalocyanines, lanthanides, food contact paper, consumer products and waste water from medical facilities were screened using e.g., OECD QSAR Toolbox, Stably Transfected Transactivation *In vitro* Assay to Detect Estrogen Receptor Agonists (OECD TG 455) and yeast-based microplate assay (in compliance with Draft ISO/DIS 19040) in order to determine the interactions with human estrogen and androgen receptors. Substances showing strong estrogenic activity exhibited parallel activity on the androgen receptor. Our results emphasize the importance of further research, in particular the determination of an appropriate battery of alternative *in vitro* methods that will include more specific receptors, such as thyroid, retinoid, liver X, growth hormone receptor, etc. With regard to recent developments in EU legislation, the onset of increasing pressure can be expected for testing chemicals mainly from the group of plant protection products.

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278

Microphysiological flux balance platform unravels the dynamics of drug induced steatosis

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Drug development is currently hampered by the inability of animal experiments to accurately predict human response. While emerging organ on chip technology offers to reduce risk using microfluidic models of human tissues, the technology still mostly relies on end-point assays and biomarker measurements to assess tissue damage resulting in limited mechanistic information and difficulties to detect adverse effects occurring below the threshold of cellular damage. Here we present a sensor-integrated liver on chip array in which oxygen is monitored using two-frequency phase modulation of tissue-embedded microprobes, while glucose, lactate and temperature are measured in real time using microfluidic electrochemical sensors. Our microphysiological platform permits the calculation of dynamic changes in metabolic fluxes around central carbon metabolism, producing a unique metabolic fingerprint of the liver's response to stimuli. Using our platform, we studied the dynamics of human liver response to the epilepsy drug Valproate (Depakine™) and the antiretroviral medication Stavudine (Zerit™). Using E6/E7LOW hepatocytes, we show TC50 of 2.5 and 0.8 mM, respectively, coupled with a significant induction of steatosis in 2D and 3D cultures. Time to onset analysis showed slow progressive damage starting only 15-20 hours post-exposure. However, flux analysis showed a rapid disruption of metabolic homeostasis occurring below the threshold of cellular damage. While Valproate exposure led to a sustained 15% increase in lipogenesis followed by mitochondrial stress, Stavudine exposure showed only a transient increase in lipogenesis suggesting disruption of β-oxidation. Our data demonstrates the importance of tracking metabolic stress as a predictor of clinical outcome.

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Impact of spheroidal age on drug toxicity and diffusivity

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The enhanced predictive power of 3D multi-cellular spheroids in comparison to conventional monolayer cultures makes them a promising drug screening tool. However, clinical translation for pharmacology and toxicology is lagging its technological progression. Even though spheroids show a biological complexity resembling native tissue, standardization and validation of drug screening protocols are influenced by continuously changing physiological parameters during spheroid formation. Such cellular heterogeneities impede the comparability of drug efficacy studies and toxicological screenings. In this paper we demonstrated that aside from already well-established physiological parameters, spheroidal age is an additional critical parameter that impacts drug diffusivity and toxicity in 3D cell culture models. HepG2 spheroids were generated and maintained on a self-assembled ultra-low attachment nanobiointerface and characterized regarding time-dependent changes in morphology, functionality as well as anti-cancer drug resistance. We demonstrated that spheroidal aging directly influences drug response due the evolution of spheroid micro-structure and organo-typic functions, that alter inward diffusion, thus drug uptake.

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Investigation of endocrine effects of sublethal cadmium doses on the reproduction system of the freshwater snail *B. glabrata*

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Endocrine Disruptors (EDs) are substances which hinder natural hormones to bind to its receptor or which inhibit or activate hormone receptors themselves. To investigate the mechanisms underlying the interaction of EDs with the hormonal system, examining the effect on isolated cells is not meaningful, as the endocrine system exhibits highly complex pathways throughout the whole organism [1,2]. To avoid animal-testing on mice or rats, genetic commonalities between vertebrates and invertebrates can be utilized to investigate the effects of EDs across species [2].

In this project, the effects of cadmium on the reproductive system of the freshwater snail *Biomphalaria glabrata* is investigated. In general, snails have the feature to accumulate pollutants in their tissue. Furthermore, *B. glabrata* is a well-known model organism due to its importance in medical research where its interaction with the trematode *S. mansoni* is studied. The fact that its genome has been recently sequenced facilitates the use of molecular methods [3].

To test the impact of cadmium on an endocrine level, the transcription levels of 4 selected genes involved in reproduction (ovipostatin, yolk ferritin, vitellogenin, estrogen receptor) will be determined and compared with a positive control (β-estradiol) using RT-qPCR. To do so, selective primers for the 4 chosen genes were designed and the amplicons were sequenced. Subsequently, the optimal melting temperatures were examined by conventional PCR. Further steps will be to expose snails to 10 µg/L cadmium and 10 and 100 ng/L β-estradiol for 21 days. The number of egg clutches will be counted, the ovotestis and midgut gland of the snails will be dissected, and RNA will be isolated and reverse transcribed into cDNA.

So far, the optimal RT-qPCR settings for the estrogen receptor primer were determined. A primer concentration of 100/50 nM (upstream/downstream) proved to be optimal. The next steps will be to determine the settings for the other primers and to acquire RT-qPCR data for all samples of the experiment.

If the exposure of snails to cadmium changes the expression levels of genes involved in reproduction, this would indicate that cadmium acts as metallohormone in snails [4]. Moreover, this project will be the first step to establish a simple invertebrate detection assay with *B. glabrata* in order to test endocrine properties of any chemicals. The gained knowledge can further be transferred to higher organisms.

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45

Ecological Threshold for Toxicological Concern (eco-TTC) – applications for environmental risk assessment in various contexts

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The Threshold for Toxicological Concern (TTC) is well-established for assessing human safety but has only recently been explored in the ecological context. Ecological Thresholds for Toxicological Concern TTC (eco-TTC) summarize the wealth of ecotoxicological information as Predicted No-Observed Effect Concentrations (PNECs) on diverse chemical substances in the form of probability distributions. These enable the prediction of untested chemicals based on a structural attribute, mode of action, or functional use. The approach may be useful for assessing chemicals at early tiers of the risk assessment process, providing hazard perspective on chemicals that lack QSARs, guiding product development discussions, and assisting read across or category justifications.

An ecotoxicological database was developed based on recent assessments of published data and international chemical management programs. This ecotoxicity data is associated with physical chemistry data and curated taxonomic information for the organisms tested, including a process to conclude acute and chronic effects as well as identify the PNEC for exposed ecosystems based on depth and breadth of data. Several mode of action schemes are also included to facilitate development of a best approach for grouping compounds.

To make these data accessible and useful to stakeholders, the dataset was transitioned from Microsoft Excel and Access into a modern MySQL format, allowing for a format that is relational and scalable, facilitating easy access, sharing, and integration with other datasets and tools. The dataset is accessed via a web-based query system that is integrated with PNEC calculator and probability distribution tools. The novel interface allows users to explore the data, upload additional datasets, derive threshold values based on specific criteria, and explore the potential use and application of the eco-TTC concept.

An international workshop was held to discuss and evaluate the feasibility of the eco-TTC approach, which included evaluation of several case-studies based on particular decision-contexts (e.g., prioritization and screening, chemical risk assessment, site specific risk assessment, mixtures, product development, criteria development). The discussions and conclusions from that workshop will be presented, including exploration of how this approach could be applied and integrated into evaluation strategies (e.g., IATA).

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204

Tomorrow today: Organ-on-a-chip advances towards clinically relevant pharmaceutical and medical *in vitro* models

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Organ-on-a-chip technology offers the potential to recapitulate human physiology by keeping human cells in a precisely controlled and artificial tissue-like microenvironment. The current and potential advantages of organ-on-chips over conventional cell cultures systems and animal models have captured the attention of scientists, clinicians and policymakers as well as advocacy groups in the past few years. Recent advances in tissue engineering and stem cell research are also aiding the development of clinically relevant chip-based organ and diseases models with organ level physiology for drug screening, biomedical research and personalized medicine. Here, the latest advances in organ-on-a-chip technology are reviewed and future clinical applications discussed. We have successfully integrated different electro-analytical, magnetic and optical detection methods in microfluidic devices to detect cell-to-cell and cell-to-matrix interactions. In course of the presentation various components including microvalves, micropumps, degassers, actuators and sensing systems for lab-on-a-chip will be presented as well as their application for live-cell microarrays and organ-on-a-chip systems.

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Swiss 3R Competence Centre: Promoting research & education on 3Rs

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Considerable progress took place in the last decade at an international level for the refinement, reduction and replacement (principles of 3Rs) of animal experimentation for regulatory purposes. On the 27 March 2018, the Swiss 3R Competence Centre (3RCC) has been founded to further promote the principles of 3R in the areas of research and education.

Under the presidency of Dr Kathy Riklin, member of the Swiss National Council, the 3RCC represents an association of academia, industry, regulators, government and animal welfare association including the eleven most important Universities and Higher Education Institutions from Switzerland, the Swiss association of pharmaceutical industry (Interpharma), the Swiss Federal Food Safety and Veterinary Office (FSVO) and, the Swiss Animal Protection. The 3RCC also benefits from an important support from the Swiss State Secretariat for Education, Research and Innovation (SERI), as it represents a scientific centre of national importance working on a non-commercial basis according to article 15 of the Federal Act on the Promotion of Research and Innovation (RIPA).

Situated in Bern, the Swiss 3R Competence Centre will subsidize scientific projects of quality and establish an educational program and communication strategy to promote the principles of 3Rs. In addition, through its educational program and communication strategy, the centre aims at making accessible to all those involved and/or interested on animal experimentation, up-to-date information on alternative methods to animal experimentation. Finally, the 3R Competence Centre will monitor progress made regarding the implementation of the principles of 3Rs in Switzerland and will offer its services to authorities, teaching bodies and other interested parties willing to gain additional information on the principles of 3Rs and on alternative methods to animal experimentation.

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33

Reverse dosimetry approaches for potential endocrine disruptors

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In animal-free risk assessments, *in vitro* obtained effect concentrations should be translated to external, e.g. oral, doses by kinetic modeling (reverse dosimetry). For this purpose, we extrapolated lowest effect concentrations (LOEC) from *in vitro* assays for potential endocrine effects to lowest observed effect levels (LOEL).

Here, we compare 1) a simple kinetic 1 compartment model versus 2) an 8 compartment PBTK model. For 1), the calculated, dose dependent steady state concentration in rat plasma for a continuous dosing scenario was related to the effect nominal concentration *in vitro* whereas for 2), dose dependent maximum plasma concentrations and nominal concentrations in the *in vitro* assays were used as dose metrics to link *in vitro* to *in vivo*. Substance specific input parameters for 1) are molecular weight, plasma protein binding (PPB) and hepatic clearance whereas for 2) additionally logP and apparent permeability through Caco-2 cells (Papp) were taken into consideration. Calculations were performed for 10 compounds (e.g. Bisphenol A (BPA), Fenarimol (FEN), 17α- Ethinylestradiol (EE), Acetaminophen (APAP), Caffeine (CAF), Ketoconazole (KET), Flutamide (FLU), Genistein (GEN), Methyltestosterone (MTT), Trenbolone (TRE)), using lowest effect concentrations from *in vitro* assays for interaction with estrogen and androgen receptors as well as steroidogenesis. To evaluate the quantitative reverse dosimetry, *in vitro* based extrapolated oral LOELs were compared to determined LOELs in the rat.

For 1) and 2), 7 and 6 out of 10 substances resulted in estimated LOELs in the same order of magnitude than *in vivo*-derived LOELs, respectively (assessed as correctly predicted). Correct predictions in both models were obtained for BPA, FEN, APAP, CAF and KET, whereas in model 1) FLU and MTT and in model 2) GEN were additionally correctly predicted. It should be mentioned that although less complex, the simple kinetic model yielded results closer to the measured *in vivo* LOELs than the PBTK model for 6 out of the 10 modeled substances (FEN, APAP, CAF, FLU, MTT, TRE). In conclusion, our results demonstrate that for reverse dosimetry, also the application of a simple 1 compartment model is possible in principle.

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241

Investigation on *ex-vivo* functional interactions between tumor cells and lymphoblastoid cells in chordoma

Federica Torricelli [1], Benedetta Donati [1], Alessia Ciarrocchi [1], Birgit Lohberger [2], Andreas Leithner [2], Bernadette Liegl-Atzwanger [3] and Beate Rinner [4]

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Chordomas are rare malignant tumors that develop from embryonic remnants of the notochord and arise only in the midline from the clivus to the sacrum. As chordomas are resistant to chemotherapy, further treatment options are urgently needed. Recently we succeeded in the establishment of a clival chordoma cell line named MUG-CC1 and a spontaneously established non-tumorigenic lymphoplastoid cell line MUG-CC1-LCL originating from the same chordoma patient. MUG-CC1 is strongly positive for brachyury, cytokeratin, and S100. During chordom cell line cultivation a lymphoblastoid EBV-positive cell line grew out, we characterized the suspension cells in detail. The immortalized lymphoblastoid cell line MUG-CC1-LCL provides material for a variety of assays and will be used as a source of biomolecules like DNA, RNA, and proteins. A new, well-characterized clival chordoma cell line, as well as a non-tumorigenic lymphoblastoid cell line should serve as an *in vitro* model for the development of potential new treatment strategies and to further understand the pathogenesis and tumor biology of skull base chordomas [1].

The interaction of tumor cells with the surrounding microenvironment including tumor associated fibroblasts and immune cells is a major research area. However, limited attention has been focused upon interactions between cancer cells and normal cells. The aim of this study was to figure out the influence of non-tumorigenic cells on chordoma cells. To this end we performed RNA sequencing gene expression profile analysis and *in vitro* experiments to assess proliferation, migration and growth behavior. We also performed metabolic analysis of metabolic profile by NMR technology. The experiment was set up in triplicates with cultivation of MUG-CC1 and MUG-CC1-LCL alone and co-cultivation of both cell types. Lysate and supernatant of each cultivation condition was investigated. We also explored by RNA-Seq changes in the gene expression profile of MUG-CC1 and MUG-CC1-LCL upon co-culture. Exposure to autologous immune cells induced profound changes in the gene expression profile of MUG-CC1. A total of 811 genes were deregulated in MUG-CC1 upon co-culture with MUG-CC1-LCL of which the vast majority was up-regulated. Ingenuity Pathway Analysis (IPA) showed that exposure of MUG-CC1 to MUG-CC1-LCL induced a strong activation of immune-related and inflammation related pathways. In particular activation of Interferon (Z-score = 8.118, p-value = 8.34e-29) and NFKB (Z-score = 6.037, p-value = 1.25e-16) signaling seemed to be crucial in this interplay.

The interaction of the chordoma cells with immune cells was clearly demonstrated and can be used in further consequence as innovative therapy options

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137

Gene edited non-human primates (NHPs) – further ethical challenges. And the responsibility of scientific journals

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There is a broad discussion about the benefits and the necessity of non-human primates (NHPs) for fundamental research. Considering the SCHEER report (2017) and other publications NHPs are still needed for research, but they state that experiments with NHPs should only be permitted if unavoidable and no alternative methods are available. A clear suggestion how to decide about necessity and who will decide about it is missing. Within all these reports the discussion about the use of gene edited NHPs comes rather short, though there is a strong interest in their use worldwide due to new methods that probably will increase its feasibility. Both the Brain/MINDS project in Japan and the China Brain Project strongly support the use of gene edited NHPs.

I assume that there is no need for gene edited NHPs in biomedical research due to many reasons e.g. aspects of epigenetics and nature via nurture something completely neglected in the ongoing discussions. But in face of the ongoing discussion that primarily support the use of gene edited NHPs I like to point at some welfare issues. If we assume that gene edited NHPs will be necessary to improve human health many questions arise, some might be valid for all NHPs and other animals as well: Who will decide which disease model to choose and based on what sorts of assumptions? How to deal with gene-edited animals – some might suffer e.g. pain or distress over long periods of time. How to establish and implement an analysis about the benefits for humans and the harm done to the animals – a task that will be quite challenging when it comes to gene edited NHPs. Here I like to focus on the role of journals: Journals need to accept their responsibility and they should report about the benefits of gene edited NHPs in medical research in a more balanced way as hitherto. Today many articles highlight and overestimate possible benefits for humans while hardly mentioning the harm done to the animals. All sorts of overestimation will harm the credibility of science. I would like to argue that journals might even try to push towards higher shared animal welfare standards and thus be a driving force for the improvement of animal welfare issues and there is no reason why they could not be beyond those required from different ethical boards or institutions.

Scientists and journals need to take their responsibility. They will face great challenges when it comes to the use of gene edited NHPs in medical research and they should start to deal with these challenges in a more realistic and down-to earth way and start to communicate and argue based on reasonable assumptions. We need to drive our ethical standards to higher grounds and not everything possible is justified.

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129

Sensitivity of CaCo-2 3D spheroids for Fresh Royal Jelly (F-RY) (M) and HuIFN-αN3

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The interest in using 3-D *in vitro* models is increasing. They are designed for quick screening of formulations and sensitivity with the possibility for employment in *in vivo* condition. Spheroid cultures possess a complex network of cell-cell contacts and advanced extracellular matrix development, as well as pH, oxygen, metabolic and proliferative conditions analogous to the vascular and avascular regions of solid tumours. They really mimic *in vivo* micro tumours. The presented experiments were aimed to analyse the sensitivity of CaCo-2 3D stellate Spheroids for Fresh Royal Jelly (F-RY) (M) and HuIFN-áN3 for volume, growth, apoptosis and cytotoxicity.

The Fresh Royal Jelly (10 mg/ml) (F-RY) (M) and HuIFN-áN3 (5000 I.U. /ml) were used. The spheroids were first treated with HuIFN-áN3 and followed with the F-RJ (M). CaCo-2 3D stellate spheroid volume was plotted over a 12-day period following a 24 h incubation with HuIFN-áN3 following with RJ. The growth of 3D spheroid was completely impeded after incubation with 5000 I.U. /ml of HuIFN-áN3 following with F-RJ (M) (10 mg/ml). Interestingly, following re-treatment on day 7 demonstrated greater inhibition of spheroid growth. The changes in the 3D stellate Spheroids volume was: from 70 ěm3 to 12-15 ěm3 after seven days and one retreatment. The cytotoxicity level after the treatment was measured. The 3D stellate CaCo-2 spheroid viability declined to 20-25%. Additionally, the apoptosis level was determined. The increase of apoptosis of the viable 3D stellate CaCo-2 spheroid in the level of 35-48% was observed.

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An *in silico* model on inflamed arthritic joints

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The joint disease osteoarthritis (OA) leads to damage of cartilage and bone, and results in painful and immobile joints. Until now, the knowledge about pathways involved in OA and their cross-talk mechanisms is limited [1,2,3]. Thus, only a symptomatic treatment of the disease is applicable. Additionally, in research towards therapeutic approaches for rheumatic disorder mainly rodents are used as *in vivo* models although the results are barely transferable to the human organism. So far, there does not exist an appropriate model which is able to simulate an inflamed arthritic joint including all signaling molecules, cells and tissue types. Therefore, we evaluate an *in vitro* and *in silico* model for OA compared to current modeling approaches as described by Catt et al. and Kar et al. [4,5]. We describe the biological processes by differential equations considering, e.g., the change of chondrocytes, collagen II and interleukin-1 over time, and estimate model parameters such as apoptosis or production rates. Optimal parameter values are determined by minimizing the difference between the *in silico* model data and the *in vitro* observations in terms of a nonlinear least squares problem. The integration of collected data into the mathematical *in silico* model also allows for refining the *in vitro* model. By combining the methods used in biological developmental research and those used in mathematical systems biology we aim at developing a valid, efficient and attractive alternative to animal experiments in the arthritis research.

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Animal protection and 3R in Switzerland

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For many years, animal protection organisations have primarily concentrated on the development and implementation of alternatives in order to reduce the consistently high numbers of animals being used in the laboratories. However, in spite of the proven commercial and scientific potential of procedures that avoid testing with animals, up to now far too little use is being made of these methods – even though it has long been known that the quality and validity of such tests is better. The transferability of results obtained from studies based on animal testing has also been highly controversial for many years. The development, recognition and application of the 3R principles were laid down in our laws on animal protection some decades ago [1], but to date we still have to view our progress in the area of alternative methods with some criticism. It is true that the year 2000 saw the lowest value ever statistically recorded for animals used in experiments (566,398). Since then, however, this number has risen continuously by about 11% to approximately 630,000 animals/year[1]. It is not simply the extreme financial imbalance between the support provided by the state for animal testing in comparison with the rather more shabby funding for the 3R and/or alternative methods that should give us pause for thought in this context[2]. The recently publicised shortcomings of many animal experiments being undertaken at Swiss higher education institutions and universities also ought to make us sit up and take notice [2,3]. A transition has taken place in the use of laboratory animals, from industry into university research. The number of animals used for animal testing in state-supported basic research has risen by about 30% since the year 2000. In view of REACH, together with nano and genetic engineering, we fear that this number is destined to grow still further.

Despite the seemingly inherent deficiencies in the "animal testing" research model, so far neither animal lovers nor those involved in animal protection have been able to identify any significant progress towards abandoning animal testing in favour of the alternatives. In Switzerland, the constant pressure from animal protection organisations, together with political commitment, have now led to the establishment of a new 3R Competence Centre (3RCC). Expectations are high for an improvement in research combined with (significantly) fewer animal experiments.

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[1] Worldwide, about 25 million animals are used for animal testing every year. In comparison with the EU, where an average of 12 million animals are used in experiments per annum, and with Germany (about 2.8 million animals per year) Switzerland plays a significant part in the area of animal testing in relation to its size.

[2] Up to 2017, the ratio was 100%:0.4%, (far in excess of 100 million CHF state funding available every year for animal testing, with only about 400,000 CHF provided for the development of alternative methods via the former 3R Research Foundation, SF3R, 1987-2017). http://www.tierschutz.com/tierversuche/docs/ersatzmethoden.html and http://www.tierschutz.com/media/230413/pdf/report_steuergelder_tierversuche.pdf

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Use of publicly available transcriptomics data to support the steatosis AOP development

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The concept of Adverse Outcome Pathways (AOPs) as tools to facilitate human risk assessment is gaining popularity. These constructs are designed to provide a clear-cut mechanistic representation of critical toxicological effects that span over different layers of biological organisation.

The main objective of this study, apart from support of AOP development, is to provide a practical example of reuse of toxicological and transcriptomics data. In brief, *in vivo* (whole liver) and *in vitro* (primary hepatocytes) rat transcriptomics TG Gates data on a set of prototypical (i.e. *in vivo* proven) steatotic and non-steatotic compounds was collected for multiple time-point and doses and re-analysed and mapped to Reactome. The outcome was then compared and integrated in the existing steatosis AOP network.

The results showed that *in vitro* data is supporting the mapping to already existing events, whereas *in vivo* data can be used to enrich the AOP with new nodes/processes. In conclusion, the created workflow for data extraction and AOP enrichment is a valuable tool for new hypothesis generation and fast re-use of public data.

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224

Lung simulation – an alternative approach to animal testing for applications in aerosol and respiratory research

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Current developments in legal provisions and humane experimental procedures have shown the necessity of increasing the number of opportunities for alternatives to animal testing procedures. This topic especially seems challenging when a generic environmental factor, like ubiquitous aerosols, are taken into account. Approaches in aerosol and respiratory research, allowing conclusions on deposition and actually inhaled and exhaled particle number concentrations, have to be developed into this area as well. The xPULM simulator shall close the gap from already existing and well established procedures and experimental setups from the field of numerical simulation, over mechanical simulation approaches to *in vitro* procedures. All of the named test methodologies allow very specific and comprehensive research on effects of aerosol inhalation, but lack information on an organ scale level. The active breathing lung simulator xPULM, aims to bridge this gap by allowing multiple lung equivalents, like latex bags and primed porcine lungs (salvaged from the meat production process), to breath under different physiological and pathological breathing patterns, taking up surrounding particles which can be analysed in terms of their number concentration and size distribution.

Several approaches of numerical models are at hand for simulating the path of particles in the human respiratory tract. (1) Most of these simulations either include simplified geometries or only include a partial section of the entire respiratory system. These models are well defined and provide good results for the calculated deposition of particles and even the geometrical particle distribution. Nevertheless, such models are limited by the complexity of the human lung. Another approach for lung simulation without the use of animals, is based on mechanical breathing simulation. (2) These models often use simplified mechanical representatives of the human lung, like polymer bags or rigid cylinder piston systems. Some also include porcine lungs for particle inhalation. (3) The third and wide spread option is to use the lab-on chip approach in order to include some few cell types in a 3D model and expose these cells to aerosols. (4) All of these simulation types are based on assumptions or simplifications, or include only a part of the respiratory system in the model. By its composition, including mathematical models as basis for the breathing patterns as well as mechanical components in order to simulate the thoracic environment and including tissue options as lung equivalents, the xPULM simulator (5) can be seen as a cyberphysical system. Next steps include the integration of fresh vivid porcine lungs (salvaged from an abattoir) using the approach of *ex vivo* lung perfusion to keep the organ alive under physiological conditions outside of the animal's body. Such a vivid organ will fully represent the complex inner geometry of a lung, combining the opportunity of breathing simulation, aerosol deposition measurements and actual local particle concentration evaluation.

This approach allows the opportunity to move respiratory and aerosol deposition research further into the field of alternatives to animal testing, in concordance with European legislation and the 3R principles of humane experimentation.

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Xenobiotic metabolism in the fish hepatic cell lines Hepa-E1 and RTH-149, and the gill cell lines RTgill-W1 and G1B: Biomarkers of CYP450 activity and oxidative stress

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The use of fish cell cultures has proven to be an effective tool in the study of environmental and aquatic toxicology, and in supporting the 3R principle. Valuable information can be obtained from comparisons between cell lines from different species and organs to select suitable models for research. In the present study, specific chemicals were used and biomarkers of CYP450 activity and oxidative stress were measured to assess the metabolic capabilities and cytotoxicity of the fish hepatic cell lines Hepa-E1 and RTH-149, and the fish gill cell lines RTgill-W1 and G1B. These cell lines were exposed to ß-naphthoflavone (BNF) and benzo[a]pyrene (BaP), the pharmaceutical tamoxifen (TMX), and the organic peroxide tert-butylhydroperoxide (tBHP). Cytotoxicity in gill cell lines was significantly higher than in hepatic cells, with BNF and TMX being the most toxic compounds. CYP1-like associated activity, measured through EROD activity, was only detected in hepatic cells; Hepa-E1 cells showed the highest activity after exposure to both BNF and BaP. Significantly higher levels of CYP3A-like activity were also observed in Hepa-E1 cells exposed to TMX, while gill cell lines presented the lowest levels. Measurements of ROS and antioxidant enzymes indicated that peroxide levels were higher in gill cell lines in general. However, levels of superoxide were significantly higher in RTH-149 cells, where no distinctive increase of superoxide-related antioxidants was observed. The present study demonstrates the importance of selecting adequate cell lines in measuring specific metabolic parameters and provides strong evidence for the fish hepatocarcinoma Hepa-E1 cells to be an excellent alternative to animal testing in assessing metabolism of xenobiotics, and in expanding the applicability of fish cell lines for *in vitro* studies.

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Organ-on-a-chip meets traction force microscopy: *In situ* characterization of forces in 3D micro-tissues

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Organ-on-a-Chip (OoC) systems are microfluidic devices which enable the cultivation of 3D tissues in a precisely controllable, physiological microenvironment. In combination with human induced pluripotent stem cells these systems have the potential to revolutionize the drug development process. Therefore, it is essential to accurately characterize the integrated tissues. An important characteristic of many tissues is the force exerted by the cells. This information is useful to characterize for instance the growth of cells and the contraction state of (cardiac) muscle cells. Traction force microscopy (TFM) is a commonly used tool to spatially resolve these forces. Here, we present a TFM system directly embeddable into OoC systems, which consists of an elastic layer with integrated fluorescent nanobeads on the surface. Based on the elastic modulus of the substrate, the force on the surface can be derived from the bead displacement. We highlight that our system directly integrates a gauging mechanism for the determination of the substrate's mechanical properties, allowing the accurate determination of forces by considering each individual sample composition. The presented system enables precise *in situ* measurements of forces exerted by different tissue types in an OoC with a simple fluorescence microscope.

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Establishment of an *in silico* model for drug-target interaction analysis, drug efficacy screening and drug design

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Especially in the field of drug design and efficacy screening, animal testing is still the state of art [1]. Digital alternatives for new treatment options and simulating the drug efficacy are promising to refine, reduce and even replace animal testing. In this regard, we developed an *in silico* model for drug-target-interaction analysis and drug efficacy screenings [2]. Our enhanced fingerprint-algorithm allows comparison and functional annotations of drug targets and provides fast, tailored information on drugs, targets and drug actions. Moreover, docking-analysis provide insight into drug binding motifs, leading to design of chemicals lead structures for potential drugs. This is supplemented by clinical and molecular data to assess the drug efficacy of a therapy [2,3]. We exemplified our *in silico* model to study treatment approaches in a patient with an aggressive molecular subtype of an adenocarcinoma in the tooth, in which resistance to standard therapies occurs. The drug efficacy screening identifies promising drug action for potential therapy. In conclusion, we established an *in silico* model for drug-target interaction and efficacy analysis. This allows finding the best drug for a therapy. We believe that our *in silico* model provides a promising alternative to animal testing according to the 3R principles for preclinical drug efficacy screening and developing novel drugs.

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24

Methodological improvements and automation for better biodetection of endocrine activities *in-vitro*

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Consumer preference for more "natural" and "health-conscious" ingredients, clean labeling and environmentally sustainable packaging is driving innovation within the food industry. These preferences advocate for the substitution of synthetic solutions and well-characterized packaging materials with essential oils, complex botanical extracts, novel flavors, fermented ingredients and recycled materials. Often the proposed substitutions are complex mixtures of hundreds, if not thousands, of unknown and toxicologically uncharacterized substances, each at low doses. As identification and large-scale toxicological testing is not possible for practical reasons, these types of samples are not without a challenge for human-health risk assessment.

To meet this challenge, Nestlé is exploiting a "safety-by-design" battery of *in vitro* toxicity tests to screen for and eliminate candidates with poor toxicological profiles, including endocrine disruption potential, as early as possible in the project life-cycle. At the center of the endocrine disruption- strategy is a series of CALUX-reporter gene assays for nuclear receptor agonism and antagonism. These assays have subsequently been modified to include a number of "upgrades". Firstly, a cell viability measure was added to better differentiate between true changes to receptor-mediated activities and non-specific effects on cell proliferation or cytotoxicity. Next, a liver S9 metabolizing system was added for the detection of samples requiring bioactivation for their endocrine activities. Thirdly, the CALUX assays for ER alpha- and AR-agonism have been coupled to an H295R assay protocol, augmenting the sensitivity of this test to changes in estrogen and androgen synthesis. This modification of the H295R assay increases the relevance of steroidogenesis testing for low-dose exposures. Lastly, the CALUX assay protocols containing these methodological improvements were automated for seed-to-read cell-based screening of samples on a Hamilton STAR microfluidics platform. Comparisons of the results from robot tests with those performed by technicians indicate no loss of assay performance as a result of this automation.

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Modelling Alzheimer's disease in three-dimensional human neural progenitor cultures

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Alzheimer's disease (AD) research currently relies on many transgenic models resulting in the widespread use of animals for the study of the disease. Unfortunately, there are a lack of suitable *in vitro* models that recapitulate the key features of AD; namely the accumulation of amyloid beta (Abeta) plaques, the formation of neurofibrillary tangles and neuro-inflammation leading to loss of neurons and synaptic connections. In our attempt to replace animal models, we took advantage of three-dimensional (3D) cell culture alternative methods to generate a neuronal Matrigel-based *in vitro* model of familial AD (FAD) with mutations in human amyloid precursor protein (APP) and human presenilin 1 (PSEN1). Our data demonstrate that the generated FAD cell line carries the expected genotypes: APP with both K670N/M671L (Swedish) and V717I (London) mutations and PSEN1 with deleted exon 9. The engineered cells differentiated into neurons and astrocytes, as detected by specific neuronal (MAP2 and Tuj1) and astrocyte (GFAP) markers. Long-term (up to 12 weeks) maintenance of these cells in 3D-cultures led to the development of AD-pathology *in vitro*, defined by increased Abeta-secretion, Abeta-deposition and tau-hyperphosphorylation. The FAD neurons grown in Matrigel-based 3D thin-layers exhibited a higher number of Abeta deposits after 6 weeks of differentiation compared to control cells as well as hyperphosphorylated Tau after 9 weeks of differentiation as evidenced by immunofluorescence studies. An imbalance on the three-repeat (3R) to four-repeat (4R) splicing ratio of Tau was also detected by RT-PCR. Taken together, our results show that our *in vitro* human FAD model successfully recapitulates both the Abeta and tau pathologies. Such a cellular tool could further help elucidate pathological mechanisms of AD and accelerate drug discovery by identifying new targets and drugs for AD patients.

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Carp stem cells growth on chorioallantoic membrane of fertilized hen's egg

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Introduction

The use of *in vitro* methods in cancer research is restricted since cultivable mammalian cells are already malignant transformed tumor derived cells or have an intrinsic teratogenic potential in case of embryonic stem cells. Thus a replacement of animal testing by *in vitro* methods in cancer research depends on the availability of "healthy" cell lines which are susceptible for carcinogenic transformation. A promising candidate cell line for the investigation of carcinogenic cell transformation are tissue regenerating stem cells isolated from carp, an animal model with more than tenfold higher live span than any mammalian model used in cancer research. In terms of gene expression these cells correspond to embryonic stem cells of mammals or induced pluripotent stem cells. A transgenic variant of this line showing stable histone 2B-associated green fluorescent protein (GFP) expression has been engineered (KCB GFP DSMACC3285). This cell line provides the advantage of self-signaling enabling kinetic live imaging beginning with the carcinogenesis triggering event to the manifestation of malignant transformation in terms of tumorigenesis and metastasis. Both cell differentiation and tissue formation as well as malignant transformation followed by tumor formation depends, to a large extent, on the interaction with blood vessels. Therefore, angiogenesis plays an important role in tissue formation whether it is healthy or malignant. The hen's egg chorioallantoic membrane (HET-CAM) model is considered an experimental alternative to animal model. The aim of the present study is to assess the relevance of KCB *in vitro* model for higher vertebrates. The effects of KCB GFP, when applied directly on HET-CAM, have been studied in order to find out, if KCB cell-signaling interaction with HET-CAM results in appropriate angiogenesis.

Materials and methods

Suspended KCB stem cells were cultivated on CAM for several days on fertilized eggs at 37°C. Colony formation, cell differentiation and blood vessels formation were analyzed through live imaging. KCB bearing CAM was separated for further detailed computer analysis of stem cells differentiation and blood vessels network structure. GFP and carp specific matrix metalloprotease gene expression was studied using PCR.

Results

On examination, there was a presence of living KCB stem cells showing normal features colony formation and differentiation. Some cells surrounded lipid vacuoles in a manner resembling typical pattern formation of KCB *in vivo*. Further KCB colonies were found adhered to or penetrated by blood vessels. Some cells were differentiated and arranged in an elongated structure surrounding blood vessels. DNA and RNA of KCB stem cells were isolated after cultivation on CAM and GFP and matrix metalloproteinase-9 (MMP-9) expression could be proved.

Conclusion

The stable fluorescence labeling of cell nuclei gives the advantage of real time live imaging of the cells in terms of differentiation, colony (tumor) formation and infiltration into CAM. Qualitative and quantitative analysis of angiogenesis provides an important approach in studying the stem cells differentiation and tissue formation. In view of this variety of *in vivo* processes performed on CAM *in vitro* there is a high potential of replacing animal testing by using the KCB HET-CAM system.

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180

Pelargonic acid – an alternative to glyphosate-based herbicides?

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Glyphosate-based herbicides are widely used in agriculture. However, over the past decades there is growing evidence on the toxicity and genotoxicity of glyphosate on non-target species. In case of a ban on using glyphosate, the availability of alternative active substances will be necessary. Nonanoic acid (a.k.a. pelargonic acid), which is used an alternative to glyphosate-based herbicides, is a biological derived substance considered as an environmental friendly herbicide. In order to assess hazards, safety or the risk of different herbicides, toxicity tests have to be performed. The use of animals for toxicity testing has raised ethical concerns, thus the 3R principle of refinement, reduction and replacement should be applied when determining the toxicity of chemicals.

During a semester course, students of the master study program "environmental management and ecotoxicology" aimed to compare the toxicity levels of the glyphosate-based herbicide Roundup (RU) to the pelargonic acid-based herbicides TopGun (TG). The application concentration of glyphosate in RU is 7.2 g/L, whereby TG contains 31.02 g/L of pelargonic acid. In accordance with the 3R principle, the selected test organisms were the freshwater snail *Biomphalaria glabrata*, zebrafish (*Danio rerio*) embryos, the nematode *Caenorhabditis elegans*, duckweed (*Lemna minuta*), and the fish cell line RTgill-W1.

Acute toxicity tests on *B. glabrata* based on the OECD guideline 243 were performed and revealed IC50 values of 0.05 g/L for pelargonic acid in TG (0.2% v/v) and 0.18 g/L for glyphosate in RU (2.5% v/v). Moreover, LC50 values of 0.03 g/L pelargonic acid in TG (0.1% v/v) and 0.28 g/L glyphosate in RU (3.9% v/v) were determined by performing fish embryo acute toxicity tests according to the OECD guideline 236. Viability and broodsize assays of *C. elegans* furthermore showed that TG had a greater impact on the reproduction of the nematodes after acute and chronic exposure compared to RU. Interestingly, *L. minuta* exposed to RU showed dark spots on the leaves, whereas treatment with TG led to dark roots and a pale leaf colour. Neutral Red Uptake assays were performed on the trout-derived gill cell line RTgill-W1 and IC50 values of 0.7 g/L pelargonic acid in TG (2.3% v/v) and 2.6 g/L glyphosate in RU (36.4% v/v) were received.

The results of all acute toxicity tests indicate that the pelargonic acid-based formulation TG is more toxic than the glyphosate based-herbicide RU. However, detailed ecotoxicological risk assessments and an evaluation of the genotoxicity of both herbicides has to be performed before any conclusion can be drawn.

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Advanced human *in vitro* blood-brain barrier disease models for cerebral ischemia

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In recent years research on the development of *in vitro* blood-brain barrier models (BBB) has increased progressively. Several model set-ups have been established, but the most commonly used model remains the transwell model. This *in vitro* model allows the co-cultivation of several cell types including brain capillary endothelial cells, pericytes and astrocytes as well as the characterization of the BBB tightness via TEER (transendothelial electrical resistance) measurements and permeability studies. Though meeting the majority of relevant parameters, transwell models still lack to resemble physiological *in-vivo* conditions. Therefore, advanced *in vitro* models including dynamic *in vitro* hollow fibre models (DIV-model), organ-on a chip models as well as 3D spheroids and organoids have been developed. These models feature several important physiological parameters such as capillary blood flow inducing shear stress as well as 3D-architecture and direct cell-cell contact. An overview of the available human BBB *in vitro* models focusing on their advantages and disadvantages will be given. A detailed comparison of data using transwell models, DIV-models and 3D-spheroids to resemble pathomechanisms during cerebral ischemia will be presented with regard to the expression and functionality of barrier markers and properties (TEER, paracellular marker permeability, expression analysis of tight junction proteins, ABC- and SLC-transporters). These data are obtained from mono- as well as co-culture models based on human cells such as brain capillary endothelial cells, pericytes and astrocytes and indicate species differences compared to mouse models.

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Persistent endocrine-disrupting chemicals found in human follicular fluid stimulate proliferation of granulosa tumor via IGF1R – 3D cell culture model

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It is well known that follicular fluid (FF) is rich in steroids, growth factors, and proteins produced in the follicles. One of them is an insulin-like growth factor 1 (IGF1) produced in the granulosa cells of follicles and plays an important role in the growth and maturation of follicles. However, epidemiological studies have found that women have also detectable levels of persistent organic pollutants (POPs) such as hexachlorobenzene (HCB), 2,2-dichlorodiphenyldichloroethylene (p,p'-DDE), polychlorinated biphenyl 153 (PCB153), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in their follicular fluid [1,2]. This evidence suggests that POPs in FF may further exert their potential direct effects on oocyte, cumulus and granulosa cell function. Moreover, these compounds can act as agonists or antagonists for hormone receptors and may activate pathways involved in the progression of hormone-related cancers such as granulosa cell tumors (GCTs). GCTs account for 5% of all ovarian tumors are classified into two subtypes based on clinical presentation and histological characteristics: the juvenile (JGCT) and the adult (AGCT) form. In this study, we analyzed the effect of mixture POPs in FF on the proliferation of two human GCT cell lines: COV434 and KGN, which represent the juvenile and the adult subtype, respectively. We further examined if mixture may modulate the effects of IGF1 on granulosa tumors proliferation. Finally, we determined if IGF1R is involved in the action of mixture.

In our study we used of *in vitro* three-dimensional (3D) cell cultures model that closely mimics the tumor microenvironment and is an important alternative for animal experiments. Moreover, two human GCT-derived cell lines, COV434 (ECACC) and KGN (Riken Cell Bank; after approval by Drs. Yoshiro Nishi and Toshihiko Yanase), were cultured in 3D architecture. To form spheroids, 6000 cancer cells were seeded in 96-well CellStar U-bottom plates. Cells were incubated for 72 h to allow spheroid formation.

First, we quantified the basal expression levels of IGF1R in 2D and 3D cell culture, both granulosa cell tumor cell lines by real-time PCR. We observed that the expression level of IGF1R was higher in adult than the juvenile GCT subtype and did not change depending on culture condition. Treatment of the cells with a mixture of the five compounds (Mix 1: PFOA 2ng/ml, PFOS 8 ng/ml, HCB 50 pg/ml, p,p-DDE 1ng/ml, PCB153 100 pg/ml; Mix0.1: 10x diluted; Mix10: 10x concentrated) exhibited the stimulation of the cell proliferation significantly. Interestingly, the adult GCT subtype is more sensitive to proliferative action of the mixtures than the juvenile subtype. We found also, that IGF1 is a very potent proliferation stimulator in KGN cells, but mixture doesn't change the action of IGF1 in granulosa tumors. However, using pharmacological inhibition of IGF1R by PPP, we found that IGF1 receptor is involved in proliferative effect of mixtures in granulosa tumors.

Taken together, our results demonstrate, that mixtures of persistent organic pollutants present in FF may induce granulosa tumor progression through IGF1R by acting as mitogenic factors in granulosa cells.

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Cellular senescence: A response pathway within AOPs?

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Cellular senescence has evolved from a phenomenon considered to be a mere *in vitro* feature of normal human cells in culture to an acknowledged co-driver of age-associated diseases. Indeed, senescent cells have been found to accumulate with aging and damage in various organs and tissues and to be associated with various lesions *in vivo*. Clearing of such senescent cells from mouse models have been very recently published to postpone the onset of several age associated diseases including cardiovascular or musculoskeletal diseases.

Cellular senescence is not only induced by telomere shortening associated with replication as a consequence of the end-replication problem, but also induced by irradiation, free radical species, or several DNA damaging chemotherapeutics, all well known to induce acute toxicity. Especially in the context of chemotherapies, clearance of senescent cells has shown beneficial effects in mouse models in regard to lung fibrosis as a potential side effect. One of the characteristics of senescent cells that seems to specifically contribute to a negative impact on tissue and organ function is the senescence specific secretory phenotype (SASP). We here will present some examples that not only cytokines, but also extracellular vesicles and their miRNA cargo form part of the SASP and impact on cell differentiation and proliferation.

Therefore, we here propose to consider cellular senescence as one cellular response pathway to toxicants also in the context of adverse outcome pathways.

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Bioprinting perfusion-enabled liver equivalents for advanced organ-on-a-chip applications

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Many tissue models have been developed to mimic liver-specific functions for metabolic and toxin conversion in *in vitro* assays. Most models represent a 2D environment rather than a complex 3D structure similar to native tissue. To overcome this issue, spheroid cultures have become the gold standard in tissue engineering. Unfortunately, spheroids are limited in size due to diffusion barriers in their dense structures, limiting nutrient and oxygen supply. Recent developments in bioprinting techniques have enabled us to engineer complex 3D structures with perfusion-enabled channel systems to ensure nutritional supply within larger, densely-populated tissue models. In this study, we present a proof-of-concept for the feasibility of bioprinting a liver organoid by combining HepaRG and human stellate cells in a stereolithographic printing approach, and show basic characterization under static cultivation conditions. Using standard tissue engineering analytics, such as immunohistology and qPCR, we found higher albumin and cytochrome P450 3A4 (CYP3A4) expression in bioprinted liver tissues compared to monolayer controls over a two-week cultivation period. In addition, the expression of tight junctions, liver-specific bile transporter multidrug resistance-associated protein 2 (MRP2), and overall metabolism (glucose, lactate, lactate dehydrogenase (LDH)) were found to be stable. Furthermore, we provide evidence for the perfusability of the organoids' intrinsic channel system. These results motivate new approaches and further development in liver tissue engineering for advanced organ-on-a-chip applications and pharmaceutical developments.

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Organotypic head and neck cancer models for drug testing

Leonie Gronbach [1], Christopher Wolff [1], Konrad Klinghammer [2], Christian Zoschke [3], Ulrich Keilholz [2] and Monika Schäfer-Korting [1]

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Head and neck cancer is the 6th most common cancer type worldwide and affects mostly elderly patients. Although a local drug therapy should be feasible in the oral cavity, no such treatment is available and current options like surgery and systemic drug therapy induce severe adverse reactions. The predictability of preclinical testing of new compounds could be improved [1] and the number of animals in oncological research reduced by a human-cell based, organotypic model. Herein, we developed and characterized a model of head and neck squamous cell carcinoma (HNSCC).

For HNSCC models, cancer cells from patient-derived xenografts [2] were co-cultured with human oral keratinocytes and seeded onto lamina propria equivalents with primary human fibroblasts. Models without cancer cells or with the SCC-25 cancer cell line [3] were built for reference. Whereas normal mucosa models showed a well-structured squamous epithelium, HNSCC models in contrast presented an increased suprabasal layer with rounded swollen cells. The morphology of HNSCC models with primary cancer cells appears closely related to the tumor grading. For first pharmacodynamic experiments, docetaxel (7 µg/ml, 100x more than the plasma value [4]) was applied three or seven times a week. Docetaxel application to either the culture medium or on the tissue surface e.g. reduced tumor size, tumor proliferation, induced cell death (increased lactate dehydrogenase secretion) in HNSCC models whereas cetuximab treatment lacked activity and did not improve the antitumor effects of docetaxel either.

In conclusion, organotypic HNSCC models with human cells capture hallmarks of HNSCC and its treatment. Moreover, testing in the models might allow insights into the signaling between tumor and a human stroma. This will help to improve the current head and neck cancer treatment as well as to reduce the number of animal tests in preclinical drug development.

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126

3D *in vitro* cardiac model established from fish larvae for application in pharmacology

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The importance of studying cardiac physiology in animal models is driven by the prevalence of heart disease in human populations [1]. It could be shown that models established from fish are very suitable for cardiac safety due to the similarities of cardiac ion channel composition of fish and humans. Previously, we reported of the possibility to develop an easy, fast and cost-effective *in vitro* cardiac model system which was established from diverse fish species, like trout, zebrafish, salmon, sturgeon and mareana [2]. It was possible to generate up to five long-term spontaneously contracting cell aggregates (SCC) from one fish larva without using differentiation medium or electrical stimulation [3]. SCCs could be kept in cell culture for up to 6 month while retaining their functionality and displaying contraction rates similar to the fish heart [2]. Electron microscopic analysis revealed that the aggregates develop from heart progenitor cells which differentiate in cell culture into fully developed cardiomyocytes with the typical sarcomere structures throughout the cells [4,5]. Molecular as well as immunochemical analysis provided the existence of muscle proteins like α-Actin, Troponin I, myosin and actinin and proteins like β-Catenin, N-Cadherin, 1&2-Desmoplakin for cell-cell-connections [2,5]. Furthermore, the presence of the protein HCN4 and Connexin45 did prove the existence of a pacemaker centre within these autonomous functional aggregates [2]. Intracellular as well as extracellular electrophysiological measurements revealed the existence and functionality of the ATP-potassium channel, L-type calcium channel and hERG potassium channel. After the addition of common ion channel blockers, contraction frequencies, action- and field potential durations changed significantly like it could be shown for human cardiomyocytes [2,6]. In conclusion, SCCs composed of fully developed cardiomyocytes with human-like electrophysiological properties. As this model system exhibit additionally a high generation.

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275

3D models and multi-organ-chips: Scaffold-free 3D tissue models – from static to microphysiological applications

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Studying and understanding the etiologies of diseases, with the goal of developing novel therapeutic approaches translated into safe and efficient drugs, presents manifold challenges. *In-vitro* cell-based assays represent one of the key group of techniques and with the evolution of complex 3D tissue models receive more and more attention and a greater share in the drug discovery process. Routine implementation of novel *in-vitro* systems requires control over the sometimes complex tissue- and disease-relevant parameters and at the same time simple and robust methods for handling, experimentation and readout. This becomes particularly true when studying organ-organ interactions involving tissue models from different types in fluidic communication.

Our new generation of readily available and screening-compatible 3D microtissues models are able to emulate the healthy and various diseased states of different organ models including human liver and pancreatic islets as well as a large set of tumors. Accessing the biology of the models in a reliable and reproducible way to a large extend depends on the platforms, in which the microtissues are cultured and handled in. We therefore specially engineered and matched our 96 and 384-well plates to the microtissue morphology considering easy, but highest quality optical inspection, reliable and efficient medium exchange and compound dosing preserving maximal functionality and allowing seamless integration into automation systems. Together with the uniform, functionally robust, and long-lived characteristics of the microtissues a complete screening platform for a wide set of efficacy and safety testing can be offered.

The next steps toward more complex *in-vitro* models includes the combinations of such advanced microtissues in a microphysiological system to study their interactions. We extended our technology platform by a microfluidic plate based on SBS standards, which enables culturing of the same microtissues also under physiological flow conditions, and with the flexibility to interconnect and culture different types of microtissues multi-tissue configurations. Up to 10 same or different microtissues can be interconnected and cultured in 8 identical or different conditions in parallel per plate.

Providing continuity of the microtissue models enables maximal translatability between the different pre-clinical applications and control over the increasing model complexity along the drug discovery process.

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274

Disease models using human cells: 3D *in-vitro* models for metabolic diseases show translation in diabetes and liver fibrosis

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NAFLD, obesity, insulin resistance, metabolic syndrome, and Type 2 Diabetes are closely connected clinical pathologies. Further, insults, as for example inflammatory stimuli, contribute to the progression of NAFLD to NASH, an increasing healthcare burden in developed countries and major indication for liver transplant. Studying the etiologies for this group of diseases, with the goal of developing novel therapeutic approaches, presents multiple challenges, such as having readily available organotypic, human tissues with inducible disease states, as well as simple and robust methods for studying organ-organ interactions.

We developed a new generation of screening-compatible 3D microtissue models that emulate the healthy and various diseased states of human liver and pancreatic islets, adaptable to a unique standard multi-well microtissue plate format and a novel microphysiological system to accommodate interconnected culture and assay of such microtissues. In our 3D microtissue models, organotypic liver and islet tissues are metabolically active, highly accessible to experimentation, and importantly, immune competent. Starting from their healthy state, we demonstrate induction of liver steatosis by exposure to high Glucose/Insulin media supplemented with dietary fatty acids (imaging/lipidomics). Treatment with inflammatory agents and dietary-induced injuries trigger molecular events leading to NASH, as shown by the induction of liver fibrosis markers (qRT-PCR, imaging, ELISA). Treatment of the tissues with free fatty acids and LPS in the diabetic medium as compared to the control increased the accumulation of lipids as well as secretion of pro-inflammatory markers such as TNF-α, IL-6, IL-8, MCP-1, MIP-1α, IP-10. Furthermore, lipotoxic stress stimuli increased mRNA and protein expression of pro-fibrotic markers such as the α-smooth muscle actin (α-SMA), a marker of activated HSCs and collagen type I. Sirius Red staining of the liver tissues demonstrated the presence and increased deposition of ECM in the NASH model. Moreover, in 3D islet microtissues, we demonstrate modulation of glucose-stimulated insulin secretion by tolbutamide and Exendin-4 under physiological flow conditions, highlighting the unprecedented insights achievable with advanced physiologically relevant *in vitro* systems.

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259

Bacterially expressed nanobodies can replace secondary antibody production in animals

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Polyclonal anti-immunoglobulin G (anti-IgG) secondary antibodies are essential tools for many molecular biology techniques and diagnostic tests. Their animal-based production (e.g. in goat, sheep or donkeys) is, however, a major ethical problem. Here, we introduce a sustainable alternative, namely nanobodies against all mouse IgG subclasses and rabbit IgG. They can be produced at large scale in *Escherichia coli* and could thus make secondary antibody production in animals obsolete. Their recombinant nature allows fusion with affinity tags or reporter enzymes as well as efficient maleimide chemistry for fluorophore coupling. We demonstrate their superior performance in Western blotting, in both peroxidase- and fluorophore-linked form. Their site-specific labeling with multiple fluorophores creates bright imaging reagents for confocal and superresolution microscopy with much smaller label displacement than traditional secondary antibodies. They also enable simpler and faster immunostaining protocols, and allow multitarget localization with primary IgGs from the same species and of the same class.

Reference

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239

Progress towards the replacement of the rabbit blood sugar assay for quality control of insulins required by US Pharmacopeia (USP <121>) by an *in vitro* test

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The US Pharmacopeia requires in its Chapter "<121> Insulin Assays" for insulins and insulin analogs marketed in the US a bioassay in rabbits to evaluate the biological activity for the release of each batch of drug substance and for the characterization of drug product in development and after major changes during production. The number of animals used in these tests depends on the requirements to meet the respective acceptance criteria.

Sanofi produces all insulins in Frankfurt and exports insulin glargine, a long-acting insulin, the drug substance in the drug products Lantus® and Toujeo®, to the US.

In 2012 Sanofi-Aventis Deutschland decided to develop and validate an *in vitro* cell-based test to replace the rabbit blood sugar assay for batch release of insulin glargine in *a first step*. This *in vitro* test in plate format determines the biological activity of insulin by its binding to the human insulin receptor over-expressed in Chinese hamster ovary cells. This cell-based test was validated to meet the requirements of the US Pharmacopeia and US Food and Drug Administration. A bridging study of *in vivo* and *in vitro* testing revealed superior precision and equivalent accuracy of the *in vitro* test compared to the animal assay. The validation data for batch release of insulin glargine drug substance were submitted to the US Food and Drug Administration and resulted in regulatory acceptance of the cell-based test as an alternative to the rabbit blood sugar assay in 2015. As of January 2016 this animal experiment is not performed in Frankfurt anymore.

As *second step* Sanofi validated this cell-based test for numerous other insulins and insulin analogs. The data were submitted to US Pharmacopeia, presented at scientific congresses and submitted for publication as invited stimuli article to the US Pharmacopeial Forum. The test protocols were evaluated in US Pharmacopeia laboratories and Sanofi made the cell line available through ATCC. Based on this groundworks US Pharmacopeia published the stimuli article [1] and the "*In vitro* Cell-Based Bioidentity Test for Insulin Glargine and Insulin Lispro" as additional method in Chapter "<121> Insulin Assays" [2] in 2017. A summary of these activities has been published recently [3]. Currently the comments to this Draft chapter [2] are discussed in the "Insulin Expert Panel" and the "Expert Committee Bio1" of US Pharmacopeia.

As *third step* Sanofi has submitted final data to US Pharmacopeia showing that this *in vitro* test is also able to replace the rabbit blood sugar when it is used for the characterization of drug product in development and after major changes during production.

The final aim is the inclusion of the cell-based test as an alternative to the rabbit blood sugar assay in the US Pharmacopeia final guideline. This change will enable all pharmaceutical companies producing insulins, insulin analogs or insulin biosimilars for the US market to shift away from the animal assay.

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47

International harmonisation: Progress in establishing VICH guidelines on waiving criteria for general batch safety tests of veterinary vaccines

Marlies Halder [1] and Esther Werner [2]

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General batch safety tests for veterinary vaccines as the Laboratory Animal Batch Safety Test (LABST) or the Target Animal Batch Safety Test (TABST) are supposed to demonstrate that a vaccine does not cause abnormal local or systemic reactions. They have been introduced decades ago during the development of the first veterinary vaccines. However, over the last 25 years, the relevance of the TABST and LABST was questioned due to the introduction of more specific safety and purity tests, strict control of starting material and the introduction of Good Manufacturing Practice. Retrospective analysis of LABST and TABST data revealed that both tests are not able to detect batches causing safety problems and therefore are no longer justifiable. The LABST (or abnormal toxicity test) had already been removed from European Pharmacopoeia monographs for veterinary vaccines in 1997, and the TABST in a stepwise approach until its complete deletion in 2013. However, outside of Europe and European Pharmacopoeia countries, general safety tests may still be required for batch release.

In 2008, Europe proposed to The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) to aim at harmonisation of general batch safety tests across the VICH regions (USA, Japan, Europe) in order to minimise the need to perform separate studies for regulatory authorities of different countries. However, due to the great divergence in requirements between the regions it was agreed to adopt a phased approach with the first step to harmonise the criteria on data requirements for waiving of the TABST for inactivated vaccines in regions where it is required, and the respective VICH GL50 came into force in 2014. A comparable guideline for live vaccines (VICH GL55) together with an updated GL50(R) were published in 2017 and came into force in May 2018.

Since 2016, the VICH experts are working on a guideline on harmonisation of criteria for waiving the LABST for veterinary vaccines.

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Development of OECD Test Guidelines and Guidance Document on determination of fish *in vitro* hepatic clearance

Marlies Halder [1], Michelle Embry [2], Kellie Fay [3], Ina Bischoff [4], John Davis [5], Jean Domoradzki [5], Jing Hu [5], Karla Johanning [6], Heike Laue [7], Diane Nabb [8], John Nichols [3], Christian Schlechtriem [4], Helmut Segner [9] and John Weeks [10]

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Chemical biotransformation represents the largest source of uncertainty in chemical bioaccumulation assessments, and model-based estimates of chemical bioconcentration in fish may be greatly improved by including biotransformation rates, as measured *in vitro*. Substrate depletion assays, using rainbow trout hepatocytes (RT-HEP) or liver subcellular fractions (RT-S9), have been successfully developed to provide estimates of fish biotransformation. A multi-laboratory ring trial, coordinated by the ILSI Health and Environmental Sciences Institute (HESI), was recently completed which demonstrates assay reliability within and across laboratories and similar performance of substrate depletion assays using the two biological systems. Based on the successful results of this ring-trial, two OECD test guidelines (TG) ("Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes" and "Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fractions") have been drafted and are accompanied by a Guidance Document (GD) under the co-lead of USA and the European Commission (Joint Research Centre). The OECD GD provides detailed information on how to conduct the tests as well as how to apply the measured *in vitro* biotransformation rates to predict bioconcentration factors (BCFs). In addition, guidance on the selection of the assay system (e.g., primary hepatocytes vs. liver S9 fractions), specific considerations for testing chemicals, use of negative and positive controls, BCF extrapolation models, and application of the two test methods beyond BCF prediction are also covered. Draft TGs, GD and the ring trial report underwent two OECD public commenting rounds during 2017 and have been approved by OECD WNT in April 2018. They will be published in June 2018.

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Recommendations of the VAC2VAC workshop on optimising the design of multi-centre validation studies

Marlies Halder [1], Hilde Depraetere [2], Frédérique Delannois [3], Carmen Jungbaeck [4], Peter Rigsby [5], Catrina Stirling [6], Coenraad Hendriksen [7] and Ruediger Raue [8]

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The VAC2VAC project (www.vac2vac.eu) aims to develop and validate non-animal based methods for the quality control of vaccines. In order to incorporate a new method into a monograph or a guideline used for regulatory purposes, the method has to be shown to be reproducible and to have the ability to detect vaccine batches of insufficient quality. Moreover, any new method introduced for the quality control of a vaccine needs to undergo product-specific validation. In the light of this,

JRC's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM; leading the work package on validation) and VAC2VAC partners organised the VAC2VAC Workshop on novel design of multi-centre validation studies covering the needs for product specific validation in January 2017.

31 experts from veterinary and human vaccine manufacturers, Official Medicines Control Laboratories, academia, translational research organisations, and vaccinology alliances discussed the current situation and possible ways of improving the design of multi-centre validation studies. The summary of the conclusions and recommendations were published in 2018 (Halder et al., 2018).

Recommendations encourage manufacturers to play a more active role by identifying suitable non-animal methods, providing relevant samples, or by sponsoring of studies. Both multi-centre validation studies and product-specific validation are technically demanding and time and resource intensive, availability of sufficient resources for validation studies and implementation of new methods is crucial. Moreover, the availability of critical reagents and reference preparations should be secured. There are several new documents available or under development which support the substitution of animal tests for the quality control of vaccines and the use of data generated in multi-centre validation studies for product-specific validation.

A number of the recommendations are addressed to the VAC2VAC project and will be followed up by the partners.

VAC2VAC – Vaccine batch to vaccine batch comparison by consistency testing. The VAC2VAC project receives funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement N- 115924. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA.

Reference

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Optimization of the Gluc-HET model

Renate Haselgrübler [1], Flora Stübl [1], Marcus Iken [2], Klaus Schröder [3] and Julian Weghuber [1]

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Insulin resistance and β cell failure are the main causes of elevated blood glucose levels in Type 2 diabetes mellitus (T2DM), a complex and multifactorial metabolic disease. Several medications to treat or reduce the symptoms of T2DM are used, including the injection of insulin and the application of insulin sensitizing or glucose production reducing drugs. Furthermore, the use of phytochemicals has attracted increasing attention for the therapy and prevention of T2DM. In order to identify and characterize antidiabetic compounds, efficient test systems are required. Here we present a modified chick embryo model (hens egg test, HET), which has originally been developed to determine the potential irritancy of chemicals, as a versatile tool for the characterization of phytochemicals with antidiabetic properties. We termed this modified assay variation Gluc-HET. More precisely, we determined the influence of variations in the incubation time of the fertilized eggs and studied the effects of different buffer parameters, such as the temperature, composition and volume, used for drug application. In addition, we tested several putative antidiabetic plant extracts, which have been identified in an *in-vitro* primary screening procedure, for their effectiveness in reducing blood glucose levels *in-ovo*. Taken together, our Gluc-HET model has proven to be a reliable and manageable system for the characterization of antidiabetic compounds

Reference

Haselgruebler, R., Stuebl, F., Essl, K. et al. (2017). *PLoS One 12*, e0182788. doi:10.1371/journal.pone.0182788

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Translational failures call for critical appraisal of animal studies

Kathrin Herrmann

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Most animal research is being justified as indispensable to furthering human healthcare. However, despite measures being taken to improve the quality of animal-based research, the translational success rate from animal studies to humans is low. Less than 12% of drugs entering clinical trials result in an approved medicine [e.g., 1]. Moreover, between 51% and 89% of preclinical studies are not even reproducible [2,3]. Thus, it has become evident that animal-based studies require critical appraisal [e.g., 4].This paper aims to provide tools for an objective evaluation of animal models for human disease and to highlight necessary steps that help further medical advances [5]. Systematic reviews (SRs) and other retrospective assessments (RAs) of animal studies have proven to be useful to assess preclinical model value. By assessing the external and construct validity of animal models in a particular field, those models with low translational value can be identified and then avoided in the future. SRs therefore help to decrease unnecessary animal studies as their application leads to an evidence-based choice of model (animal and non-animal). Article 39 of Directive 2010/63/EU has already made retrospective assessments (RAs) mandatory in certain situations, namely for studies using non-human primates and for all studies that are expected to induce severe pain and/or suffering. However, the competent authority may assess the outcomes of any authorized animal study. Hence, RAs could become extremely effective in facilitating a thorough review of the use of animals in scientific procedures, consistent with the European Commission's aim of using such retrospective assessments to identify 3Rs improvements and the provision of information for future research studies. The conduct of both SRs and RAs, as well as specific examples of identified failures of animal models to mimic clinical disease in humans, will be described, such as mouse models of sepsis, acute ischemic stroke, and Alzheimer disease. Finally, future measures on how to improve preclinical research and thus meet patients' expectations will be discussed.

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53

Human buffy-coat derived platelet lysate: Comparison of single-patient and pooled units for the cultivation of different human cell lines

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Since the beginning of eukaryotic cell culture techniques in the early 20th century, animal sera such as fetal bovine serum (FBS) plays a major role as source for all essential nutrients needed for metabolism, growth and proliferation of cell cultures. FBS is widely used as a growth additive in cell culture media [1,2]. The harvesting of FBS is very cruel and painful for the fetuses and the composition can vary significantly depending on the origin. FBS is xenogenic and can be contaminated by unknown pathogens, therefore its use for the development of new approaches in tissue engineering and stem cell therapies is undesirable [2,3]. For this reasons, a major goal of the 3R principles is an appropriate replacement of FBS in cell cultures.

Human platelet lysate (hPL) is an animal-free serum FBS-replacement, which provides a number of growth factors released from temperature-shocked platelets2. In principal, it is suitable for all human cell types because of its allogeneic origin and can be easily obtained by buffy-coats, which result as waste products from centrifuged whole blood in blood banks [1,3]. For patient-derived tissue models (e.g. from induced pluripotent stem cells), it would be a benefit to cultivate the cells in autologous culture media. For this approach, it is urgent to investigate in a first step, whether it is possible to use the in house produced hPL derived from the buffy coat of one single donor instead of pooled hPL units from several donors.

In this study the effects of single hPL and pooled hPL on the human barrier forming cell lines Caco-2 (intestine), HepG2 (liver) and A549 (lung) were compared regarding cell proliferation, metabolism and cell morphology. Additionally, barrier formation of Caco-2 and spheroid formation of HepG2 were investigated and compared under hPL- and FBS-conditions. The barrier formation of Caco-2 was observed over 21 days via transport studies of the paracellular marker fluorescein sodium as well as with transepithelial electrical resistance (TER) measurements. HepG2 is a suitable hepatic cell line for metabolism studies, which forms three-dimensional microtissues in hanging drops. We investigated the forming efficiency of these microtissues under the different culture conditions. A549 cell line is often used to perform aerosol exposure studies at an air-liquid-interface culture, which we have also investigated in its characteristics.

First results indicated that cells treated with single patient hPL do not differ to those treated with pooled hPL regarding metabolism and proliferation. Moreover, the characteristics of the barrier models cultivated with single hPL have not strongly changed compared to FBS. These results are a first indicator of the qualification of single-patient hPL as FBS replacement in a patient-derived *in vitro* model and the development of autologous culture media.

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Predictive toxicology, strategies for implementing new approaches outcomes of the 7th annual meeting of the American Society for Cellular and Computational Toxicology

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Significant efforts are underway in the United States (US) to maximize the use and regulatory acceptance of new, human relevant, predictive toxicology tools. Much of this momentum was initiated by the creation of the Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States; a document developed with input from the sixteen US federal agencies comprising the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Subsequently, individual US regulatory agencies, such as the Food and Drug Administration (FDA), have published their own strategic plans for the review and acceptance new toxicological approaches. The American Society for Cellular and Computational Toxicology (ASCCT) designed its 7th annual meeting to review the opportunities and challenges to implementing new approaches within various centers of the US FDA. Representatives of the NIH's National Toxicology Program (NTP), and the US FDA Centers for Food Safety and Applied Nutrition (CFSAN), Tobacco Products (CTP), Drug Evaluation and Research (CDER), and Devices and Radiological Health (CDRH) discussed their regulatory review processes and how new approach methodologies might fit into their respective decision-making. Representatives from the industries regulated by these agencies and other stakeholders described ongoing efforts to apply these approaches for internal and/or regulatory decision making. The meeting concluded with a panel discussion to explore the importance of public – private partnerships as an effective means to implement these strategies. This poster will provide a summary of the presentations and discussions held at the meeting and will highlight critical next steps to ensure maximum application of new toxicological approaches within US regulatory agencies.

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A human lung *ex vivo* infection model

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Host properties and pathogen characteristics together form the manifestation of an infectious disease. Species barriers in zoonotic disease such as influenza virus (IV) or coronavirus infection are typical examples. Some pathogens, such as *Streptococcus pneumoniae* (*S.p.*), naturally infect humans only. In some cases, infections by wild-type (wt) pathogens could not be studied in genetically or otherwise unmodified animal models; and researches modify pathogens to make them virulent in animals, thereby changing pathogen biology. Therefore, in infectious diseases it is of outmost importance to study wt pathogens in the respective natural host of interest. Since pneumonia is the third leading cause of human death worldwide, and emerging respiratory pathogens could spread rapidly globally, we established a human lung *ex vivo* infection model. Lung slices obtained during regular surgery were processed, cultured and infected *ex vivo* with different IV strains [1-3], Middle East respiratory syndrome coronavirus (MERS-CoV) [4], or *S.p.* [5-8]. Pathogen replication and critical tissue responses such as pro-inflammatory mediator liberation were investigated. For example, emerging IV strains were compared with respect to the susceptibility of the human host to the different strains. Spectral confocal microscopy allowed for analysis of pathogen tissue tropism and live tissue microscopy of pathogen-host interaction despite high autofluorescence of human lung tissue down to the organelle level. In conjunction with primary cell isolation procedures, we identified individual cell responses and intercellular regulation circuits. Overall, this and related models [9,10] model allows for the investigation of critical aspects of host-pathogen interaction in original living three-dimensional human lung tissue.

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91

Inconsistent effects of chemical filters in UV/ROS stress cell models

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As a barrier organ, skin is continuously exposed to insults from the environment. If adequate countermeasures fail or are absent, oxidative stress may arise. UV-radiation can increase reactive oxygen species (ROS) dramatically. The formation of ROS is triggered mainly by UVA/B light (290-400 nm), while UVC (100-290 nm) -radiation mainly leads to DNA damage in form of photo-adducts.

To protect from detrimental effects of sun light exposure, chemical UV-filters are frequently applied as active ingredients in sunscreens. Regardless, many of these compounds are not well characterized concerning their protective capacity against UV-induced ROS stress on cellular and molecular level. Official testing protocols only retrieve UV-absorption by cell-free physical measurements as well as a grading of erythema formation in humans.

In this study, we investigated the capacity of several chemical UV-filter compounds to counteract UVA/UVB stress in spontaneously immortalized human keratinocytes and E6/E7 immortalized human fibroblasts by using the reduction of ROS formation as primary endpoint. Additionally, the effect of treatment on the cells' metabolic activity was analyzed as an indicator of viability. To differentiate between antioxidative capacity and UV-protection, the protective effect of compounds was investigated on cells treated with an azo-based radical generator in the absence of UV. Well known antioxidant compounds were used as positive controls.

Data show remarkable differences in the mode of action of chemical UV-filters in the proposed test setting, ranging from protective, inactive to pro-oxidative properties. The different cell types showed largely coherent responses despite different sensitivity. The use of these *in vitro* results for the assessment of the real world *in vivo* exposure situations needs further consideration and evaluation. However, the obtained results support considering a more detailed mode of action based analysis for chemical UV-filters.

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Rapid development of anti-idiotypic binders using a novel affinity scaffold

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The Affimer® scaffold is a versatile next-generation non-antibody platform that offers great potential for both novel biotherapeutics as well as research and diagnostics tools. The Affimer® scaffold has been engineered to accept peptide loops so target-specific Affimer® proteins can be selected from highly diverse libraries, with specific binders being selected using phage display methods. Affimer® reagents have been designed for use in a wide range of applications where antibodies and aptamers have limitations.

Affimer® reagents are highly specific, possess chemical and thermal stability, and can be easily formatted for various functions and purposes. Without the use of animals, Affimer® reagents are rapidly and cost-effectively manufactured, displaying minimal to no batch-to-batch variation. Because no animal host is required to generate or produce the reagents, it is possible to isolate binders to challenging targets such as toxic or non-immunogenic targets. Moreover, there is no risk of contaminating products with animal-derived viruses.

Using the Affimer® platform, we have successfully developed binders to a wide range of targets to facilitate or improve assays for targets such as biomarkers (Toxin B/CRP), as well as anti-idiotypic binders to therapeutic antibodies to more effectively and ethically facilitate drug development pipelines. In addition to developing research and diagnostic tools, we are also pursuing the use of the Affimer® platform to develop therapeutic candidates. In summary, the Affimer® technology is a versatile, animal-free platform that allows to rapidly discover and validate specific binders that are suitable tools in a multitude of therapeutic and diagnostic applications.

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10

Systematic assessment of well-being in mice for procedures using general anesthesia

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In the scope of the 3R Principle (Replacement, Reduction, Refinement), appropriate refinement strategies should be applied to minimize pain, suffering, and distress in laboratory animals, when animal experimentation cannot be replaced by alternative methods. Depending on the degree of pain, suffering, and distress, animal experiments must be classified as non-recovery, mild, moderate, or severe according to Directive 2010/63/EU. In order to reduce the severity of a procedure, refinement measures have to be applied. Since it is crucial to evaluate whether those measures were effective, we need scientific tools to objectively assess well-being in a scientific and animal-based way.

Therefore, we designed a well-being-assessment protocol for procedures using general anesthesia in laboratory mice, which can be carried out in the postanesthetic period [1,2]. The goal was to develop a protocol that is easy to perform and includes behavioral, physical and biochemical indicators for well-being in mice. At the same time, additional stress caused by data collecting should be minimized. Our protocol included the Mouse Grimace Scale, luxury behaviors such as burrowing and nest building behavior, food intake, home cage activity, a test for trait anxiety-related behavior and the analysis of fecal corticosterone metabolites. The latter served as an indicator of acute stress over the 24-h post-anesthetic period. High scores on the Mouse Grimace Scale, a poor performance in burrowing behavior and low nest scores indicate that well-being of mice is impaired. Moreover, changes in food intake, home cage activity, trait anxiety levels or concentrations in fecal corticosterone metabolites when compared to control point out reduced well-being. If any parameter of the protocol reveals a difference in comparison to control levels, this parameter should be tested again at a later time point. Thereby, it can be determined when the animal fully recovers from the procedure.

The protocol can be easily integrated into other studies to evaluate both, the impact of a procedure on the well-being of mice and the efficacy of refinement measures. In addition to score sheets, it provides systematic information on well-being of mice following procedures using general anesthesia, which can help estimate the degree of severity of a procedure according to the Directive 2010/63/EU.

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254

Avoiding false positive results of the *in vitro* micronucleus assay in order to reduce follow-up studies *in vivo*

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The assessment of the genotoxic potential of compounds requires a series of *in vitro* assays, which address gene mutation, clastogenicity and aneugenicity. If further clarification of the *in vitro* results is required, additional *in vitro* assays, but also *in vivo* genotoxicity tests are performed. The *in vitro* micronucleus assay, as introduced as an OECD guideline test since 2010, assesses the clastogenic and aneugenic potential of compounds. Positive results in the *in vitro* MNT assay usually trigger *in vivo* testing of micronucleus induction in the bone marrow. A survey of the *in vitro* MNT assays performed in our laboratory showed, that a total 221 assays have been performed using either V79 cells (mixed population method, or cytochalasin B (CytB) method), TK6 cells or primary human lymphocytes (both CytB method). Of these 48 (22%) studies were positive, whereby the rate of positive results was highest in the TK6 model (62.5%). A retrospective analysis of *in vivo* follow-up studies with 30 of these test substances showed only one positive (a tertiary amine). An assessment of the 29 test substances which resulted false positive results *in vitro*, disclosed a significant number of test substances (9) which increased micronucleus rates only after longer preparation intervals (corresponding to 3-4 cell cycles). The longer preparation interval is recommended for test substances which induced cell cycle delays, e.g. nucleotide analogues. None of the nine test substances was delaying the cell cycle and all were negative in the *in vivo* MNT. Hence, the mechanism behind the delayed appearance of micronucleated cells *in vitro* is not likely to be compound related. Unjustified testing at longer preparation intervals *in vitro* may increase false positive results and trigger unnecessary *in vivo* follow-up studies.

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Application of human multi-organ-chips to enhance safety and efficacy assessment in drug discovery

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Microphysiological systems have proven to be a powerful tool for recreating human tissue- and organ-like functions at research level. This provides the basis for the establishment of qualified preclinical assays with improved predictive power. Industrial adoption of microphysiological systems and respective assays is progressing slowly due to their complexity. In the first part of the presentation examples of established single-organ chip and two-organ chip solutions are highlighted. The underlying universal microfluidic Multi-Organ-Chip (MOC) platform of a size of a microscopic slide integrating an on-chip micro-pump and capable to interconnect different organ equivalents will be presented. The second part of the presentation focusses on the challenges to translate a MOC-based combination of four human organ equivalents into a highly predictive tool for ADME profiling and toxicity testing of drug candidates. This four-organ tissue chip combines intestine, liver and kidney equivalents for adsorption, metabolism and excretion, respectively. Furthermore, it provides an additional tissue culture compartment for a fourth organ equivalent, e.g. skin or neuronal tissue for extended toxicity testing. Issues to ensure long-term performance and industrial acceptance of such complex microphysiological systems, such as design criteria, tissue supply and on chip tissue homeostasis will be discussed.

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63

Benchmarking study to support regulatory acceptance of the application of *in vitro-in vivo* extrapolation to predict the bioaccumulation potential of fragrance chemicals

Lu Hostettler [1], Heike Laue [1], Anita Hintermeister [1], Karen Jenner [2], Gordon Sanders [3] and Andreas Natsch [1]

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Bioaccumulation in aquatic species is a critical endpoint in the regulatory assessment of chemicals. To evaluate the bioaccumulative potential of a chemical, the bioconcentration factor (BCF) is used, which is usually determined in fish according to OECD Test Guideline (TG) 305. This test requires a large number of animals, is expensive and time consuming. *In vitro* systems measuring biotransformation rates of chemicals have been established as alternative methods to refine predictive BCF models which are based on hydrophobicity (i.e. log K_{ow}). The reproducibility and reliability of these *in vitro* assays was proven recently in a multi-laboratory ring trial [1]. In April 2018, two Test Guidelines and a Guidance Document on the determination of *in vitro* intrinsic clearance using cryopreserved primary hepatocytes (RT-HEP) and liver S9 subcellular fractions (RT-S9) from rainbow trout were approved by OECD.

To support wide regulatory acceptance of these new *in vitro* methods, we determined *in vitro* intrinsic clearance in RT-S9 of 21 fragrance chemicals and known bioaccumulative chemicals from other categories and compared BCF predictions with available *in vivo* BCF data. Substrate depletion was analysed by GC-MS. The measured biotransformation rates then served as input for an *in vitro-in vivo* extrapolation (IVIVE) model to extrapolate a whole-body biotransformation rate constant which is used to predict BCFs [5]. The majority of fragrance chemicals ($logK_{ow}$ values ranging from 3.9 to 6.5) were moderately to rapidly transformed with *in vitro* intrinsic clearance rates ranging from 0.2 to 25.3 ml/h/mg protein. No significant turnover was observed with the control chemicals with a reported high bioaccumulation potential. BCFs predicted with the IVIVE model were similar to *in vivo* BCFs when the correction factor f_u, an estimate of the unbound chemical fraction, was assumed to be 1. In general, the predicted BCFs did not underestimate the bioaccumulation potential, but avoided strong overpredictions which are a problem of BCF models solely based on hydrophobicity (i.e. $logK_{ow}$) not taking into account metabolism.

In conclusion, the newly approved *in vitro* method for determination of *in vitro* intrinsic clearance using RT-S9 is applicable to fragrance chemicals allowing a refinement of BCF predictions using IVIVE models and therefore presents a promising alternative to *in vivo* testing.

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62

Prediction of acute fish toxicity of fragrance chemicals: The RTgill-W1 cell assay as a simple, accurate and reliable alternative to the fish *in vivo* test

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Acute toxicity to fish is currently being tested according to OECD Test Guideline (TG) 203, where fish are exposed to a test substance for 96 hours. Considerable effort has been invested in recent years in the development and validation of testing methods aimed at fulfilling the principles of the 3Rs (reduction, refinement and replacement) of animal tests. The OECD TG 203, Fish Embryo Acute Toxicity Test had been developed as alternative, but is currently only accepted as part of a weight of evidence approach. An *in vitro* assay using the fish gill cell line RTgill-W1 was proposed as a true replacement based on the fact that in fish the gills are the organ which is most directly exposed to dissolved organic chemicals [1]. The RTgill-W1 assay was recently validated in an international ring trial involving 5 laboratories [2] required as a pre-requisite for adoption as a draft OECD TG.

For the test, gill cells are grown as a monolayer culture in microtiter plates and exposed to different concentrations of the test substance in a serum-free mineral medium. Cell viability is measured with 3 different endpoints: metabolic activity, plasma membrane integrity and lysosomal integrity. At the start and the end of exposition, the actual test concentration in the medium is analytically determined and the toxicity can be related to the mean measured instead of the nominal concentration.

The test was recently applied to 38 fragrance molecules with a wide range of physicochemical properties and *in vivo* lethal concentrations (LC50). A strong correlation between *in vivo* LC50 values, and *in vitro* median effect concentration (EC50), based on cell viability, was observed [3].

A challenge often encountered, especially for highly hydrophobic or volatile chemicals, is a significant decrease of the test concentrations during exposure. In a small study, 6 previously tested fragrance chemicals which showed a loss of > 80% within 24 h incubation, were retested to optimize the test towards more stable exposure concentrations. The standard polystyrene plates were replaced with plates made from Polyether ether ketone (PEEK) and exposure concentrations at 0 and 24 h were analysed by GC. Exchanging the material of the plates did not result in an improved retention of chemical concentrations, whereas replacing the adhesive foil with aluminium foil had a more significant impact resulting in only a ~30% loss of parent chemicals within 24 h. EC50 values based on nominal exposure concentrations were lower with the aluminium foil compared to the adhesive foil. However, maintaining a more stable exposure concentration has little effect on the EC50 values based on mean measured concentrations.

In conclusion, the EC50 value based on mean measured concentrations is a robust parameter not significantly affected by different test conditions and reproducible over time in the same laboratory. The gill cell assay is a possible full replacement of the OECD TG 203 for fragrance chemicals by fulfilling the 3R principle and providing a prediction accuracy that is in the same range as for the currently accepted *in vivo* test.

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Engineering of three-dimensional tissue constructs using our novel MagneTissue bioreactor as alternative to animal models

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Tissue engineering approaches for the generation of three-dimensional *in vitro* models of both healthy and pathological tissues have gained great interest in the scientific community. Not only can they potentially be used to replace damaged tissues, but they also serve as platforms for drug screening as well as for investigating various pathologic conditions. With the establishment of the MagneTissue bioreactor system, we are able to rapidly engineer biomimetic tissue constructs resembling native tissues in terms of structure, gene expression profile, and maturity. Tissue constructs are created by embedding cells into ring-shaped fibrin hydrogels and subjecting them to mechanical strain via magnetic force transmission. The versatile use of different strain parameters (i.e. cyclic, static, ramp, and frequency) and cell types allows us to engineer different tissue-like constructs, including tendons, ligaments, vasculature, skeletal muscle and peripheral nerves. The overall goal of these *in vitro* tissue models is to create a promising alternative to animal models in research.

With the main focus being skeletal muscle tissue engineering, we have successfully engineered highly aligned myofibers along the axis of strain with increased fiber length and size as well as more pronounced sarcomeric patterning compared to unstrained controls. Moreover, structural genes involved in muscle function and contractility were significantly upregulated. These mature skeletal muscle-like constructs could be further used to create muscle disease models related to exercise-induced damage, sarcopenia or exercise-induced damage [1].

In order to more closely mimic the complexity of native skeletal muscle tissue, we furthermore strive to engineer muscle-like constructs with proper vascularization to ensure oxygen and nutrient supply to the scaffold interior. First results using endothelial cells from umbilical veins already showed formation of aligned tube-like structures along the axis of strain. Besides, we also plan to innervate these muscle constructs to further increase the functionality of our constructs in terms of contractility. Ultimately, we seek to obtain mature vascularized and innervated muscle-like tissues to be able to more accurately translate the findings obtained *in vitro* to the *in vivo* situation.

Consequently, according to the '3R's principle, the generation of different tissue models with our MagneTissue bioreactor system may provide a promising strategy to replace animal experiments in the future.

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3Rs in education – systematical evaluation of simulators for rats and mice

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Practical education in laboratory animal science is mainly performed on living animals and is therefore legally classified as an animal experiment according to the Directive 63/2010/EU. Up to now, there is no adequate replacement. In order to refine experiments for educational purposes and reduce the number of animals used, simulators that imitate a real mouse or rat are integrated in training courses. Using these dummies, training attendees can practice technical procedures before working with real animals. Currently, five rat simulators and one mouse simulator for handling and procedures and one rat simulator for microsurgical techniques are commercially available. As it seems that not many of them have prevailed in common practical education so far, a team of scientists consisting of anatomists, epidemiologists and laboratory animal scientists evaluates all current rat and mouse simulators with the objective of creating a new model that is best possible suited for laboratory animal training courses.

In a first use-oriented questionnaire, course trainers are asked about their utilization of simulators and the practical applicability of the simulators they work with. Based on this, pros and cons of the existing simulators as well as the trainers' requirements for an ideal simulator are determined.

For the second evaluation, course participants, who have the possibility to train with all simulators are asked to assess their learning success in a survey. They shall summarize their claims and demands for better training as well as for improved simulators.

In a third evaluation, laboratory animal scientists examine all simulators and determine their functionality for skills training. Specific attributes to optimize the simulators' educational function are defined.

All results will feed into a specification analysis for the construction of a new 3D-printed, anatomically and haptically realistic simulator that combines all requirements at its best.

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A new approach for animal free carcinogenicity testing using MN as biomarker (PMNvit)

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The *in vitro* micronucleus assay (MNvit, OECD TG 487) is recognized as a reliable method for assessing the genotoxicity of samples through cytogenetic evaluation. It is usually performed either in primary cells (e.g. lymphocytes) or immortalized cell lines (e.g. CHO, V79), and MN scoring either by visual counting of fixed, stained cells, or by fluorescence staining followed by flow cytometry.

With the discovery of the reintegration of micronuclei (MN) into the main nucleus of the cell without passing repair mechanisms (chromothripsis), micronuclei are seen as one of the initial steps of carcinogenic transformation [1]. The characteristic features of reintegrated DNA are multiple clustered gene translocations, which is also the most frequent cytogenetic alteration found in cancer cells. Thus, the forecasting power of micronucleus testing became strengthened, since for the first time a causal relationship between the biomarker micronucleus and carcinogenic pathway could be proved. By employing healthy stem cells from the Koi carp (*Cyprinus carpio haematopterus*) brain with self-signaling H2B-GFP labelled nuclei (KCBGFPDSMACC3285) instead of tumor-derived cell lines, it is possible to visually score micronuclei in healthy living cells by fluorescence microscopy. This cell line shares many basic features on the molecular level with embryonic vertebrate stem cells. The sensitivity of the KCBGFP could be proved for various carcinogenic test compounds known to cause micronuclei via different modes of action.

Since the intrinsic metabolome of a cell line is restricted to a certain cell type, it is crucial to broaden the spectrum of the metabolic competences of the used cell type. Thus, the *in vitro* investigation of substances that become toxic after transformation is enabled by the supplementation of *in vitro* test systems with rat liver S9 fraction as the source for liver enzymes. According to standard protocols, isolation of S9 fraction with enhanced enzymatic content requires poisoning of animals prior to liver extraction, for instance by treatment with Aroclor-1254 or with a combination of sodium phenobarbital and beta-naphthoflavone [2]. Therefore, large numbers of animals suffer for the production of a supplement for *in vitro* tests. This is controversial in terms of the ethical use of animals in testing (3Rs). Thus, replacement of animal-derived S9 fraction represents the crucial step towards de facto animal free comprehensive *in vitro* toxicology. ewoS9R is a non-animal-derived source for various Phase I/II biotransformation enzymes (i.e. *Rattus norvegicus*) used in *in vitro* metabolization and directly substitutes the rat liver-derived S9 fractions. ewoS9R has been tested in various genotoxicity tests with very promising results. *In vitro* micronucleus test results comparing the conventional metabolic activation technology with animal free ewoS9R will be presented. This data shows that ewoS9R represents a highly adequate surrogate for animal-derived S9 fraction.

Taken together: i) the usage of healthy stem cells that can still suffer carcinogenic transformation, ii) the nondestructive recording of micronuclei enabling downstream analyses of carcinogenic cell transformation and iii) the animal free metabolic activation, represents a breakthrough in *in vitro* toxicology, towards complete replacement of animal testing in field of cancer research and chemical testing.

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Co-cultivation of myofibroblasts and adipocytes provides new insights into hypertrophic scar regeneration

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Introduction

Hypertrophic scars result from aberrant scarring after elective surgeries or burn wounds. Characterized by persistent myofibroblast activity with excessive collagen accumulation, hypertrophic scars cause cosmetic problems combined with pain, pruritus and contractures. Following autologous fat grafting in plastic surgery, significant improvements in morphology and function of hypertrophic scar tissue have been observed repeatedly [1]. However, the underlying molecular mechanisms are largely unknown. Existing studies include animal models despite the fact that skin physiology and therefore wound healing processes are markedly different [2]. For instance, Plikus and coworkers postulated fat cell regeneration from myofibroblasts during mice wound healing due to hair follicles secreting bone morphogenetic proteins (BMPs) [3]. However, if those findings are transferable to the human situation remains to be elucidated. Therefore, our study covers analyses of both adipose and scar tissue based on primary human cells aiming to establish a suitable 3D model.

Methods

Myofibroblasts (TGF-β induced and from hypertrophic scars) were stimulated for 24h with conditioned medium from adipose-derived stem cells (ASC) or adipocytes. Following this, protein expression analyses of alpha smooth muscle actin (α-sma) and extracellular matrix (ECM) proteins were performed. Since nuclear receptor PPARɣ interferes with TGF-β-signaling, the contribution of PPARɣ was tested. Therefore, myofibroblasts were treated for 1 h with the PPARɣ antagonist GW9662 (1 µM) followed by 24 h incubation with adipocyte-conditioned medium. Aiming to identify the key mediator in conditioned media, secretion of BMP-4 was analyzed by ELISA and myofibroblasts were treated with BMP receptor antagonist LDN-159189 (200 µM). Additionally, BMP downstream signaling including SMAD 1/5/9 was analyzed.

Results

After 24 h, exposure to conditioned medium from adipocytes but not ASCs induced a significant downregulation of the myofibroblast marker α-sma on protein level. Notably, this effect was even more pronounced in fibroblasts derived from hypertrophic scar tissue. When myofibroblasts were pre-treated with GW9662, no downregulation in α-sma could be induced. Analyses of conditioned media identified BMP-4 as a potential mediator. Similar to GW9662, no changes in α-sma expression were detectable when myofibroblasts were incubated with LDN-159189. Indeed, adipocyte-conditioned medium activated SMAD 1/5/9 on protein level in myofibroblasts.

Conclusion

Downregulation of α-sma and ECM proteins may indicate a modification of the myofibroblast differentiation state. Unlike the Plikus group, we present adipocytes as the main responsible cell type for myofibroblast reprogramming. In line with this reprogramming, PPARɣ as well as BMPs seem to play an important role. Currently, we investigate how BMP signaling and PPARɣ activation may be linked. However, a myofibroblast re- or dedifferentiation would provide a plausible explanation for the observed scar regeneration by adipose tissue.

In addition to the described data, first experiments were conducted to establish a 3D model with myofibroblasts to investigate adipocyte stimulation in a more complex environment. Providing more suitable human cell based tools for studying scarring processes could reduce experiments using animals

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Pre-validation of an acute inhalation toxicity test using the EpiAirway *in vitro* human airway model

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Knowledge of acute inhalation toxicity potential is important for establishing safe use of chemicals and consumer products. Inhalation toxicity testing and classification procedures currently accepted within worldwide government regulatory systems rely primarily on tests conducted in animals. The goal of the current work was to develop and pre-validate a non-animal (*in vitro*) test for determining acute inhalation toxicity using the EpiAirway™ *in vitro* human airway model as a potential alternative for currently accepted animal tests. The *in vitro* test method exposes EpiAirway tissues to test chemicals for 3 hrs, followed by measurement of tissue viability as the test endpoint. Fifty-nine chemicals covering a broad range of toxicity classes, chemical structures and physical properties were evaluated. The *in vitro* toxicity data was utilized to establish a prediction model to classify the chemicals into categories corresponding to currently accepted GHS and EPA systems. The EpiAirway IC75 prediction model identified *in vivo* rat based GHS Acute Inhalation Toxicity Category 1-2 and EPA Acute Inhalation Toxicity Category I-II chemicals with 100% sensitivity, and specificity of 43.1% and 50.0%, for GHS and EPA acute inhalation toxicity systems, respectively. The sensitivity and specificity of the EpiAirway prediction model for identifying GHS Specific Target Organ Toxicity – Single Exposure (STOT-SE) Category 1 human toxicants was 75.0% and 56.5%, respectively. Corrosivity, electrophilic and oxidative reactivity appear to be the predominate mechanisms of toxicity for the most highly toxic chemicals. These results indicate that the EpiAirway test is a promising alternative to the currently accepted animal tests for acute inhalation toxicity.

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"ICar3R" – a new interdisciplinary centre for 3Rs in animal research of the Justus Liebig University Giessen

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Supported by the state of Hessen an interdisciplinary centre for the 3R Principle (Russel and Burch, 1959) at the Justus Liebig University Giessen was established. The international research campus of central Hessen (Germany) was founded in 2016 as a union of the Justus Liebig University Giessen, the Philipp's University Marburg and the Technical College of Hessen (THM). Thereby, Hessen intends to create a complex scientific infrastructure in order to promote top research, focussing on lung and infectious diseases, microbiology, virology, psychology and neuro sciences. A part of the strategy is to promote the protection of animals in applied biomedical sciences by strengthening 3R research. The centre "ICar3R" (read "I care 3 R") – Interdisciplinary Centre for 3Rs in Animal Research of the Justus Liebig University Giessen, is allocated in the faculties of veterinary medicine and medicine. Within two newly appointed 3R professorships contributing to refinement and replacement strategies the centre's aim is to improve animal welfare and to reduce the number of laboratory animals.

Concerning refinement strategies a main focus will be set on education and qualification in laboratory animal sciences of personal involved in animal testing. Furthermore, on behavioral aspects and improvement of housing of laboratory animals. A strong contribution to replacement and reduction can be seen in the second focus of the centre, the development of *in silico* models. Robust computer based simulations already exist concerning neurological structures and are expanded continuously. Additionally, the centre is collaborating with a third 3R professorship at the Goethe University in Frankfurt, specialized on alternative drug testing. The strength of the centre is given through a high degree of interdisciplinarity. This is not only displayed by the fact, that the centre is a true collaboration between two medical university departments, additionally 3R research is supported by other natural sciences like faculty of experimental physics and material testing. Furthermore, the centre is in close contact to the department of philosophy and humanities in order to reflect ethical issues and aspects of human animal relationships in the critical context of animal testing. The infrastructure of the research campus in Giessen and the associated universities in Marburg and Frankfurt, together with the entitlement of a high degree of interdisciplinarity are a robust precondition in order to fulfill the basic idea of Russel and Burch's concept. During the last years many precious approaches in the field of 3R research became obvious, although the number of applied experimental animals was not reduced significantly. Therefore, we still see a great necessity for improvement in this field and believe that our novel concept of the centre anchored in the scientific surrounding of the campus structure of Central Hessen will strongly contribute to 3R research.

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A novel co-culture system on skin sensitization for oil products

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Chn-CALT and SHELL are developing a new skin sensitization test method combining the Episkin model and h-CLAT for oil products. Based on the consideration of oil products, we topically applied onto the Episkin, and the THP-1 was cultured beneath the Episkin. After a different exposure time (e.g. 15, 30 and 60 min), Episkin was rinsed thoroughly and an 18 and 42 h post-incubation were conducted. Tissue viability of Episkin was measured using MTT test and the cell surface expression was detected following the protocol of h-CLAT by using a flow cytometer. Besides that, the 5 genes relative to skin sensitization were measured by RT-qPCR. 20 test articles were tested on different concentration. The CD86 or CD54 significantly express increasingly when the viability were > 50% in sensitizers. In the meantime, the MTT showed that the sample in the non-irritation concentration could induce CD86 or CD54 overexpression which it is a sensitizer. According to the exit data on skin sensitization on human or animals, we found that our test show a good accuracy (18/20). The result of gene can be used to distinguish the degree of sensitizers as well. After the proof of concept of the co-culture system previously presented, the aim of this study was to establish an optimize method for oil-products. Our result shows that it may be a promising tool for oil-solubility sample such as oil-products, plant extraction or chemicals for its skin sensitization potential identification.

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3Rs at the University of Zurich

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In connection with the establishment of the Swiss 3R competence center (3RCC), 3R node coordinator positions have been implemented at eleven Swiss universities.

The University of Zurich is the largest university in Switzerland and offers the widest range of subjects and courses of any Swiss higher education institution. With the creation of a full-time 3R coordinator position the University Zurich emphasizes its commitment to advance the 3Rs.

In this talk I will present the role and tasks of the academic 3R node coordinators within the executive board of the Swiss 3RCC. Moreover, I will introduce the implementation of national and local 3R initiatives at the University of Zurich in the fields of communication, education & training, funding and research.

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Tramadol: Paracetamol in drinking water to treat post-surgical pain in laboratory mice

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In the search for stress-free analgesia administration for laboratory mice suffering pain, oral delivery of Tramadol:Paracetamol (T:P) shows great promise.

Here, we monitored the analgesic efficacy and side effects of a T:P combination administered solely in the drinking water of female C57BL/6J mice after moderate-impact surgery (sham embryo transfer), using clinical and behavioral pain and recovery parameters. Animals underwent anesthesia and surgery with T:P treatment (OP +T:P), received no pain relief after surgery (OP), underwent anesthesia only with T:P treatment (An +T:P) or T:P treatment only (T:P). Indicators of pain and constraint were assessed at several time points during 24 h after surgery.

The animal housing and experimental protocols were approved by the Cantonal Veterinary Office, Zurich, Switzerland, under license no.ZH 181/2012 and ZH 059/2017, and were in accordance with Swiss Animal Protection Law.

T:P-containing drinking water was consumed readily in amounts to assure sufficient drug levels. No obvious detrimental side effects of analgesia were observed. General condition of animals differed only slightly and non-significantly between treatment groups, with comparable post-procedural weight loss, water and food intake as well as home cage activity. Mean nest scores differed significantly between T:P and both surgery groups ($p = 0.002$, $p < 0.0001$) but revealed no significant difference between OP and OP+T:P groups. Nevertheless, pain scores showed significant differences between the treatment groups at 1, 3 and 6 h after surgery ($p = 0.001$, $p = 0.014$, $p = 0.003$). OP animals scored highest, while scores of OP+T:P animals were comparable or lower than scores of the AN+T:P group. Same was true for burrowing latency that was significantly increased in OP animals compared to An+T:P and OP+T:P ($p = 0.032$, $p = 0.019$), but comparable between An+T:P and OP+T:P. These results hint on a clear post-surgical pain effect after surgery that could be significantly reduced with T:P treatment towards a level of the control group receiving anesthesia and T:P only.

In conclusion, we assume that orally administered T:P offers pain relief with no obvious side effects after mild-to-moderate impact surgery in female C57BL/6J mice

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Relevance of *in vitro* test results to human data in safety and efficacy testing of cosmetics

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The EU ban on animal testing requires to rely exclusively on the predictive capacity of *in vitro* methods for safety and efficacy assessment of cosmetics and their ingredients. Relevance of experimental *in vitro* data with human exposure tests is essential for reliable prediction of human skin and eye irritation hazard.
The presented study focused on prediction of safety in terms of potential of eye irritation and beneficial effects of commercially available shampoos intended for dry and damaged hair accompanied by in-use self-assessment of products by volunteers. The study comprised a group of 10 shampoos evaluated by 200 human volunteers (twenty volunteers for each shampoo tested) under professional hairdressers' supervision.

With the aim to assess the potential of eye irritancy and compare regular and bio/natural products, a battery of *in vitro* tests has been employed, i.e. eye irritation test on corneal model EpiOcular™, cytotoxicity tests using 3T3 Balb/c cells and HET-CAM assay.

The accompanying biomedical study in a group of volunteers, selected by a professional hairdresser to suffer from dry and damaged hair, comprised repeated in-use test for 3 weeks and evaluation of the subjects' hair by sophisticated methods of microscopy and image analysis.

The samples were anonymized. One week before the beginning of the study the volunteers used a neutral shampoo for hair washing, under the same conditions as the tested product samples were applied during the study, in order to unify baseline hair quality. The test shampoo was applied for hair washing 3 times a week. Before and at the end of the experimental hair washing period, a professional hairdresser graded the hair quality of the volunteers based on a 9 point scale, from extremely dry to healthy strong shiny hair. At the end of the study volunteers were asked to provide subjective evaluation of product parameters, e.g., easy to use application, olphactory characteristics, cleansing properties, effect on hair quality and appearance, possible adverse effects and pros and cons of the product.

The *in vitro* methods identified differences between the formulations, resulting in different expected eye irritation potential, highlighting the general mildness of bio/natural shampoos.

All volunteers completed the biomedical study. The products were generally well tolerated. However, a significant positive effect in terms of improving the quality of hair has not been observed either by volunteers and expert evaluators, or by objective methods of microscopy and image analysis.

The bio/natural mild formulation was not identified by the volunteers as beneficial for hair quality improvement and did not lead to their decision to buy the product, moreover, these samples were frequently reported to have unpleasant scent and/or consistence.

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265

Inter-and intra-laboratory reproducibility of the *in vitro* photo-toxicity test using 3D reconstructed human epidermis model EpiDerm

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Assessment of the phototoxicity hazard and phototoxic potency (i.e. phototoxic risk) of compounds and mixtures is a crucial step in the safety assessment of cosmetic, pesticide and pharmaceutical products absorbing UV and visible light. The validated and regulatory accepted *in vitro* assay, the 3T3 NRU PT (OED TG 432), provides high level of sensitivity, however, it has been reported that it also generates high rate of false positive results due to the lack of barrier properties naturally appearing in the human skin or other targeted tissues. *In vitro* reconstituted human skin models are increasingly being investigated for their usability in hazard identification and safety testing, because of their organotypic structure with a functional stratum corneum that allows for assessment of bioavailability of topically applied compounds and mixtures. An *in vitro* phototoxicity test using the human reconstructed epidermis model EpiDerm™ (EpiDerm™ H3D-PT) has been developed and pre-validated almost 20 years ago [1,2] and can be used either as standalone method for the phototoxicity testing of topically applied materials, or in combination with the 3T3 NRU PT, to minimise the potentially false positive results from this assay. In the current study we internally validated the method with six reference substances, of which four were known phototoxins (chlorpromazine hydrochloride, two types of bergamot oil and anthracene) and two compounds were UV-absorbing, but without phototoxic potential (cinnamaldehyde, p-aminobenzoic acid). In the next step the method has been transferred into two other laboratories and the test was repeated using the same materials. The high reproducibility of the predictions between the test laboratories confirmed the robustness of the protocol and the prediction model. Extended validation study with coded materials is planned for autumn 2018. Submission of the results from the current project to the OECD, together with the review of the existing datasets related to EpiDerm phototoxicity test [3] is planned for 2019.

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Successful development and validation of the EpiDerm *in vitro* skin irritation protocol for the assessment of medical devices extracts

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Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. Reconstructed human epidermis (RhE) models have replaced rabbit skin irritation testing for neat chemicals (OECD TG 439); however, medical device (MD) extracts are dilute solutions with low irritation potential, therefore the validated RhE-methods required modification to reflect the needs of ISO 10993.

A protocol employing RhE EpiDerm was optimized in 2013 using known irritants and spiked polymers [1]. In 2014, a second laboratory assessed the transferability of the assay. After the successful transfer and standardization of the protocol, 17 laboratories worldwide were trained in the use of the protocol in preparation for the validation. All laboratories produced data with almost 100% agreement of predictions for the selected references [2].

In 2016, an international round robin validation study was conducted to confirm the ability of the RhE models to correctly predict the intra-cutaneous irritation of extracts from MDs. Four irritant polymers and three non-irritant controls were tested blindly following extraction in sesame oil or saline per ISO 10993-12. Positive and negative solvent controls were also included in the study.

EpiDerm tissues were able to correctly identify nearly all of the irritant polymer samples when extracted in either saline or sesame oil or in some cases both solvent extracts. Our results indicate that RhE tissue models can detect the presence of skin irritants at low concentrations in dilute medical device polymer extracts [3]. The use of the reconstructed tissue models, as replacements for the rabbit intra-cutaneus test is currently being implemented into the ISO 10993 standards used to evaluate medical device biocompatibility. The work will be published in a special issue of *Toxicology In Vitro* in 2018 [4].

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Having a successful career in *in vitro* toxicology: Pillars of success

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The definition of successful career is very personal matter. For one it may be the achievement of a prestige position at the university, multiple scientific degrees, recognition in the academia circles, while for other it may be the amount of money earned or simply enjoying the job one is doing. A successful person usually possesses a good mix of healthy ambition, courage, endurance, determination, optimism, patient efforts and high stamina. However, success usually does not happen overnight and is a journey that requires time. It has multiple lows and highs and can be compared to the marathon. Resiliency therefore also belongs to the toolkit of a successful individual.

Having success in *in vitro* toxicology has one additional and highly important dimension. While we are achieving our personal goals and building our successful careers in this field, our personal journeys are also simultaneously creating good, because our efforts aim into replacement of animal testing by novel *in vitro* technologies we develop. With our small every-day efforts, we contribute to a better and humane-based science.

Nothing great can be achieved alone – we live in a connected world. Scientific collaboration and knowledge sharing with colleagues and friends on the same journey is an essential part of the success. It is essential to have possibility to learn from others, to collaborate on projects, to be able to present the results as freely as we can, and together to pursue for a common goal to replace animals whenever possible. This highly ethical mission, together with a personal dedication and endurance may ultimately ensure a success and recognition to any individual stepping on this path.

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The Slovak National Platform for 3Rs – SNP 3Rs

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The idea for establishment of 3Rs center in Slovakia was under the discussion amongst the Slovak scientist, regulators and industry already since 2015. First strategy concept of a national 3Rs center was presented at the TOXCON 2016 conference, organized by the Slovak Toxicology Society SETOX in High Tatras, and further discussed between several members of the National Committee for Alternative Methods (NOVS) at the EUROTOX 2017 Congress in Bratislava.

As a follow up, the Ministry of Agriculture and Rural Development of the Slovak Republic organized in February 2018 an informal meeting of NOVS members, invited experts in toxicology and pharmacology, representatives of Slovak Academy of Science and academia, industry partners and governmental organizations involved in the implementation of the EU legislation on animal welfare and protection in order to discuss the collaboration amongst the parties and to express an interest and formal support to the national 3Rs center. The attendees of that meeting fully endorsed an establishment of the Slovak National Platform for the Three Rs (SNP 3Rs) that should stimulate development and implementation of the alternative methods in Slovakia.

The platform was officially launched at the annual meeting of the Slovak Toxicology Society SETOX on June 21st 2018 at the Toxicology Conference TOXCON 2018. The SNP 3Rs will operate with support of SETOX and its mission will be to provide information, resources, and practical guidelines on the 3Rs principles of Russell and Burch in science, education, research and development.

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Integrating knowledge of carcinogenicity Adverse Outcome Pathways (AOPs) with experimental data

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The assessment of carcinogenicity and related toxicity endpoints is a principal area of research in the development of *in silico* prediction systems. In recent years, these systems have become embedded in regulatory guidance, where they may be used to replace or augment other testing methods.

Previously, we have described how knowledge relating to the carcinogenicity endpoint contained in the expert rule-based prediction system Derek Nexus (DX) was rearranged to generate a network of adverse outcome pathways (AOPs) that share common key events (KE) and which can be interrogated at different levels.

In this work we outline how this network can provide the basis for carcinogenicity predictions for individual compounds and that presenting knowledge in this way will allow the user to more intelligently combine *in vitro* and *in vivo* data with hypotheses for a predicted mode of action (MOA).

It is hoped that this approach will allow the user to build a weight of evidence (WOE) to predict the carcinogenicity of a query compound and determine the most appropriate next steps in the testing of a hypothesis. Where the WOE is strong enough, animal testing may be avoided completely.

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Integration of emerging *in vitro* assays into skin sensitisation assessment strategy

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The use of laboratory animals has been traditionally involved in skin sensitisation assessment for a long time. Although *in vivo* assays like Local Lymph Node Assay (LLNA) have been extensively used to predict sensitization potential, non-animal approaches became a concern when the new the 7th Amendment to the European Union Cosmetics Directive (2003/15/EC) was set to ban all *in vivo* testing of toiletry and cosmetic ingredients by 2013 and when the new frame of EU-Chemicals directive (REACh) has been established. Moreover the pressing social need to replace animal sensitization models, and the scientific need to more precisely control and evaluate the mechanistic progression of hypersensitivity have also contribute to the growing interest in *in vitro* models [5]. Non-animal alternatives for assessing skin sensitisation includes *in chemico* and *in vitro* assays.

A skin sensitiser is defined as a substance that will lead to an allergic response following skin contact by the UN GHS. The chemical and biological mechanisms associated with skin sensitisation has been summarised as the Adverse Outcome Pathway and has been addressed by the combination of several *in chemico* and *in vitro* assays as a substitution to the LLNA [1]. Direct peptide Reactivity Assay (OECD 442C) addresses the first key event, when the covalent binding of electrophilic substances to nucleophilic centres in skin proteins occurs [2]. The second key event is addressed by the ARE-Nrf2 Luciferase Test Method (KeratinoSens™) (OECD 442D), focusing on the inflammatory responses induced in keratinocytes to change their gene expression associated with specific cell signalling pathways (ARE-dependent pathways) [3]. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, addressed by the human Cell Line Activation Test (h-CLAT) (OECD 442E) [4].

To demonstrate the reliability and skin sensitisation prediction potential of the *in vitro* assays compared to *in vivo* methods, ten proficiency substances were tested in each of the above mentioned assays. The proficiency chemicals with high quality *in vivo* reference data were recommended by the OECD guidelines to represent wide range of responses for skin sensitisation hazards. To differentiate between sensitisers and non-sensitisers LLNA uses the EC3 value as the measurement of the relative potency which is the concentration causing three-fold stimulation in draining lymph nodes compared to vehicle controls. The measurement of relative potency in DPRA is the mean percentage of the established covalent bonds between the used chemicals and the synthetic peptides. In the KeratinoSens™ assay the EC1.5 value is used as the relative sensitisation potency, which indicates 1.5-fold luciferase induction. In the h-CLAT two effective concentrations the EC150 and the EC200, which mark the extent of cell surface marker expression compared to isotypic controls, are the threshold values used when categorizing sensitisers. Using the respective prediction models from the OECD guidelines, the obtained *in chemico* and *in vitro* data for each substance corresponded to the *in vivo* LLNA based predictions regarding the identification of sensitisers.

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Estrogen and androgen receptor binding affinity of bisphenols estimated by QSAR

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Chemicals might interact with proteins such as the estrogen receptor (ER) or androgen receptor (AR) resulting in initiation of a cascade of biological effects and perturbation of the endogenous hormone system. Despite the complexity of the endpoints for reproductive impairment, it has been long appreciated that chemical binding to the ER or AR is one of the important mechanisms of interference with the reproductive process. Since reprotoxicity tests *in vivo* are very expensive and time-consuming, alternative procedures are being developed.

It is also known that the ER or AR are much less of a lock-and-key interaction than highly specific receptors. The ER and AR are 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. The 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 ER and cause subsequent gene activation. For AR the most important parameter seems to be distance between nucleophilic sites in the molecules, the oxygen atom connected to a cyclic carbon atom (associated with the 3-position in the steroidal skeleton) and the oxygen atom in the hydroxyl group (associated with the 17-position in the steroidal skeleton). Also partition coefficient log Kow (n-octanol-water) of binding chemical plays significant role.

Defining the boundaries of these chemicals is the challenge for (Q)SAR and computational chemistry. The aim of the presented work was to characterize the groups of bisphenols with potential to bind to ER or AR and cause adverse effects in the human organism. All the evaluated bisphenols showed high and even very high affinity to the ER binding subpockets. These compounds may act as a gene activators and cause adverse effect in the human, especially during the pregnancy, breast-feeding and developing.

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Re-epithelialization in focus: Non-invasive monitoring of epidermal wound closure *in vitro* using organotypic skin models

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Cutaneous wound healing is of essential interest considering a patient's health, above all when it comes to skin diseases impairing a proper wound closure. Up to today, *in vivo* studies are the gold standard for testing of new wound therapeutics. Alternative *in vitro* studies are mostly limited to two-dimensional cell cultures and thus only poorly reflect the complex physiological wound situation.

Here we present a new three-dimensional wound model based on a reconstructed human epidermis (RHE). Furthermore, we introduce impedance spectroscopy as a test method to determine the efficacy of wound healing non-destructively with a high sampling rate and thus allowing for rapid screening of test substances and wound therapies. With this technique we are focusing on the barrier function of the RHE as a main feature of intact skin. By applying a sinusoidal alternating perturbation signal over a frequency range of 1 Hz to 100 kHz impedance spectra are measured. The skin barrier can then be assessed quantitatively and qualitatively either by calculating the transepithelial electrical resistance (TEER) or by fitting an equivalent circuit and analyzing capacitive as well as resistive parts of the impedance spectra. Fully matured epidermal models of three different donors resulting in TEER values between 6.3 and 11.4 kΩcm² were included in this study. Upon wounding using a dermal punch of 2 mm in diameter, the impedance dropped significantly to 5% of the initial value. Subsequently, epidermal wound healing was monitored on-line over fourteen days showing recovery of the barrier indicated by significant increases in TEER values back to levels between 7.9 and 14.8 kΩcm². Microscopy and histology images correlate with these findings, revealing an active wound closure mostly completed by day seven after wounding. Interestingly, the models of three donors showed different progressiveness both in microscopy imaging and in impedance spectroscopy.

These wounded epidermal models can now be applied in therapeutic screenings studying the impact of substances on re-epithelialization. For including more stages of cutaneous wound healing, the epidermal models can be expanded by a dermal component either consisting of a collagen type I hydrogel or a porcine matrix with primary human dermal fibroblasts to increase the complexity of the model and allowing for observation of remodeling processes of the extracellular matrix. With the help of rapid screening by impedance spectroscopy, expensive and time-consuming imaging and histological methods as well as the use of animal models can be reduced.

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Establishment of a new cholangiocarcinoma cell line MUG-CCArly1

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Background

Cholangiocarcinoma (CCA) is a malignant tumor of the biliary tree, with a five-year survival rate between 5-18% due to late diagnosis and rapid disease progression. A special form of extrahepatic CCA is called Klatskin tumor, occurring in the confluence of the right and left hepatic bile ducts. These tumors tend to be late in symptoms and are mostly unresectable. Resection is still the most promising treatment option. Lack of animal models, variations of genetically different cell lines and the rarity of this tumor, make CCA hardly accessible for research. Therefore, we are focusing on the establishment of new alternative biomodels for the study of this rare tumor type.

Methods

Under the approval of the Ethics Committee and patient's informed consent, a grade 2 Klatskin tumor tissue was collected from a 72 years-old Caucasian patient directly after surgery. The tumor was mechanically and enzymatically digested, and cultured in DMEM/F12 supplemented with 10% FBS and 2mM L-glutamine. After attachment of cells, the tumor cells were separated from cells of the connective tissue by different detachment times. DNA (QIAamp DNA Mini Kit, Qiagen, The Netherlands) was isolated from pure tumor cultures to perform STR analysis (PowerPlex 16HS System, Promega, Madison, USA). Genetic characterization was performed by genome-wide copy number aberrations (CNA) profiling using low-coverage whole genome sequencing. To this end shotgun libraries were prepared using the TruSeq DNA LT Sample Preparation Kit and sequenced on an Illumina MiSeq.

Results

Primary culture of the tumor tissue resulted in cultures of epithelial cells and fibroblast. The epithelial-like tumor cells grow in islands and are multi-vacuolated. After several months in culture, pure tumor cultures were achieved and DNA was isolated. STR analysis proved the same profile of the cell culture and the fresh frozen tumor tissue. Copy number analysis showed a variety of copy number aberrations including gains of chromosomes 1p, 2, 6, 9, 11 and 17p as well as losses on chromosomes 1q and 18. Moreover, focal amplifications on chromosomes 8 and 18 including highly relevant driver genes such as FGFR1, MYC and GATA3 were observed, which confirms the origin from malignant tissue.

Conclusion

We were successful to culture and isolate tumor cells from a Klatskin tumor. The new established cell line will help to further understand the pathogenesis and tumor biology of cholangiocarcinoma, and it will facilitate development of further treatment strategies.

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The 3Rs Database Programme: Humane Endpoints website, Interspecies Database and FCS-free Database

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The European Union has taken an important step forward in the Replacement, Reduction and Refinement of animal experiments by adopting Directive 2010/63/EU. Researchers are obliged to consider the 3Rs when designing and performing procedures involving animals. To accomplish this, the latest information about the 3Rs has to be identified. The large amounts of information available online and in literature can be an obstacle for researchers to find the desired data.

The availability of databases with specific 3R information may save time in this search for information. To be successful, these databases should be easily found, accessed, managed and updated. Designing, building and filling a database is a time and money consuming activity. Often, financial support can only be obtained in the development stage, whereas continuous updating and maintenance is essential for the success, usability and sustainability of the websites and databases. Continuous financial support is therefore crucial.

The 3Rs-Centre Utrecht Life Sciences (ULS) has initiated the 3Rs Database Programme, which aims to provide up-to-date 3Rs information free of charge, thereby contributing to the implementation of the 3Rs in research. The programme currently comprises three databases: the Interspecies Database, the Humane Endpoints website and the FCS-free Database.

The Interspecies Database (www.interspeciesinfo.com) provides insight into physiological, anatomical and biochemical parameters of different animal species and humans. With the database, researchers can design their experiments smarter with respect to the choice of an animal model. This could lead to a reduction in the number of experimental animals.

The Humane Endpoints website (www.humane-endpoints.info) provides information and training modules on how to recognize and apply humane endpoints in laboratory animals. This helps to prevent unnecessary pain and distress in the animals. Therefore, the website contributes to refinement.

The FCS-free Database (www.FCS-free.org) was launched in 2017 to raise awareness about the scientific and ethical problems related to the use of fetal calf serum (FCS), and to provide an overview of FCS-free media for specific cell types. Furthermore, the comment function in the database allows researchers to exchange information on the applicability of FCS-free media. This website will increase the reproducibility of *in vitro* methods and contributes to the replacement of animals used for research.

To guarantee a sustainable future for these websites and increase their usage, the 3Rs Database Programme is inviting partners who are willing to cooperate and support its activities.

For more information, visit www.uu.nl/en/3Rsdatabases or contact 3RsCentreULS@uu.nl.

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HIF-1 activation by oxyquinoline derivative in BeWo b30 monolayer as model of placental barrier hypoxia

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Objectives

There is conflicting evidence that Hypoxia-inducible factor (HIF)-1 activation in hypoxia in the trophoblast can be both a pathogenetic mechanism of placental barrier dysfunction and an adaptive mechanism that protects the trophoblast [1,2]. It was found that oxyquinoline derivatives can increase HIF-1 by inhibition of HIF prolyl-hydroxylases mimicking hypoxic conditions and activation of hypoxia pathways [3,4]. We studied the effect of the oxyquinoline derivative on the activity of HIF-1 and changes in the transepithelial resistance (TEER) and transmembrane permeability of BeWo b30 cell monolayer as a model of placental barrier.

Methods

BeWo b30 cell line was grown in the DMEM with L-glutamine, 4,5 g glucose/L and Earle's salts containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 96-well Transwell plate. Cells were seeded with a density of 3×10^4 cells per insert (growth area 0.143 cm^2, recommended medium volumes are 70 and 235 µL for upper and lower chambers). After 2 days of cultivation, a 10 µM solution of the oxyquinoline derivative in the medium was applied to the cells for 24 h. TEER was measured before and after 6 and 24 h of drug application and permeability for 70 kDa FITC-dextran and glucose were measured in the end of experiment. Whole transcriptome analysis of the BeWo b30 cells after oxyquinoline and in control conditions was performed with aid of Affymetrix Human Transcriptome Arrays 2.0. The change in gene expression was considered significant when the fold change was ≥ 2 and FDR p-value was ≤ 0.01.

Results

After 6 h of incubation with oxyquinoline derivative there was significant increase of TEER in BeWo b30 monolayer comparing to control conditions (90.3 ±10.5 vs 69.2 ±13.4 $\Omega\cdot cm^2$), but 24 h incubation with drug leaded to a significant decrease of TEER comparing to control conditions (60.4 ±3.5 vs 95.9 ±16.8 $\Omega\cdot cm^2$). Using the 70 kDa FITC-dextran permeability method it was showed that the integrity of the monolayer was preserved under both conditions. Glucose transport analysis with concentration gradient from 4.5 to 1.0 g/L revealed that after 24 h oxyquinoline does not influence glucose uptake from apical side but decreases 1.5 times ($p < 0.05$) transepithelial transport to basal chamber of Transwell system. Expression of *GLUT3* gene was elevated 2 times but this does not influence glucose uptake. Elevated glucose consumption can be explained by signs of metabolic reprogramming [5] including upregulated *BNIP3*, *PDK1* and *NOS3* genes and downregulated *PPARGC1B* gene suggesting activated mechanisms of a direct inhibition of mitochondrial oxygen consumption, a diversion of carbohydrates away from oxidative pathways and a reduction in mitochondrial mass in cells.

Conclusion

The oxyquinoline derivative causes HIF-1 activation in the BeWo b30 cell monolayer decreasing transepithelial electrical resistance without compromising integrity. This compound triggers metabolic reprogramming in cells which increases glucose consumption.

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Phasing out the use of experimental animals within a 10-year period?

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In closing the 8th World Congress in Rome, 2008 I made the statement that by 2020 the regulatory safety assessment of chemicals will be realized without experimental animal use. Today, we are approaching that date still using animals. However, progress since Rome has been tremendous and technologies such as novel stem cell technologies, organs on a chip and many more developments have found their way to numerous applications. In Spring 2016, the Minister of Agriculture decided to burden the "Netherlands National Committee for the Protection of Animals Used for Scientific Purposes" (NCad in short) with the assignment: "to develop a roadmap for the phasing out of the use of animals in scientific research". He further urged the Dutch scientific community to aim at becoming a world leader in animal-free innovations. He marked his footprint just before his political party lost the elections and the Minister left the political scene. We stayed behind with this assignment and were facing a scientific community that was hopelessly divided. My keynote lecture will not be about fundamental science as I am too old for keeping up with all the new developments in life sciences. Instead I will skate on the surface where I feel comfortable. But I will also enter into the deep forest to address the concept of transition, politics, extremely hard-working Commission staff, not so hard-working box tickers, visionaries, target setting front-runners, fundamental and applied science practitioners, transparency and international cooperation. But most importantly, I will address the future with the goldmine of big data.
At the end I will thank a few people.

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An *in silico* lung tumor model for drug efficacy screening and optimal clinical therapy as alternative to animal testing

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Lung cancer is the main cause of cancer-associated mortality worldwide. The five-year survival rate is less than 15% [1,2]. Targeted therapies has led to some treatment improvements, but unfortunately, lung tumor patients respond differently as they have a different mutation profile. This requires patient-tailored animal testing for best drug efficacy according to the patient driver mutations [3-5]. To overcome this, we developed a generic and rapid *in silico* lung tumor model for calculation of drug efficacy and optimal targeted tumor therapy [4,5]. Based on *in vitro* and clinical data we generated patient-specific *in silico* models correctly mirroring the clinical treatment responses including resistance mechanisms upon gefitinib and the HSP90 inhibitor 17AAG in EGFR- and KRAS-activating lung tumors. Next, a machine learning approach performs a patient stratification, on which a prediction model of matching drug efficacy calculates the optimal clinical treatment suggestion according to the patient driver mutations. For example, we found that individual drug targeting of HIF1A and LKB1 in two different KRAS-mutated lung tumors is promising, whereas the combined PI3K and MEK inhibition is the best strategy to break gefitinib resistance in an EGFR-mutated lung tumor [4,5]. In conclusion, our *in silico* lung tumor model refines patient therapy and is an innovative alternative to reduce animal testing for drug efficacy screening. Moreover, our *in silico* model is already implemented in the molecular tumor board of the comprehensive cancer center Würzburg for targeted therapy decisions. In the long term, we expect that our *in silico* model can be applied to several diseases to fully replace animal experiments for drug efficacy screening and patient-tailored therapies.

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Characterization of chemical metabolism in combined skin and liver models over extended and repeated exposure in a multi-organ chip device

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New *in vitro* methods and testing strategies for animal-free toxicity testing are important to support risk assessment of potential new cosmetic ingredients. To guarantee consumer safety, Cosmetics Europe is committed to endorse and actively contribute to the development of alternatives to animal testing. To date, no *in vitro* systems have been validated to assess systemic toxicity, leaving an assessment gap pertinent to compounds that are bioavailable after skin permeation, oral uptake, or inhalation. Dynamic microphysiological systems (MPS) that integrate biological 3D tissues models have emerged as potential future *in vitro* testing platforms for complex toxicological endpoints such as systemic toxicity. Several approaches of "organs-on-chip" and dynamic co-cultures ("multi-organ chips") aim to emulate the *in vivo* physiology of single organs or the interactions between organoids, respectively. To explore the use of MPS to provide information about the influence of different application routes on the bioavailability and metabolic fate of chemicals, we employed TissUse's two organ chip (MOC) to connect reconstructed human epidermis (RhE) models (i.e. EpiDerm) and liver organoids (consisting of HepaRG and stellate cells). Applying a nested testing approach in two labs to evaluate the systems reproducibility, we assessed the metabolism of topically and systematically applied, single and repeated application, of three model chemicals: retinoic acid, permethrin and hyperforin. We first determined viability and functionality of single organoids over time and identified optimal non-toxic concentrations of test chemicals for the ultimate metabolism studies in the MOC. Pretests included i) kinetics of RhE models penetration, ii) toxicity towards RhE models and liver organoids, iii) chemical stability and binding to chip material. For each application scenario, parent and metabolites were analyzed by mass spectrometry in MOCs over five days. Transcriptional qPCR analyses of relevant xenobiotic metabolizing enzyme genes were performed in liver organoids after two and five days of exposure to determine potential autoinduction and modulation of compound metabolism. Our results show that 1) metabolic capacity in RhE and liver organoids is maintained over 5 days; 2) RhE model barrier function remains intact (according to histology and Trans Epithelial Electrical Resistance measurements); 3) repeated application of compounds resulted in higher concentrations of parent chemicals and some metabolites compared to single application; 4) compound-specific gene induction e.g. induction of CYP3A4 by hyperforin is dependent on the application route and frequency; 5) different application routes impact systemic concentrations of both parents and metabolites in the chip over the course of the experiment; 6) there was excellent intra- and inter-lab reproducibility, indicating a high robustness of the MOC for metabolism investigations and its transferability. In summary, the MOC provided important information on parent and metabolite disposition that may be relevant to risk assessment.

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Decision making on the possible handling of laboratory animals after a trial

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Animals that have been bred for or used in animal experiments have not necessarily to be euthanized and disposed safely at the end of a trial. Humane killing might be mandatory for example a) if organs or large amounts of blood have to be removed for further examinations, b) if they have infections, c) if a further life would only be possible while enduring more than minor pain, suffering or damage (according to Directive 63/2010/EU) or d) because keeping animals with genetical modifications outside a genetic engineering facility is forbidden [1].

If survival is within the realm of possibility, the restoration of health has to be confirmed by a veterinarian. A thorough decision has to be made, if animals can be re-used in an additional trial, if none of the procedures already completed or planned is classified as "associated with severe pain or suffering" [2]. If re-use is not applicable, rehoming could be an additional alternative. Animal facilities should develop programs with the advice of their animal welfare committees [2]. These programs may comprise strategies to identify appropriate animals and to prepare these animals for the challenges of their new living conditions and environments as well as for the detection and choice of suitable owners who provide adequate housing, nutrition and veterinary care. It may be helpful to cooperate with animal protection organizations that are experienced in the rehoming and adoption of animals. However, it has to be ensured, that the animals do not pose a risk towards other animals or humans.

When farm animals are delivered to private owners, it has to be guaranteed, that products derived from them, which may enter the food chain are proven to be safe for consumers, other animals and the environment [4].

For animals without genetical modifications [5] that have been bred for but never been used in animal experiments, the use as feed animals for predators or raptors [3] can be seen a reasonable justification for humane killing.

In addition to the thoroughly planning of interventions and procedures, the considerations concerning the whereabouts of animals after the trial must already be taken into account when an animal experiment is applied for, because some of the options listed above require time-consuming preparation and official permissions [1].

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240

Alternative approaches in education provide excellent refinement measures

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Animals are used for educational purposes in different contexts, which may relate to an apprenticeship or a university degree in general or to a certain scientific project. However, the requirements of competence and skills are constantly increasing in each of the mentioned contexts, but explicitly in veterinary medicine and biomedical research. According to the Directive 63/2010/EU on the protection of animals used in scientific purposes, higher education and or training for the acquisition, maintenance or improvement of vocational skills is categorized as a "procedure", which may cause the animal suffering, distress or lasting harm [1]. Therefore, it has to be applied for and it has to be ethically justified. This justification includes the presentation of indispensability with respect to the project itself, but also in terms of the degree of pain, suffering or damage inflicted on animals. In accordance with the 3R principle, efforts should be made to minimize potential harm and suffering of animals used in education and training. There are several methods available that can be used to provide basic knowledge about interventions without demonstration on a living animal, such as images or films, computer simulations, plastinates, tissues and organs from abattoir, each of which has strengths and limitations. The use of simulators is also a good way to exercise certain manual skills and courses of action. Good simulators enable training under realistic conditions. The learner's attention can be fully focused on the procedure and at the same time it is allowed to make mistakes and to take them as a chance to enhance the learning process by repeating the procedure, since no living animal has to suffer from the lack of experience of the person doing. When it comes to a first application to a living animal, students are better prepared and can easier concentrate on the patient. Some simulators are commercially available, but at the department of veterinary medicine of the *Freie Universität* simulators even for farm animals are also developed as an initiative in cooperation with the students and their use is integrated in curricular teaching [2].

The use of animals in education, especially in professions that aim to work with living animals, may not be totally replaceable, but the implementation of alternative methods for education, especially of realistic simulators, contributes substantially to Reduction and most of all to applied Refinement.

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[2] Veterinary Skills Net, Department of Veterinary Medicine, Freie Univerversität Berlin, Berlin, Germany http://www.vetmed.fu-berlin.de/studium/skills-net/index.html

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38

Sharing is caring – update on innovative approaches to share organs and tissues to reduce the lab animal usage

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The reduction of lab animal usage can be achieved by e.g. improved statistical and experimental planning, in-depth pre-analysis in suitable cell culture models or conscious handling of biological resources and materials that are left during the animal experiments. Therefore, sharing of organs and tissues of animals sacrificed for scientific purpose, surplus animals or those used for organ collection under anaesthesia or educational purposes provides the great opportunity to sustainably reduce the animal number in a short-term setup. This has also been taken into account by the European directive 2010/63/EU which includes the request that "Member States shall facilitate, where appropriate, the establishment of programmes for the sharing of organs and tissues of animals killed." (Recital 27 and Article 18 2010/63/EU). Therefore, several small initiatives can be identified in Europe which are mostly focusing on biobanking of one specific animal species (EU PRIMNET, MIDY), tissue (NERD) or disease model (SEARCHBreast, SharmUK). A more general approach was launched under the name AniMatch (www.animatch.eu) – an innovative web-based platform that allows scientists to register and publish or search for offers to facilitate the multiple use of freshly killed animals. The overall number of people is convinced of the resource-sparing and morally sustainable approach of those initiatives. Nevertheless, challenges are seen in the additional effort needed as well as in the lack of incentives which need to be addressed and discussed. Within the presentation, the goals, advantages and limitations of those initiatives will be presented, in accordance with a current update on acceptance and challenges within the scientific community. Besides the moral exculpation for scientists, sharing initiatives provide a cost efficient way to use existing infrastructure and to conserve resources in accordance with reducing lab animal usage.

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The horse as model for osteoarthritis research – research tools vs. stakeholders

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Today, *a priori* small animal models are used in osteoarthritis (OA) basic research, whereas large animal models are used for validation purpose as well as for the improvement of surgical or therapeutic interventions. However, the applicability to human OA, especially with regard to the small animal models is questionable. The horse as a patient of considerable economic value also offers the opportunity to serve as a naturally occurring model of OA which parallels the human form disease in many aspects.

In Germany approximately 33.7% of treated horses in the clinics suffer from diseases affecting the musculoskeletal-system and are incident to high costs. In North America the costs reach the total of $1 billion annually and the incidence for lameness lies between 8.5 up to 13.7%. Especially racing thoroughbreds are susceptible to fetlock pain and lameness (25%) leading to the early retirement of equine athletes and a high economic loss to the equine industry. Therefore, up to 60% lameness incidents are caused by osteoarthritic disorders demonstrating an undeniable need for OA research not only in men but also in horse.

Moreover, when comparing the cartilage thickness among different species, the stifle joint of horses provides the closest approximation to the thickness of human articular cartilage of the knee. This comparability in size allows the applicability of comparative studies such as the examination of postoperative parameters immediately after surgery using specific scores.

In addition, the horse provides the opportunity for arthroscopic interventions to obtain synovial fluid, to assess the cartilage shape macroscopically and to harvest small pieces of cartilage for molecular biological investigations. Of note, one horse provides enough material to address most research purposes whereas lots of small animals would be needed to end up with enough material. Economic efficiency could be reached by cooperation between researchers and equine industry.

In our own work, we use the horse as *in vivo* as well as *in vitro* model to effectively reduce the number of needed animals and to gain maximum knowledge for the human system.

In vivo, we developed a model based on the creation of full-thickness defects with an average diameter size of 1 cm in the femoropatellar joint. The defects are filled directly after the creation. The specific protocol depends on the research question or therapeutic approach. Furthermore, the availability of tissues and cells from equine donors is much easier compared to human resources. Therefore, we developed *in vitro* models for OA and bone healing based on equine cells. The gained knowledge can be used for the transformation to human system as well as a replacement strategy for the small animal model in OA research.

In conclusion, as an OA patient, the horse is a suitable model for OA research. Considering animals as beneficiary and not only as research tools could give a major impact on translational medicine in the future.

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69

Comparability of the *in vitro* RTgutGC (rainbow trout) intestinal cell line to native gastrointestinal tissue

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Intestinal derived cell lines are useful *in vitro* models, which allow for focused investigations of metabolism and other responses in the intestine. The development of the first immortalized intestinal cell line derived from the rainbow trout (RTgutGC) offered an opportunity to explore intestinal uptake without the need for the use of numerous animals. Recent work using numerous compounds has acknowledged its potential as a replacement tool for animal-based laboratory studies, however there is still a lot to be explored before its widespread incorporation as a toxicity tool. Cell lines are known to acquire additional mutations or modifications while in culture, and it is important to understand to what extent this cell line retains the genetic landscape of primary intestinal tissue.

In this study, RNA-Seq sequencing of the RTgutGC cell line was used to establish gene expression in this potential animal replacement model. Over 94% of the sequences were mapped to the genome. Following filtering for transcript abundance using TPM (transcripts per million), 24,890 contigs were identified and blasted against the NR database. In comparison to the sequenced intestinal tissue, over 93% of contigs were shared between the two systems with 412 unique to the cell line. Following normalization, a significant proportion of genes were identified as differentially expressed and can be broken down as 9,852 genes upregulated and 6,746 downregulated in the cell line compared to the tissue. KEGG pathway analysis revealed 103 major biochemical pathways in this system and the presence of significant metabolism pathways (< 40%).

This study provides the first in-depth sequence data of any rainbow trout cell line and identifies many commonalities between the 3D model and native tissue. Characterization of the RTgutGC transcriptome and genes and enzymes expressed in this model will greatly help in building realistic *in silico* models of exposure when integrated with other available chemical data.

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58

Tissue specific response of organoids to the model compound pyrene in rainbow trout 3D co-cultures

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Generally, the liver is considered the principal side of xenobiotic metabolism immediately after absorption from the gastrointestinal tract, or via the circulatory system. On the other hand, the gill is the principle site of xenobiotic transfer to and from the aqueous environment. Together, these two organs contributed substantially to the bioactivation and elimination of contaminants from fish. Advances in aquatic animal alternatives have seen the emergence and support of equivalent *in vitro* cell lines and *in silico* models, which is advancing knowledge of organ specific toxic response. Nonetheless, the complexity of these systems is limited by the prevalence of one cell type in immortalized cell lines and the lack of connectiveness with other organs involved in the absorption, distribution, metabolism and elimination (ADME) chain. Primary culturing methodology of the liver and gill of rainbow trout *Oncorhynchus mykiss* are well established and known to generate functional physiologically relevant organoid models. In addition, they are considered a replacement model in line with the 3Rs principles.

The aim of the study was therefore to advance aquatic animal alternatives by not just identifying organ specific response to a model toxicant (pyrene) but also investigate variations in response incurred through the co-culture of the organs in a more physiologically relevant model. In a three-phase experiment, gill and liver organoid response was established separately and then under co-culture (together) using GC-MS, RT-PCR and western blot over 24 h. Under single culture and co-culture conditions, only the gill organoid demonstrated the conversion of pyrene to hydroxpyrene (1-OH) indicating the gills as the most important route of waterborne pyrene absorption and metabolism. Six genes (*CYP1A, AHR-α, AHR-β, UGT, GST and SREBP1*) were used to establish variation between native tissue and the organ specific organoids with no significant differences in MNE in either organoid system (under single or co-culture scenario). In addition, there was no significant difference in gene expression over 24 h following pyrene exposure or between the single and co-culture exposure routes, although dose response curves are visible. The findings support the hypothesis that co-culturing these organoids is suitable for modelling the role of gill and liver biotransformation and transport processes in the toxicological potential of aqueous contaminates.

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200

Modeling sex differences *in vitro*

Judith Lechner [1], Georg Kern [1], Elisabeth Feifel [1], Anja Wilmes [2], Paul Jennings [2] and Gerhard Gstraunthaler [1]

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Women are not small men. Every cell in the body is female or male depending on its chromosomal make-up. Sex and sex hormones affect all the cells in the body resulting in different susceptibility to cellular stressors and disease development. Since women and men were found to potentially respond differently to therapeutics, inclusion and analysis of women in clinical trials is mandatory to prevent unpredicted effects. Researchers engaged in basic research and preclinical studies are increasingly challenged by regulatory bodies, major funding agencies, and high ranked journals to also include sex as a biological variable into their study designs [1]. At present, these requirements are mostly met – if at all – through animal experimentation. *In vitro* models suffer from cells loosing their sex memory with time in culture as shown for established cell lines.

Human induced pluripotent stem cells (iPSC) generated from human somatic cells present a new trajectory to develop a novel and ethically acceptable *in vitro* model system for modeling sex differences. As detailed in [2], sex differences originate from differences in gonad development giving rise to the specific lifelong exposure with sex hormones *in vivo*. Sex hormones display organizational (persistent) and activational (transient) effects. In addition, XX and XY cells are distinct regarding their epigenomic imprinting, transcriptome, and proteome, thus being different independent of sex hormone exposure, but also responding differently to sex hormones. For example, microarray data of liver, adipose, muscle and brain tissues revealed between 14 to 70% of transcripts as having sex dimorphic expression. On a physiological level, differences in resistance of female-derived cells compared to male-derived cells to oxidative stress have been described [3].

This presentation will outline a possible approach to integrate sex as a biological variable into basic and preclinical research designs through a human iPSC-based *in vitro* model.

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287

Benefits of combining positive reinforcement training and social observation in laboratory rats

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Cage cleaning is a routinely performed husbandry procedure, which is known to induce stress in laboratory rats. As stress can have a negative impact on well-being and the comparability and reproducibility of research results, stress of laboratory animals should clearly be avoided. Further, the direct contact between rat and animal caretaker during the cage change bears a hygiene risks and therefore possibly negatively impacts the well-being of the rats and the quality of research.

Our protocol aims to improve this routinely performed procedure of cage changing. For this reason we here show a feasible protocol, which enables rats to learn via clicker training and observation to voluntarily change to a clean cage. This allows avoiding stress caused by the physical disturbance and handling associated with the cage change, and concurrently enables the reduction of direct contact between animal and animal caretaker after the training phase is completed.

The implementation of clicker training to rats is fast and easy. Rats are generally interested in the training and learn the desired behavior "cage change through a pipe" efficiently. Even without training the rats learned to perform the desired behavior by observation, as 80% of the observational learning group showed the cage-change when tested. The training further helps to establish a relationship of trust between trainer and animal. As hygiene and well-being are both very important in animal experiments, this protocol might additionally help to improve high quality research.

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111

Teaching 3R principles in ecotoxicology by performing a problem-based learning project

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In ecotoxicology, one major focus lies on the development of toxicity test methods, which replace, reduce or refine the use of laboratory animals. Consequently, important educational goals for ecotoxicology students are, on the one hand, the ability to plan and analyze scientific study designs under consideration of social and ethical principles and legal frameworks, in particular the application of 3R principles already in the early stages of research, and on the other hand an intensive training of practical laboratory skills and methods. For that purpose, in the master study program "environmental engineering and ecotoxicology" a semester project using a problem-based learning (PBL) concept has been implemented and further improved in the study years 2016 and 2017.

Starting from an open-ended PBL-case, small student teams applied a stepwise problem solving process to design a concrete semester project. This included the thorough elaboration of the theoretical background including the 3R concept, ethical and legal standards and the relevant OECD guidelines, the definition and exploration of research questions and the concretisation and performance of practical experiments. In the study year 2017, the common topic for all teams was to test the toxicity of glyphosate based herbicides and alternatives thereof (e.g. pelargonic acid (PA)). Each student team worked on specific model systems (zebrafish embryos, snails, worms, plants, or cell lines) and thereby learned about and applied a set of alternative methods to animal experiments. Findings were discussed, and compared with the other student teams, supervisors and invited experts at a final student conference.

In order to illustrate the general outcome, one of these semester projects is presented here in detail. To compare the acute toxicity of PA and its formulation TopGun to glyphosate and its formulation Roundup, *in vivo* (fish embryo acute toxicity test as an alternative animal method) and *in vitro* (RTgill-W1 fish cell line cytotoxicity assay) trials were performed.

Based on our experience and evaluation results, the PBL concept provides a suitable context for the students to learn theoretical background of the 3R principles and to apply them practically in their semester projects in combination with training of essential research skills. Furthermore, working on PBL-projects is a motivating experience for students and lecturers.

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249

Assessment of contact sensitization potential of 20 chemicals using *in vitro* reconstructed normal human epidermal model EpiDerm: Impact of the modality of application

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Assessment of skin sensitization potential has traditionally been conducted in animal models, such as the Mouse Local Lymph Node Assay (LLNA) and the Guinea Pig Maximisation Test (GPMT). However, a growing focus and consensus for minimizing animal use have stimulated the development of *in vitro* methods to assess skin sensitization. Interleukin-18 (IL-18) release in reconstructed human epidermal models has been identified as a potentially useful endpoint for the identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability [1,2].

The purpose of this study was to investigate the impact of the modality of chemical exposure on the predictive capacity of the assay. EpiDerm tissue viability assessed by MTT assay and IL-18 release assessed by ELISA were evaluated after 24 h topical exposure to test chemicals directly applied to the surface of EpiDerm. DPBS and/or acetone:olive oil (4:1) were used as vehicles. A total of 20 chemicals were tested. The testing set included 11 senzitizers and 9 non senzitizers. Two independent dose-response experiments were conducted, resulting in correct prediction of the sensitizing potency of test chemicals.

The assessment of IL-18 release using *in vitro* reconstructed normal human epidermal model EpiDerm appears to be a promising tool for *in vitro* determination of contact sensitization potential.

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209

Animal centric strategies for severity assessment and refinement

Lars Lewejohann

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Severity assessment in animal experimentation is a complex ethical issue with no straight forward solutions at hand. Suffering is an internal mental state, which is experienced by an individual and, thus, cannot be measured directly. Therefore, a major challenge for severity assessment is to find reliable criteria unbiased by the "gut feeling" of the experimenter. A proposed solution presented here is to include the animals' perspective into severity assessment. Although animals cannot fill in questionnaires, they can be asked by means of behavioral tests for their opinion about the severity of experimental measures. For example traces in internal affective states due to former experience can be revealed using tests of cognitive bias. Preference tests are a straightforward approach in testing the value of different goods. However, if a preferred good is withheld it does not necessarily indicate suffering. Moreover, it is not appropriate to conclude animal suffering from the mere dislike of an animal to participate in a certain test. Therefore, there is a demand for more sophisticated tests to gain a better understanding of the valence of the choices made. In this talk I will outline our methodological approach for combining preference tests, conditioned place aversion, and cognitive bias tests for asking the animals' point of view. Results will not only lead to a better understanding of how experimental procedures are perceived by the animals, but will also help to refine these procedures in order to improve animal welfare

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276

Application of 3D liver microtissues for assessment of drug-induced liver injury (DILI) and for studying liver steatosis

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Primary hepatocytes in 2D culture (PHH) are widely used for prediction DILI, however their predictivity is limited due to rapid de-differentiation enabling only testing of acute toxicity. 3D InSight™ Human Liver Microtissues (hLiMT) consisting of primary multi-donor hepatocytes and Kupffer cells have shown to preserve liver specific function and metabolic cytochrome activity over five weeks in culture. We investigated the utility of the 3D hLiMT for short- and long-term drug toxicity assessment and for development of an *in vitro* model of liver steatosis. The assessment of the toxicity using ATP as a cell viability marker has been performed of 110 marketed drugs with known DILI potential on 2D PHH and 3D hLiMT. The hLiMT, exhibited a more than 2-fold increased sensitivity for detection of DILI compounds, depending on the threshold employed. For the development of the *in vitro* liver steatosis model the tissues were incubated with various concentrations of free fatty acids and nile-red staining was performed using confocal microcopy at several time points. Lipid accumulation was observed up to 3-fold upon 7 days of treatment with oleate, palmitate, or both in a physiological relevant 2:1 (oleate:palmitate) ratio as compared to the control.

Our data demonstrated that 3D hLiMT are a suitable model for assessment of DILI and for development of liver steatosis model for drug efficacy testing.

Reference

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18

Focus on severe suffering: How reducing suffering can be a gateway to replacement

Elliot Lilley

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All laboratory animal suffering is a concern, but the RSPCA believes that ending "severe" suffering should be a top priority. There are a number of reasons to do this: (i) the ethical obligation to avoid or reduce suffering, (ii) the legal requirement to minimise suffering set out in Directive 2010/63 EU and (iii) the scientific benefits – it is widely acknowledged that good quality science goes hand in hand with good welfare.

As a scientific animal welfare organisation with a high level of national and international liaison with scientific and regulatory communities, we have been able to establish an integrated programme of work aimed at reducing and ultimately ending severe suffering. Our approach is well supported by the scientific community and the UK Government.

Our pioneering initiative has so far included the organisation of two major international conferences, the convening of several expert working groups, and the production, publication and dissemination of a range of resources to help reduce and ultimately end severe suffering.

Our approach has always been to challenge the necessity and justification for severe models and procedures and to apply the principles of refinement to reduce suffering. In many cases, severe suffering occurs when attempts are made to recapitulate severe human disease in animals. We suggest that this approach is fundamentally flawed – it is unrealistic to expect an animal model to mirror human disease with sufficient fidelity. We argue instead for mechanism-based approaches, which not only allow for early humane endpoints but offer the possibility of transition away from animal models towards mechanism-based *in vitro* models.

This talk will explain this concept in detail as well as providing more information on the RSPCA's resources on "severe" suffering.

Reference

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107

An improved *in vitro* model of the oral mucosa epithelium for biomarker research and transport studies: Optimization by media supplements and airlift cultivation

Grace Lin [1], Tamara Leitgeb [1], Alexandra Vladetic [1], Lisa Koch [1], Lisa Bierbaumer [1], Nadine Rhodes [1], Johanna Gostner [3], Angela Rossi [2] and Winfried Neuhaus [1]

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Over the past 60 years saliva has gained popularity as a diagnostic fluid as it offers advantages over serum such as non-invasive collection, easier handling and storage. The proteomics, transcriptomics and microbiological composition of saliva have already been intensively investigated and were used to screen for biomarkers for the detection of diseases such as Sjörgen's Syndrome, oral cancer and breast cancer. However, the appearance of those molecules in saliva and the link between the concentrations of biomarker molecules in blood and saliva is still unclear. With rising applications of saliva for diagnosis and pharmaceutical applications in the buccal cavity, *in vitro* models representing the blood-saliva barrier are of necessity to understand why a proposed biomarker appears in saliva and how it is transported from blood to saliva or *vice versa*.

The blood-saliva barrier is defined as the sum of epithelial cell layers from the oral cavity and salivary glands, whereby the cell layers can be infiltrated by other cell types, depending on their location. At AIT an *in vitro* model representing the oral mucosa based on the epithelial cell line TR146 from a buccal carcinoma has been established as a transwell model. The transport of molecules is assumed to cross the oral mucosa epithelium layers using the passive paracellular transport route. Therefore, the integrity of tight junctions was assessed rigorously by the measurement of TEER (transepithelial electrical resistance) over time. After a maximum resistance value was reached, the paracellular tightness was evaluated by measuring the permeability of carboxyfluorescein. To improve the barrier integrity various media supplements in different composition were tested systematically in different set-ups (airlift or submerged). These different set-ups of the model were additionally characterized by applying high-throughput qPCR (Fluidigm) and real time PCR using defined markers for keratinization, tight junction, mucins and aquaporins. For morphological characterization HE and immunofluorescence stainings were performed.

Surprisingly, for the first time, airlift cultivation showed a significant improvement regarding the barrier forming properties using media with supplements, which also coincided with a thicker monolayer upon HE staining. The optimized model was used to investigate the transport of tryptophan, a marker for Alzheimer's disease and depression, whereby results suggested an active transport into the saliva compartment. Future studies include the evaluation of biomarkers for cardiovascular diseases and the establishment of cell models of the salivary gland epithelium to investigate its influence on the transport of biomarkers across the blood-saliva barrier.

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206

Two novel human myxofibrosarcoma cell lines represent a cellular model for tumor heterogeneity

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Objectives

Human cancers frequently display substantial intra-tumoural heterogeneity in virtually all distinguishable phenotypic features, such as cellular morphology, gene expression metabolism, and metastatic potential. Myxofibrosarcoma (MFS) comprises a spectrum of malignant neoplasms with prominent myxoid stromata, cellular pleomorphism, and distinct curvilinear vascular patterns. These neoplasms mainly affect patients in the sixth to eighth decades of life and the overall 5-year survival rate is 60-70%. Novel MFS cell lines, which enrich the bank of publicly available cell lines, are of extraordinary importance.

Methods

After the establishment of a novel MFS cell line with two well defined sub-clones called MUG-Myx2a and MUG-Myx2b, cells were characterized using short tandem repeat (STR), next generation sequencing (NGS) mutation analysis, and copy number variation (CNV). The growth behaviour and migration potential of the cells were analysed with the xCELLigence system and an MTS assay. The tumourigenicity of MUG-Myx2a and MUG-Myx2b were proved in female nu/nu Foxn1 mice.

Results

The frozen primary parental tumour tissue and both MUG-Myx2 cell lines showed the same STR profile. The facts that MUG-Myx2a showed higher proliferation activity, faster migration, and enhanced tumourigenicity were of particular interest. NGS mutation analysis revealed corresponding mutations in the FGFR3, KIT, KDR, and TP53 genes. In variation from this, the MUG-Myx2a cell lines had additionally a PTEN mutation. Analysis of copy number variation (CNV) revealed a highly aberrant karyotype with frequent losses and gains in the tumour sample. The two MUG-Myx2 cell lines share several CNV features of the tumour tissue, some CNV are only present in both cell lines and each cell line also harbours private gains and losses.

Conclusion

The well-characterised novel MFS cell lines MUG-Myx2a and MUG-Myx2b will be a useful tool to gain further insights into the pathogenesis and tumour heterogeneity of MFS and explore new treatment options.

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Merging high-content and higher-throughput screening: Microphysiological organ-on-a-chip systems integrating human retinal, cardiac and adipose tissue

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Drug discovery and development to date has relied on animal models, which are useful, but fail to resemble human physiology. The discovery of human induced pluripotent stem cells (hiPSC) has led to the emergence of a new paradigm of drug screening using human patient- and disease-specific organ/tissue-models. One promising approach to generate these models is by combining the hiPSC technology with microfluidic devices tailored to create microphysiological environments and recapitulate 3D tissue structure and function. Such microphysiological organ-on-a-chip systems (OoCs) combine human genetic background, *in vivo*-like tissue structure, physiological functionality, and "vasculature-like" perfusion.

Using microfabrication techniques, we have developed multiple OoCs that incorporate complex human 3D tissues and keep them viable and functional over multiple weeks, including a "Retina-on-a-chip", a "Heart-on-a-chip" and a "White adipose tissue(WAT)-on-a-chip". The OoCs generally consist of three functional components: organ-specific tissue chambers mimicking *in vivo* structure and microenvironment of the respective tissues; "vasculature-like" media channels enabling a precise and computationally predictable delivery of soluble compounds (nutrients, drugs, hormones); "endothelial-like" barriers protecting the tissues from shear forces while allowing diffusive transport. The small scale and accessibility for *in situ* analysis makes our OoCs amenable for both massive parallelization and integration into a high-content-screening approach.

The adoption of OoCs in industrial and non-specialized laboratories requires enabling technologies that are user-friendly and compatible with automated workflows. We have developed technologies for automated 3D tissue generation as well as for the flexible plug&play connection of individual OoCs into multi-organ-chips. These technologies paired with the versatility of our OoCs pave the way for applications in drug development, personalized medicine, toxicity screening, and mechanistic research.

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Impedance spectroscopy as a method to discriminate between all GHS categories for eye irritation *in vitro*

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For the toxicological endpoint of eye irritation, the first alternative test systems based on *ex vivo* or *in vitro* models have been developed and validated. However, besides all efforts, the Draize eye test is still not completely replaced by alternative animal-free methods because the alternative methods cannot distinguish between the globally harmonized system for the classification and labelling of chemicals (GHS) category 1 serious eye damage and category 2 eye irritation [1]. To develop a single *in vitro* test to identify all GHS categories for eye irritation, we combined organotypic cornea models based on primary human cells with an electrical readout system that measures the impedance of the test model. First, we showed that employing a primary human cornea epithelial cell based models is advantageous in native marker expression such as cytokeratin 3 and 12 to the primary human epidermal keratinocytes derived models. Secondly, by employing a non-destructive measuring system based on impedance spectroscopy, we could increase the sensitivity of the test method. Moreover, the impedance measurement allowed for the first time to detect the persistence of irritative effects by repeated measurements in an *in vitro* model and thus to distinguish between all GHS categories. Substances that do not need to be labeled stayed above 60% normalized to the negative control. Category 1 substances reduced the tissue integrity after application below 6% and the effect did persist over a period of 7 days. Category 2 substances however, could be identified by a decrease below 60% after the application of a category 2 chemical such as ethanol and increased again above 50% after 7 days. Thereby, all GHS categories of eye irritation could be identified by repeated measurements over a period of 7 days. Based on a novel prediction model we achieved an accuracy of 78% with a reproducibility of 88.9% to determine all three categories of eye irritation in one single test. This could pave the way according to the 3R principle to replace the Draize eye test

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Directive 2010/63/EU – progress, challenges and future directions

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Directive 2010/63/EU [1] took effect five years ago. A review was required to be carried out on its effectiveness in 2017. The review confirmed that the level of progress which has been made since the adoption of the Directive varies across the EU. This is largely due to widely differing starting points and speed of implementation of the respective national legislation.

Nevertheless, there are some aspects which are already developing and working well, such as the introduction of Animal Welfare Bodies (AWBs). This is considered a welcome addition, and these AWBs are already contributing positively to animal use and care practices within establishments. Other positive effects include raising standards in research practices, improving Three Rs awareness, promoting a culture of care, and growing recognition within the research community of the important link between animal welfare and good science.

However, there remain a number of challenges to achieve the Directive objectives. A particular area of focus should be the national processes for project evaluation and authorisation, which play a crucial role if further harmonisation objectives are to be attained.

Finally, a recent Commission proposal to streamline environmental reporting also includes Directive 2010/63/EU. The proposal has, through greater transparency and timely access to key data, including statistics on animal use and non-technical project summaries of authorised projects, great potential to benefit not only the general public but the whole scientific community at large.

Reference

[1] The European Parliament and the Council (2010). Directive 2010/63/EU on the protection of animals used for scientific purposes. *OJ L 276*, 33

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Culture of Care – from words to action

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Culture of Care is a central theme within Directive 2010/63/EU [1] inter alia through the requirements for continuous application of the Three Rs, and for education, training and competence of staff. The Directive recognises that animal welfare considerations should be given the highest priority in all animal care and use practices. To enable this, each establishment should have an Animal Welfare Body. One of the tasks of the Animal Welfare Bodies is to foster a climate of care.

The theme of Culture and Care is further elaborated in the guidance developed by Member States and stakeholders to facilitate the implementation of the Directive. Culture of Care is featured in the guidance on Animal Welfare Bodies and National Committees, Education Framework, and Inspections and Enforcement.

However, simply meeting the legislative requirements will not ensure appropriate welfare, care and use practices. Although Culture of care can be anchored in legislation and supported by guidance, it can only be achieved through individual commitment which itself can only be enabled through institutional support. Most of the roles involved directly or indirectly in animal use and care have a role to play in building the desired Culture of Care.

The crucial element to achieve the desired outcome rests inside each establishment: a structured management approach to Culture of Care. The talk will explore, with examples, concrete ways to help assess and address Culture of Care – moving from words to action.

Reference

[1] The European Parliament and the Council (2010). Directive 2010/63/EU on the protection of animals used for scientific purposes. *OJ L 276*, 33.

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National Committees under Directive 2010/63/EU and Three Rs Centres – complementing one another for a win-win

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All Member States are required to have a National Committee for the protection of animals used for scientific purposes, in line with Directive 2010/63/EU [1]. The main functions of the National Committee are to ensure coherent approach to project evaluation, advise competent authorities and animal-welfare bodies in establishments on matters dealing with the acquisition, breeding, accommodation, care and use of animals in procedures, and ensure sharing of best practice.

Access to information on the Three Rs is therefore central to the good functioning of the National Committees, and to enable them to fulfil their advisory role. Furthermore, there is an opportunity to play a key role in the identification and dissemination of new Three Rs ideas and initiatives.

Three Rs centres, on the other hand, are not mandated by law. However, owing to the supportive political climate and increasing ethical considerations, several Three Rs centres have been established in Europe in recent years, both by national governments and by private initiatives such as by universities. Supporting Three Rs centres, in part, also meet the Member State obligations to contribute to the development and validation of alternatives, and to promote alternative approaches at national level. The roles, tasks and means available to the centres vary greatly.

As such centres share common goals and objectives, it is of interest to all involved to call for a closer collaboration and strategic thinking. Investing scarce resources in a manner that complements each other's activities has the potential to truly advance the Three Rs for all.

Reference

[1] The European Parliament and the Council (2010). Directive 2010/63/EU on the protection of animals used for scientific purposes. *OJ L 276*, 33.

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Different cultures of care for lab animals – a comparative study of practices at universities and private research institutions in Denmark

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Background
The notion of a culture of care comprises, on one hand, the actual treatment of laboratory animals, and, on the other hand, the organizational pre-requisites that are necessary for implementation of this. Chiefly speaking, the pre-requisites relate to the organization's focus on and communication about the importance of the welfare of lab animals and the 3Rs. It is poorly understood whether organizational arrangements influence the culture of care. In most countries, lab animals are used in universities as well as in private research institutions. The steering principles of these two institution types, however, differ. This may influence the culture of care.

Research aim
The aim was to compare the culture of care for lab animals and the focus on the 3Rs in universities and private research institutions in Denmark. We only compared the pre-requisites, and not the actual treatment of the animals.

Methods
A mixed method sociological design was employed. First, a questionnaire-based survey of animal researchers working in Danish research institutions was carried out in 2015 (N = 234; response rate 37%). We examined the influence from university and private sector employment on within-organizational communication; knowledge of local animal welfare bodies, reluctance towards implementing animal welfare concerns as well as knowledge of and focus on 3R. Secondly, to support the interpretation of the questionnaire findings, data from a qualitative study (carried out in 2017-2018) was drawn on where managers and animal researchers from university and private research institutions were interviewed (N = 12).

Results
The survey showed clear differences between university and privately employed researchers. After socio-demographic adjustment researchers in private institutions had more frequent communication about the 3Rs (Spearman's r 0.328; $p < 0.000$), and less reluctance towards implementing animal welfare concerns (Spearman's r 0.360); $p < 0.000$) than university-based researchers. More privately (96%) than university employed researchers (68%) knew of the local animal welfare body ($p < 0.000$) and found it helpful. Privately employed researchers had better textbook knowledge of refinement (Spearman's r 0.278; $p < 0.000$) and a higher degree of focus on the 3Rs (Spearman's r 0.357; $p < 0.000$). The interviews shed light on likely reasons for these differences by pinpointing that researchers in private institutions experienced more support from management with respect to facilitating animal welfare and 3R implementation, and clearer rules of conduct. Researchers in universities experienced less pressure from management, one reason being that university managers expected local research groups to make changes themselves.

Conclusion/discussion
Institutional differences influence the culture of care for lab animals and focus on the 3Rs in Denmark. The market-driven management and decision-hierarchy of private research institutions appears to be better equipped to implement an animal-welfare friendly culture. Universities have historically been characterized as "organized anarchies" which could explain the less strong and concerted culture of care for lab animals. New public management principles, inspired by market and centralization thinking, has surfaced in universities. We discuss why this management trend may have failed to promote a culture of care for lab animals, and what to do about it.

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The impact of fibroblasts from atopic dermatitis patients on the epidermal differentiation of full-thickness skin equivalents

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects 15-20% of children and 1-3% of adults worldwide. Notably, although AD one of the most common skin diseases especially in industrialized countries, its pathogenesis is still not fully understood. During the past decades, most studies on pathological mechanism have been conducted in mouse models. However, the predictivity of these models for the human situation is controversially discussed due to distinct interspecies-related differences and the fact that mice do not develop AD spontaneously [1,2]. Therefore, the development of human-based AD models is of increasing interest, whereas the generation of skin equivalents using AD patient-derived cells appears particularly promising.

Hence, the aim of this study was the generation of full-thickness skin equivalents using fibroblasts from AD patients to investigate the impact of the fibroblasts on epidermal differentiation and skin barrier formation. Importantly, the fibroblasts were isolated from plucked scalp hair follicles of healthy volunteers (n = 6) and patients with AD (n = 10) according to a previously published procedure [3]. The cells were subsequently screened for the presence of common mutations in the filaggrin gene (FLG mutation R501X and 2282del4) and rare FLG variants R2447X and S3247X using Taqman allelic discrimination assays followed by a restriction fragment length polymorphism analysis. Subsequently, skin equivalents were generated using hair follicle-derived fibroblasts (HFDF) from AD patients (n = 6), from healthy donors (n = 6) and normal interfollicular keratinocytes (NHEK). The skin equivalents were characterized with regard to epidermal differentiation and proliferation. Therefore, the protein expression of FLG, involucrin (IVL), loricrin (LOR), thymic stromal lymphopoietin (TSLP) and Ki67 were analyzed using immunofluorescence staining and western blot. Additionally, the skin lipid organization was determined by ATR-FTIR and lipid composition was analyzed by HPTLC. To assess the skin barrier function, skin absorption studies were performed with the radioactively labeled standard compound testosterone. Regardless of mutations in FLG gene, skin equivalents generated with fibroblasts from AD patients showed epidermal thickening, increased expression of the proinflammatory keratinocyte-derived cytokine TSLP and increased cell proliferation rates as indicated by the expression of Ki67. Skin lipid analysis showed a more disordered skin lipid organization, lower amounts of ceramides EOS and the most polar ceramides (AS/NH and AP/AH) in the stratum corneum of skin equivalents grown from AD fibroblasts. Although, no statistically significant differences were found in the expression of the skin differentiation markers LOR, IVL and FLG, a slight trend toward a reduced LOR and increased IVL expression was seen for the skin equivalents grown from AD fibroblasts. Interestingly, all skin equivalents showed a similar barrier function. Our data show, that skin equivalents with AD fibroblasts exhibit characteristics of atopic dermatitis independently of mutations in the FLG gene. In conclusion, the skin equivalent presented here provides a valuable non-animal platform to investigate the contributions of fibroblasts in atopic dermatitis.

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21

In silico tools to assist the development of physiologically-based kinetic models: Identification, characterisation and use

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Physiologically-based kinetic (PBK) models are used to derive the concentration-time profiles of chemicals of interest in individual tissues, or organs, so enabling more accurate prediction of potential biological (toxicological) effects. In PBK modelling, the body is represented as a series of compartments connected via the blood supply. For input, the models require physiological information, such as organ volumes and blood flow, as well as chemical specific information, such as partitioning properties, solubility etc.; models can be adapted for different exposure scenarios or individual variation. Generating data for PBK models can be intensive in animal use, however, there are several *in silico* resources that can assist in the development of PBK models and so reduce animal testing. The need to identify, characterise and promote the potential of these *in silico* resources has been recognised as an essential step in increasing their uptake and application by PBK model developers [1]. One significant resource is the Knowledgebase of existing PBK models (307 unique chemicals collated by Lu et al. [2]) that can serve as templates from which to derive models for new "similar" chemicals. Whilst there is currently no consensus as to the best method of defining similarity between chemicals, there are many *in silico* methods that can be applied (such as similarity in physico-chemical properties, maximum common substructure or chemical fingerprints). The work presented here includes an evaluation of the availability and applicability of a range of *in silico* tools of relevance to PBK modelling. In particular, the results of a study demonstrating how the use of different measures of chemical similarity influenced which chemicals were selected to serve as templates for the development of new PBK models are presented.

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Modelling oxygen consumption in brain organoids

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Organoid technology has emerged as a novel method to realise physiologically relevant constructs, thanks to the self-renewing ability of stem cells, which differentiate into multiple, organ specific cell types. However, further strategies to guarantee the nutritional needs of organoids are required. Not only do local oxygen concentration levels influence cell fate, but the gas is also widely considered as the limiting nutrient for 3D cell cultures and its deprivation leads to a necrotic centre, compromising tissue function and structural arrangement. In this work we combine computational models with images of mature mid-brain organoids cultured in bioreactors to identify a minimum oxygen concentration below which cells are non-viable.

Mid-brain organoids were generated from human neuroepithelial stem cell (NESC) lines as described in Monzel et al. [1] and Berger et al. [2]. They were cultured in a *Quasi-Vivo* bioreactor with a medium flow rate of 240 µL/min for 30 days. At the end of this period, the organoids were fixed, sectioned and stained with Hoechst nuclear dye. The middle section was analysed with a confocal microscope and the area of the dead core and the area of the entire section was measured using image processing software. In parallel, computational models combining oxygen reaction and diffusion through the organoid and fluid-dynamics in the bioreactor were developed to determine the oxygen concentration profile in the system. To determine the critical viable oxygen concentration, we compared the computed cross-sectional areas at the center of the organoids with those obtained from image processing of Hoechst-stained slices. The results indicate that cells below 0.040 ±0.011 mole/m^3 are unlikely to survive in the core, which is also the critical value needed for mitochondrial ATP production [3]. The results can be used to optimise organoid preparation protocols and design organoid culture systems which guarantee sufficient nutritional resources to maintain physiological form and function and enhance the predictive value of this promising technology.

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Organotypic *in vitro* human airway models can recapitulate aspects of pulmonary fibrosis

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Introduction

Pulmonary fibrosis (PF) is a debilitating, typically fatal condition that may be caused by a variety of factors, including occupational and environmental exposures, drugs such as amiodarone and bleomycin, radiation exposure, and genetic predisposition. However, in 20-30% of cases the cause is unknown (i.e. idiopathic pulmonary fibrosis, IPF). Currently approved IPF drugs (pirfenidone, nintedanib) have only limited efficacy, and lung transplantation remains the best treatment option for IPF patients. Despite intense research, many of the molecular mechanisms involved in the initiation and progression of IPF remain unknown. Current IPF research relies on animal models and *ex vivo* lung tissues, which are expensive and are not always predictive of clinical trial results. Currently available *in vitro* models produced from immortalized cells also do not adequately replicate IPF. The goal of the current work is to develop *in vitro* organotypic, 3D airway models from primary human cells which can be used to study IPF.

Methods

In vitro models composed of differentiated primary human bronchial or alveolar epithelial cells and pulmonary fibroblasts were cultured at the air-liquid interface to replicate the *in vivo* microenvironment. The tissue models were treated with 10 ng/mL transforming growth factor beta (TGF-β) for at least six days to induce a fibrotic phenotype which was investigated using histological, immunohistochemical and gene expression analyses.

Results

Treatment of the models with TGF-β induced changes characteristic of PF, including increased expression of alpha-smooth muscle actin and deposition of Type III collagen and vimentin protein in the stromal compartment. In addition, qRT-PCR revealed increased mRNA expression of other fibrotic markers; matrix metalloproteinase 2, fibronectin and Type III collagen.

Conclusions

By recapitulating aspects of PF, these co-culture tissue models provide an experimental system to study the cross-talk between the injured epithelium and fibroblasts, which is believed to be a key factor in PF development. Furthermore, these models can be utilized to investigate the molecular events contributing to pulmonary fibrosis and to evaluate new therapeutic agents.

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Unique animal free 3D culturing of human cancer cells and normal cells

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Preclinical studies to unravel the biology of human cancers and to develop new treatment strategies have traditionally been carried out in conventional two-dimensional (2D) cell monolayers. However, these cancer models have proven inadequate as reliable preclinical tumour models due many to inherent limitations among them the three-dimensional (3D) tissue architechture *in vivo*. Hence, novel 3D cell culture models are needed, which in many aspects can mimic the native human extracellular matrix with embedded cells. In this work, we present a 3D electrospun polycaprolactone (PCL) mesh mimicking the collagen network of tissue. PCL is naturally hydrophobic and the mesh was subjected to plasma treatment to obtain hydrophilic fibers. Biocompatibility tests performed using L929 mouse fibroblasts according to ISO standards 10993-12 and 10993-5 show that the PCL scaffold and its support made of polylactic acid were non-toxic. The human breast cancer cell lines MCF-7 and JIMT-1, the normal-like human breast epithelial cell line MCF-10A and human adult dermal fibroblast were cultured on PCL scaffolds and cell proliferation was monitored using the AlamarBlue assay. Confocal microscopy analysis and cryosectioning showed that the cells of all cell lines penetrated deep into the filters within one week of cell culturing. The cancer cells formed tumour like clusters with the cells attaching mainly to each other and partly to the fibers. The human adult dermal fibroblast mainly stretched out along the fibers while the MCF-10A cells also stretched between fibers. In conclusion, we believe that these results prove the strong potential of electrospun 3D scaffolds as a suitable *in vitro* tool to culture various normal and cancer cell lines and may minimize large-scale nonhuman use of laboratory animals in future while providing a better drug efficiency prediction.

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Cytotoxic activity of six seaweed compounds, alone and in combination with doxorubicin and cisplatin in a panel of human breast cell lines

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Breast cancer (BC) is the most diagnosed malignant tumor among women in Western countries [1] and a leading cause of cancer death among females [2]. One major problem about BC treatment is the multidrug resistance coupled to the toxicity of some chemotherapics [3]. The combination of drugs with bioactive compounds of natural origin can represent a new approach in cancer treatment. Despite we still know little about this, some natural products have been studied as combinatorial therapy with anti-cancer drugs, revealing efficacy in lowering anticancer drugs doses or acting in a synergistic way, potentiating the drugs's effect, or even reducing toxicity to normal cells [4]. The aim of this study was to evaluate the cytotoxicity of six seaweed bioactive compounds (Astaxanthin, Fucoxanthin, Fucosterol, Phloroglucinol, Laminarin and Fucoidan) alone, at different concentrations, and in combination with two anticancer drugs (cisplatin and doxorubicin), at two concentrations (previously tested to gauge the cytotoxicity concentration for each cell line), in a panel of three human BC cell lines with different characteristics in terms of estrogen receptors and her2 expressions (MCF7, MDA-MB-231, SKBR3) and one normal breast epithelial cell line (MCF12A). To achieve the cited aim, the cytotoxic effect of tested compounds, alone and combined, were assessed by the MTT assay after 72 h of exposure. The results showed that Fucoxanthin alone (at 20 µM) decreased viability in all tested cell lines. Fucosterol negatively influenced the viability at 10 µM in MDA-MB-231 and SKBR3 cells. When in combination (seaweed compound + drug), we found that Fucosterol seems to increase the cytotoxicity of drugs in the BC cell lines but not in the cell line MCF12A. Further studies are warrant, not only to assess the possible mechanisms involved in the cytotoxic effects of these two compounds but also further exploring the suggestive differential behavior of Fucosterol.

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Chronic lymphocytic leukemia cells as an *in vitro* model in preclinical drug evaluation

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Reliable disease and healthy control models predictive of human responses are of vital need in preclinical evaluation of efficacy and safety of novel drugs. Chronic lymphocytic leukemia (CLL) is a malignancy of mature B cells which are highly dependent on interactions with the tissue microenvironment. In search of novel drugs for treatment of CLL, establishment of reliable *in vitro* models is of high importance. Availability of primary B cells obtained from patients is a good approach. However, despite the long survival of malignant B cells *in vivo*, they do not proliferate and die *in vitro*. The aim of this study was to establish CLL endogenous growth conditions as an *in vitro* disease model. Hence, we stimulated primary CLL cells with a) synthetic oligonucleotide DSP30 containing a CpG motif that activates immune cells by augmenting the expression of interleukin 2 (IL-2) receptors in combination with IL-2; b) Ionomycin, a calcium ionophore, which increases intracellular calcium and in combination with PMA activates protein kinase C, mimicking B cell receptor activation; and c) LPS, which activates TLR4.

Malignant B cells obtained after informed consent from patients diagnosed with CLL were incubated with IL-2/DSP30, ionomycin/PMA and LPS for 24 h and 48 h. Cell viability was measured by resazurin-based metabolic activity PrestoBlue assay. Stimulation of CLL cells (N = 19) by IL-2/DSP30 increased cell viability by 2-fold (206%) after 24 h and 4-fold (389%) after 48 h. When CLL cells (N = 16) were stimulated by ionomycin/PMA, cell viability increased to 171% after 24 h and 360% after 48 h. Incubation of CLL cells (N = 16) by LPS resulted in 129% and 136% increased cell viability after 24 h and 48 h, respectively. The same trend for increased cell viability was observed in all tested CLL samples treated with IL2/DSP30 or ionomycin/PMA, however, inter-individual differences were observed, which is in agreement with the fact that CLL is very heterogeneous disease. Stimulation of CLL cells with IL-2/DSP30 and ionomycin/PMA was thus identified as a valuable *in vitro* model.

Next, we evaluated prostaglandin EP4 receptor as a target in CLL. A selective EP4 receptor agonist, PgE1-OH, previously shown to induce apoptosis in malignant B cells [1,2] was chosen as a model drug in the present study. PgE1-OH was cytotoxic in a dose-dependent manner in CLL cells stimulated by IL2/Cp, ionomcyin/PMA as well as LPS. Moreover, ionomycin/PMA activation resulted in increased levels of TNFα, which is important growth factor for CLL cells. The pre-treatment of CLL cells (N =10) with PgE1-OH decreased the production of TNFα, indicating beneficial immunomodulatory properties of PgE1-OH in CLL. This is in agreement with our previous study, in which we evaluated immunomodulatory properties of PgE1-OH in human lymphoblastoid cell lines, a predictive *in vitro* model for immunotoxicity screening of compounds [3].

In conclusion, primary CLL cells stimulated by ionomcyin/PMA were identified as a valuable *in vitro* disease model for preclinical drug evaluation and were utilized for evaluation of novel drug candidate PgE1-OH.

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262

EpiIntestinal on a Chip: Label-free microphysiometry of intestinal epithelium

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Microphysiometry showed to be a useful tool to monitor the energy metabolism of living cells and its interaction with living cells. In the past the technique was mainly used for monitoring of 2D monolayers of living cells [1]. Recently, our group showed that it is also possible to monitor the extracellular acidification rate (EAR) and transepithelial electrical resistance (TEER) of 3D skin constructs in an automated assay maintaining an air liquid interface (ALI) with the IMOLA-IVD technology.

In this work we present an intestine-on-a-chip by monitoring EAR and TEER of the MatTek 3D-small intestinal tissue model (EpiIntestinal) for 12 h. A periodic cycle of 96 min ALI, 10 min TEER measurement and 15 min washing step was used. The test substance (0.2% sodium dodecyl sulfate) was applied after 8 h of measurement. After application of the tests substance a reduction of the EAR and the change in TEER could be monitored. To be able to monitor the EAR a low buffered basal medium was used. The presented work shows a proof of principle of automated monitoring of EAR and TEER at a 3D intestine model maintaining an automated ALI. The EpiIntestinal model on the IMOLA-IVD chip is a promising research tool for the use in the field of toxicology, cellular metabolism studies or drug absorption research

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Reconstructed 3D model of human small intestine for prediction of gastrointestinal toxicity and drug absorption

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Small intestine is an important gateway through which many nutrients, drugs and other substances enter blood flow. In fact, about 90% of orally administered drug absorption occurs in the small intestine. Thereby there is a need of good and reliable *in vitro* model capable to predict drug toxicity and absorption/metabolism patterns. However, currently available *in vitro* intestine models are neither organ nor species-specific, relying predominantly on the use of cell lines generated from the colon or kidney. Others lack a proper 3D architecture and functionality, which in turn affects their ability to properly predict drug absorption and toxic effects.

Here we present the reconstructed 3D human small intestine model – EpiIntestinal, which mimics morphology and cell-type composition of normal human small intestine. As opposed to organoid models, EpiIntestinal is polarized and allows studying bidirectional drug penetration through intestinal wall. It expresses proteins involved in active drug transport and metabolism at physiological levels, which makes it ideal for modelling of complex drug absorption profiles, including the permeation, metabolism, drug-drug interaction and adverse effects of drugs on epithelium. Current studies revealed that EpiIntestinal mimics the *in vivo* drug absorption profile much closer than the Caco-2 model. EpiIntestinal was also shown to predict toxicity with much higher specificity and sensitivity than the animal model. All in all, this model represents a promising tool to model complex processes occurring in small intestine.

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Development of a human gut-on-chip model to study phagocyte dependent inflammatory response

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Introduction

The intestinal epithelium forms an essential barrier to prevent translocation of microorganisms, toxins or other potentially harmful molecules into the blood stream. In particular, dendritic cells of the intestinal epithelium facilitate essential tolerance signals to commensal microbiota of the gut flora and regulate an adapted immune response against invading pathogens. Sepsis is typically associated with a dysregulation of this adapted immune response and is accompanied with a disruption of the epithelial and endothelial gut barrier which enables dissemination of pathogens within the human body.

To emulate these processes we established a microfluidically perfused three-dimensional gut-on-chip model to elucidate the pathophysiological mechanisms underlying the inflammation-associated gut barrier breakdown.

Methods

MOTiF (multi organ tissue flow) biochips made from polystyrol were used for the establishment of the gut model. The biochips are composed of two chambers separated by a micro-porous membrane. Each chamber is connected to inlet and outlet channels allowing independent perfusion of the individual channels and application of microfluidic shear stress. Human umbilical vein endothelial cells (HUVECs), monocyte-derived macrophages and intestinal epithelial cells (Caco-2) were assembled on the biochip membrane and grown for 7-14 days in presence of physiological flow conditions.

Results

Within one week of perfusion the epithelial cells formed self-organized and well-polarized villus- and crypt-like structures that resemble essential morphological characteristics of the human intestine. In the epithelial tissue, dendritic cells were differentiated that specifically respond to bacterial lipopolysaccharide (LPS) challenge. LPS was well-tolerated at the epithelial side corresponding to the gut lumen without signs of immune activation and a significant release of pro-inflammatory cytokines. In contrast, LPS stimulation at the endothelial side of the gut-on-chip model triggered a release of the pro-inflammatory cytokines TNF, IL-1b, IL-6 and IL-8 by activation of macrophage residing in the endothelium.

Conclusion

The gut-on-chip model represents a promising tool to gain more detailed insight into the communication processes of commensal bacteria with their host in the human gut. Thus, a gut model facilitating a long-term co-culture with probiotic bacteria such as *Lactobacillus rhamnosus* is currently being established. In addition, facultative pathogenic microorganisms (e.g. *Candida albicans*) will be integrated to study host-pathogen interactions *in vitro*.

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Emulating the gut-liver axis – organ-on-chip as translational tool in sepsis research

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Introduction

Sepsis is a life-threatening disease, associated with a severe infection and organ dysfunction causing mortality of more than 50%. So far, no effective specific anti-sepsis treatments are available, and management of sepsis patients relies mainly on early recognition allowing correct therapeutic measures. During disease progression, a disruption of epithelial and endothelial barrier function is a typical pathological change. In particular, the gut represents a major site of the microbiome – host interaction that requires tight regulation of immune tolerance to commensals versus acute immune response to pathogens. Deregulation of these processes i.e. due to dysbiosis could result in gut barrier breakdown and dissemination of bacteria and fungi into the circulation.

Main section

Two major mechanisms have to be considered to be responsible for barrier breakdown: Signals generated by the deregulated and overreacting immune system, as well as signals or activities from pathogenic bacteria and fungi directly interacting with epithelial or endothelial cells are assumed to contribute to gut barrier breakdown. As a result, microbial translocation causes systemic inflammation and leads to the development of acute sepsis with the liver among the first organs affected. To investigate the underlying molecular and cellular mechanisms of an inflammation-associated organ dysfunction we developed a human gut-liver axis based on a microfluidically perfused organ-on-chip platform. The *in vitro* gut-liver axis comprises tissue resident as well as circulating immune cells emulating essential components of the human immune system. Biochip-integrated sensors allow an instant monitoring of oxygen saturation and integrity of endothelial and epithelial layers by TEER measurement under continuous perfusion. Current work focusses on the integration of bacterial and fungal microorganisms into the gut-liver model to emulate human microbiome-host interaction under homeostatic conditions. Further, acute and chronic infection models are established to allow investigation of molecular and cellular processes related to sepsis-associated multi-organ failure *in vitro*.

Conclusion

The multi-organ platform closely resembles microphysiological conditions of the human gut-liver axis and holds the potential as powerful tool for translational clinical research and alternative to animal experimentation.

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Establishing endothelial cells derived from iPSCs for systemic sclerosis disease modeling and for *in vitro* vascular toxicity testing

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Systemic sclerosis has been considered as a connective tissue disease, characterized by immunological abnormalities, vascular injury and fibrosis of the skin and various internal organs. The pathogenesis of scleroderma is unknown, but it seems that endothelial apoptosis and vascular alterations are significantly involved. In order to get deeper insights into disease progression, researchers depend on relevant and standardizable disease models. However, isolation of specific, differentiated somatic cells from patients in reasonable cell numbers is not always possible. In this regard the use of pluripotent stem cells and subsequent differentiation of these cells towards the cells of interest in an unlimited cell number has led improved disease modeling. Especially induced pluripotent stem cells have an advantage as they can be generated from any consenting individual and are not afflicted with ethical concerns, as are human embryonic stem cells.

Therefore, we isolated fibroblast from systemic sclerosis patients and induced them toward pluripotency stage followed by a detailed characterization. Thereafter, the generated iPS cells are differentiated to endothelial cells through a novel protocol we developed using different small molecules and growth factors. QPCR and immunostainings demonstrated some deficiency in ve-cadherin expression tube formation in patient-derived endothelial cells. Therefore, we succeeded in the establishment of a patient-specific *in vitro* model system that will allow studies of scleroderma in more detail as well as the development of novel therapeutic strategies. Additionally, a protocol was developed that allows the fast and efficient generation of endothelial progenitor cells. We suggest, that these iPS derived cells will also be a powerful *in vitro* test system useful in the context of endothelial cell specific toxicants

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Assay ready frozen THP-1 cells can be used like a reagent in a human Cell Line Activation Test (h-CLAT) to measure the skin sensitizing potential of chemicals

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Recently a cell based *in vitro* model has been approved by the OECD (Test N° 442E) to assess the skin sensitizing hazard of chemicals. One of the tests applied is the human Cell Line Activation Test (h-CLAT) which uses monocytic THP-1 cells as a surrogate for dendritic cells. These cells express CD86 and CD54 upon activation through sensitizing molecules at subtoxic concentrations.

A major obstacle of the assay is the cultivation of the THP-1 cells. The concentration rage at which skin sensitization can be measured without significantly reducing viability, is very tight. A healthy and highly viable culture of THP-1 cells is therefore of the essence. THP-1 are known to recover badly from suboptimal cultivation or cryopreservation. It usually takes at least a week of intensive care until the cells regain an acceptable viability. Because the overall fitness of the cells has a significant impact on their sensitivity to sensitizers, reproducibility of the h-CLAT very much depends on the cell culture quality and is therefore difficult to control.

We here present an optimized cryopreservation protocol for THP-1 cells. These assay ready cells recover from cryostock at a high and stable viability of greater than 90%. They can be applied in a h-CLAT skin sensitization testing immediately after resuscitation. No prior cultivation or passaging is required which eliminates the cell culture factor from the assay. By applying different reference substances like NiSO4 or DNCB we demonstrate the assay ready frozen THP-1 are equally susceptible to skin sensitizers like cells from a continuously passaged maintenance culture and provide a better reproducibility.

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Development of an *in-vitro* testing battery to assess biocompatibility of medical devices

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For assessment of safety towards the end-users of medical devices and other solid products, such products have to be tested for biocompatibility before market authorization. According to standing regulations, animal testing is still state of the art, but there is a drift towards newly developed *in-vitro* and *in-chemico* methods.

During our latest research we focused on establishing an *in-vitro* testing battery to examine the biocompatibility of medical devices that get in contact with the skin or mucosa of the patients. Such tests cover at least endpoints like cytotoxicity, skin irritation and skin sensitization.

Cytotoxicity is assessed via *in-vitro* methods. However, irritation and sensitization are still tested on animals.

Various assays including chemical methods, 3D skin models and others have already been developed by the cosmetic industry and implemented as OECD guidelines for testing of pure chemicals. During our research we established such methods for the assessment of extracts from medical devices.

To identify a possible sensitization potential, a screening method to cover the adverse outcome pathway of the skin sensitization process was currently developed. The molecular initiation event, the binding of so called haptens (small molecules that become allergenic when binding to a protein) to a peptide can be assessed by the chromatographic method DPRA (Direct Peptide Reactivity Assay). The molecular and cellular responses include the activation of an antioxidant pathway in keratinocytes and the activation and hence phenotypical deformation of dendritic cells, which can be determined via the reporter gene assay ARE-Nrf2 and h-Clat (human Cell Line Activation Test), respectively.

So far, various samples have been examined in the different *in-vitro* assays. The extracts were successfully tested in the DPRA and ARE-Nrf2 assay. Spiking of sample extracts was used to prevent false negative results. Additionally, samples were examined with animal testing in order to compare the results which showed more sensitive responses in the *in-vitro* assays. For 11 out of 13 pure chemicals the *in-vitro* assays showed a response when testing concentrations of a factor more than 1,000 times lower than those of *in-vivo* experiments.

Further, it could be shown, that not only materials themselves but also the manufacturing process plays an important role in the assessment of biocompatibility. In this context, production processes of medical devices were improved with the help of these alternative methods, as due to the costs and ethnic reasons more samples can be screened with *in-vitro* assays.

Summarizing, these assays are developed not only with a sufficient sensitivity, but also to be robust, simple to use, ethically responsible and inexpensive in comparison to current animal testing.

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hAELVi cells as an *in vitro* inflammation model

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We present the proof of principle of a simplified *in vitro* lung test system that enables the screening of anti-inflammatory drugs on human alveolar epithelial cells. The hAELVi (human Alveolar Epithelial LentiVirus immortalized) cells were used as a cell line mimicking the alveolar epithelium [1]. Through their ability to form a stable epithelial barrier and their unlimited availability as a cell line in contrast to primary cells, these cells are highly suitable for the establishment of an inflammatory model and have many advantages, when compared to the complicated handling of models established from primary cells [2,3].

Using this model, we are able to mimic an acute lung inflammation characterized by a massive drop in barrier tightness through TNF-α exposure, which is known to activate the NF-κB pathway [4]. The addition of TNF-α led to a significant drop in the transepithelial electrical resistance (TEER) and an increased transport rate of marker substances such as sodium fluorescein. This effect can be reversed by addition of typical anti-inflammatory drugs (e.g. hydrocortisone), suggesting the utility of hAELVi cells as a translational model for the impact of drugs on alveolar permeability.

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193

P-glycoprotein modelling: *In silico* classification model of P-glycoprotein interacting compounds

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P-glycoprotein (P-gp) is a transmembrane protein that actively transports a wide variety of chemically diverse compounds out of cells. It is highly associated with the ADMET properties of drugs and drug candidates and it plays a main role in the multidrug resistance (MDR) phenomenon, which leads to the failure of chemotherapy in cancer treatments. Moreover, it has been showed that P-gp contributes to decrease toxicity by removing compounds from cells in mammals and preventing intracellular accumulation. Therefore, in the drug discovery and toxicological assessment process it is advisable to pay attention whether a compound under development could be transported by P-gp or not, since this contributes to reach the intended target or to be removed from the cell before exerting the action. In the early phase of drug discovery process, *in silico* models for predicting the probability of interaction with P-gp are of great importance.

In this study, an *in silico* classification model able to predict the probability of a compound to interact with P-gp was developed using the Counter-propagation artificial neural network (CP ANN), a set of 2D molecular descriptors and an extensive dataset of 2512 compounds (1,178 P-gp inhibitors, 477 P-gp substrates and 857 P-gp non-active compounds). The model provided a good classification performance producing Non Error Rate (*NER*) values of 0.93 for the training set and 0.85 for the test set. The average precision (*AvPr*) was of 0.93 for the training set and 0.87 for the test set. An external validation set of 385 compounds was used to challenge the model performance. On the external validation set the *NER* and *AvPr* values were of 0.70 for both indices. The presented classifier could be used as a reliable virtual screening tool for identifying potential ligands of P-gp.

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Validation of an *in vitro* assay for the detection of residual viable rabies virus in inactivated rabies vaccines

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Rabies is a zoonotic lethal disease characterized as a severe encephalitis caused by a RNA virus able to infect all mammals, transmitted primarily by saliva of infected animals [1]. This disease is responsible for nearly 60,000 human deaths annually, with dogs being accountable for almost 99% of its transmission to humans [1]. However, human rabies is preventable using vaccines, specially inactivated vaccines for domestic animals and humans [1,2,3]. In order to attest the quality and safety of rabies vaccines various assays are demanded, most of which require large quantities of laboratory animals [3]. Therefore, several organizations engaged with animal welfare encourage policies for the development of alternative methods following the Three Rs initiative (reduce, replace, refine) [3,4]. This study aims to validate an *in vitro* assay for the detection of residual viable virus in inactivated rabies vaccines combining the direct immunofluorescent assay (DIFA) with a quantitative duplex RT-PCR assay. For DIFA, the established conditions were: 3,22x104 BHK-21 cells/cm^2 cultured for 24 h in 96 well plates with DMEM/HAM-12 medium supplemented with 2.5% FBS were then inoculated with virus samples and incubated for 2 h at 37ºC. At the end of 2 h, the medium containing virus samples was removed and new medium was added to the cells for 72 h incubation at 37ºC. After the incubation period, the medium was removed, cells were fixed with cold acetone 80% for 15 min then stained with fluorescein isothiocyanate (FITC)-labeled anti-rabies mAb for 1 h at 37ºC. Cells were then washed 2 times with TBS and plates were examined using a fluorescence microscope. Samples at 0 and 72 h of incubation were collected for the quantification assay. The protocol for the duplex RT-qPCR consisted in 15 µL of reaction mixture containing 200 nM PCR primers and 100 nM TaqMan probes and 5 µL of viral RNA, the reactions was carried out following the SuperScript III Platinum One-Step qRT-PCR kit and were analyzed using the ViiA 7 Real-Time PCR System software. The first assay quantifies rabies virus targeting the RABV nucleoptrotein gene and the second targets BHK-21 β-actin mRNA as an internal endogenous control. The efficiency of the duplex assay was 103% with r2 of 0,999. For the validation analysis, the determined LOD for RABV was 101 TCID50/mL, repeatability of 9,2% CV and reproducibility of 4,0% CV. Clinical samples positive for other encephalitis causing virus were tested to confirm specificity of the assay showing negative amplification for the non-rabies virus. The virus samples analyzed were considered inactivated when the DIFA was negative and the 72 h viral quantification was equal or lower than the 0 h. The next step in this process will be to compare the DIFA and RT-qPCR with the standard *in vivo* assay to confirm the *in vitro*'s test sensitivity. The validation of this method will allow a significant reduction in the use of animals in the different stages of vaccine production to rule out the presence of viable residual virus.

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Liver bud formation using human upcyte cells

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Liver organoids (LOs) are of interest in tissue replacement, hepatotoxicity and pathophysiological studies. For the *in vitro* generation of liver organoids, we used differentiated adult human upcyte liver cells. Upcyte cells are derived from primary human cells that underwent targeted genetic modification (upcyte process) in order to transiently induce cell proliferation resulting in expandable cells that maintain their differentiation potential. [2]

By co-culturing a defined mixture of differentiated human upcyte cells (hepatocytes, liver sinusoidal endothelial cells (LSECs) and mesenchymal stem cells (MSCs)) on a layer of Matrigel, the cells self-organized to form liver bud-like structures within 24 hours. [1]

We found that liver organoid formation is modulated by the nature of the substrate – specifically by the interplay between stiffness and biochemical ligands – and that a high initial oxygen consumption rate of LOs may lead to hypoxia within the core unless a continuous flux of nutrients is supplied through a fluidic system. When cultured for another 10 days in a bioreactor, these liver buds revealed typical functional characteristics of liver cells including basal and induced CYP3A4 enzyme activities. [3]

Our results further indicate marked differences in enzyme modulation between liver organoids and monolayer cultures. Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in many polyvinylchloride medical devices and is washed out easily. Interestingly, DEHP modulated the expression of xenobiotic metabolizing enzymes, reduced the formation of bile canaliculi and cell polarity and inhibited CYP activity in hepatocytes. DEHP had a toxic effect on LX-2 and induced the fibrogenic activation of HSC. The mode of action of DEHP was different in monolayer cultures compared to 3D-liver organoids, which were more sensitive to DEHP. [4]

In conclusion, we describe the generation of 3D functional liver structures composed of primary human upcyte cells. These liver buds can be cultured for a prolonged period of time, and potentially represent an *ex vivo* model to study liver functions. We postulate that 3D tissue-like structures simulate the *in vivo* situation much better than conventional monolayer settings and should therefore preferentially be used for toxicity studies *in vitro* in the future.

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The alternatives in education and academia: What is happening across EU

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After almost 60 years from developing the concept of the 3Rs the aim that was the "Replacement" of animals has been unaccomplished. In 2018 we are still using animals and we need to seriously focus on Replacement, and non-animal methods, to completely avoid, one day, the use of animals in science, research and education. Research and development, education and training, courses on alternatives and conferences on 3Rs have increased in number and complexity of content with the advancement of science and technologies, but this seems to have just resulted in an increased number of known methods to do experiments, both on animals and without, with the number of animals used increasing every year and many available methods just not being used.

The latest EU statistics reported the number of animals used in 2011 in the Seventh report (2013), to be 11.5 million [1]. National EU statistics for 2016 show an increase in previous years for many countries, with total number of procedures on animals: UK 3,887,296; Germany 2,798,463; France 1,769,618; Spain 821,570; Italy 698,059; The Netherlands 563,769 [2]. Independent statistics [3] show the number of animals used in 2016 worldwide to be: 20 million in USA, 16 millions in China, 11.5 in EU, 11 in Japan; 6.7 millions in Japan; 2.9 millions in Canada, 0.27 million in New Zealand. However, the goal of the 3Rs, according to point 10 of the Preamble of the Directive 63/2010/EU, is still: "this Directive represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes ... and promote the advancement of alternative approaches." Have scientists across EU achieved this goal?

Animals still used that the focus should be no longer to just develop new Alternative methods, but to use available new Alternative methods in the laboratories, communicating them effectively, teaching and showing the new methods, strategies, ways of thinking, to apply the new available instruments to old and current research problems.

Today the number of animals used in research is no longer acceptable, it means only one thing: we are not doing enough to Replace animals in Research. This fact can only mean one thing, the progress in our society is unacceptably slow in considering the life of species other than human, or the legislation is not pushed enough by the society. Animals are still treated as materials to use in a lab, not reflecting our human and intellectual evolution and the knowledge we have now about the differences between species and the respect we should owe to every single specie on Earth. We have technology and intelligence, we don't need more intelligent technologies but to use the technologies we have with intelligence, and to do that we need to communicate, teach and spread the use of alternatives in education and academia, so that what we have we can use. Advanced courses need to be developed and financed in order to train scientists, researchers and laboratory heads on the latest development in Alternatives.

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Human induced pluripotent stem cell based *in vitro* models of the blood-brain barrier: A promise for the future of drug testing, toxicity and disease modelling

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There is an urgent and tremendous need for human disease models in drug development in order to improve preclinical predictability. In the case of brain disorders drugs have to cross the blood-brain barrier (BBB) to enter the central nervous system (CNS). It was estimated that more than 95% of the drugs cannot cross the BBB. In the case of biopharmaceutics, it seems to be even more difficult for them to overcome the BBB and reach their target sites. The functionality of the BBB is altered in chronic as well as acute diseases of the central nervous system such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), epilepsy, pain, brain tumor, stroke, and traumatic brain injury. In addition, data revealed that changes at the BBB are causally linked to the disease progressions. Thus, research focuses on the BBB to develop strategies to overcome the BBB for drug CNS delivery, but also to reestablish BBB functionality in diseases to milden disease progression. In this regard, it is a huge obstacle that currently no standard *in vitro* model of the human blood-brain barrier (BBB) with *in-vivo* like properties is established. Novel developments applying brain endothelial cells differentiated from human induced pluripotent stem cells (hiPSCs) for BBB *in vitro* models are very promising, because these models exhibit *in vivo*-like high paracellular tightness and expression of functional active transporters [1]. Since hiPSCs could be generated from several cell types, it seems that there is no limit for the establishment of personalized, human, disease BBB *in vitro* models. Currently, several national as well as EU projects are initiated or running to develop hiPSC-based BBB *in vitro* models. Next to these initiatives, the potential of hiPSC-based BBB models will be underlined by presenting the hallmarks of hiPSC differentiation protocols to cells of the neurovascular unit (brain endothelial cells, astrocytes, pericytes, neurons), characterization of established *in vitro* BBB models as well as their applications and already proven usefulness for disease models for e.g. Huntington's disease, MCT8-deficiency syndrome or stroke. The question will be discussed in how far current hiPSC-based models reflect the human *in vivo* BBB and the mechanisms during diseases, and which parameters might be considered for further improvement of these models (e.g. epigenetic, gender or environmental influences). In conclusion, hiPSCs-based BBB models possess enormous potential for human preclinical disease models especially to elucidate and reflect disease and species dependent differences.

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Reference

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The Danish National Committee for the protection of animals used for scientific purposes

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The Danish national committee (DNC) was established in 2013. The DNC members are also board members of the Danish 3R-Center (www.3rcenter.dk). The DNC and the 3R-Center have a joint secretariat with The Danish Animal Experiments Inspectorate. This structure is ideal for knowledge sharing.

The Danish national committee (DNC) hosts an annual network meeting for the Animal Welfare Bodies (AWBs). The meeting includes a workshop where AWB representatives exchange experience.

The meeting also hosts a popular market place for presenting and sharing 3R-ideas from the everyday work with the animals at the facilities. No 3R-initiative is too small to be shared. Several times participants have taken home an idea from one meeting only to develop it further at their facility and present the improved version at the next meeting.

In collaboration with The Animal Experimentation Council the DNC has written guidelines describing standardised procedures, which are often part of applications to carry out experiments on animals. These procedures and their impact on the animal can be expected to be approved within the described limits as part of an animal experiment, unless the application contains other special conditions which would militate against approval, such as, for example, the existence of a less harmful method of achieving the same objective.

The members of the DNC and the 3R-board are: Chairman, Christine Nellemann, Erwin L. Roggen, Lisbeth E. Knudsen, Adrian Smith, Jan Lund Ottesen, Axel Kornerup Hansen and Peter Bollen.

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The Danish 3R-Center

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3R-News
The 3R-Center's website focus on disseminating research and news of particular relevance to persons in the field of laboratory animal science www.en.3rcenter.dk/

Projects
An important part of the Danish 3R-Center's activities is to fund research projects that can result in improvements in the field of animal testing.

Symposium
Our annual international symposium is a significant event in efforts to promote the development of the 3Rs in Denmark, as it affords the laboratory animal community a chance to become acquainted with both national and international 3R-research and take home inspiration and valuable lessons.

Educational Material
Prepared for use in Danish lower- and upper-secondary programmes. The materials are available free of charge from our website including teacher manuals (in Danish).

3R-Surveys
A significant part of the Danish 3R-Center's work is dedicated to support the implementation of the 3Rs in practice by disseminating knowledge to relevant stakeholders. In order to reinforce the activities and assess their effect, the center commissioned a study of stakeholders' knowledge and experience of the 3Rs in Denmark.

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The Danish 3R-Center – supported projects

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The Poster show a list of projects the Danish 3R-Center has supported in the last five years.

The Danish 3R-Center will promote the 3Rs by initiating research projects focusing on one or more of the 3Rs. Every year 1.5 million DKK is reserved for research Funding.

Researchers affiliated to organizations, institutions or businesses in Denmark. Researchers with their main affiliation outside Denmark may not be principal applicants but can be included as collaborators.

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A dynamic model of the alveolar interface for the study of aerosol deposition

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Nano and micro particles are largely used in industrial, household and medicinal applications. These dispersed particles can cause inflammation and the stress in lung tissue, leading to the development of disease, such as asthma or chronic obstructive pulmonary disease (COPD) [1].

However, their effect on human tissue is not well understood. In fact, the interaction between particles and epithelial cells is complex, and it is mediated by different factors, such as the humidity of the alveolar environment and the rhythmic contraction of the diaphragm. This movement generates a periodic change in alveolar volume, the displacement of the alveolar wall, and it is thought to influence nanoparticle deposition, the uptake by the epithelial wall and the inflammatory process. To understand this complex interaction between particles and lung tissue, an *in vitro* model that mimics the alveolar microenvironment is needed [2].

Measurements of small molecule transport across epithelial cell monolayers in tissue culture are routinely performed *in vitro*; however, those systems cannot reproduce the entire complexity of the alveolar interface, because they are not able to apply a mechanical cyclic strain [3-4]. In fact, a cyclic stress leads to the increase of the pulmonary blood flow and the movement of drug across the alveolar membranes due to the opening of the epithelial cell tight junctions [5].

In order to reproduce this cyclic motion, we developed an air-liquid interface bioreactor with a mobile elastic membrane to simulate physiological lung muscle stretching. This system, named DALì (Dynamic model for ALveolar Interface), consists of an aerosol generator and a bioreactor with a moving membrane placed between an air-liquid interface to study drug and nanoparticles deposition and passage.

Moreover, this bioreactor will be sensorized: in order to measure the amount of aerosolized particles, a quartz crystal micro-balance will be integrated; for evaluating the integrity of the cellular barrier and so the formation of tight junctions, a system that measures the Trans Epithelial Electric Resistance (TEER) at air-liquid interface will be integrated.

It is also important to say that the developing of this *in vitro* model paves the way in studying the effects of nanoparticles on alveolar barrier reducing the use of animal experiments. This aspect is very important for different reasons. First of all, different species react in different way to drugs or nanoparticles due to the different physiology. So, there is a high probability that animal models are not predictive of the effect on human beings. Secondly, animal experiments are expensive, due to the higher amount of nanoparticles necessary during tests and the costs in maintaining the animals. Finally, there are ethic problematic related to animal testing. For these reasons, our research is based on the 3R's Statement: Replace the experiment with methods which avoid or replace the use of animals, Reduce the usage of animals, and Refine experimental procedures to minimize pain and distress.

To conclude, in this work is presented a sensorized dynamic *in vitro* model for the study of the effect of nanoparticles on alveolar barriers, which allows the reduction of animal experiments.

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288

Introduction of a silicone embryo model for teaching in biomedical research

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In 2016, more than 2.1 million animals were used in biomedical research in Germany, the number of mice used for research being 1.4 million (BMEL, Versuchstierzahlen 2016). The principles of 3R – reduce, refine, replace – give us an ethical guideline for experimenting with animals, with the replacement of animals being the final goal.

With 3D-printing technology coming up in the last years, we see new possibilities to replace animals in research and training. One instance being the teaching of surgical techniques such as the In-Utero-Elektroporation (IUE), a technique which is commonly used in Neurosciences that allows genetic modification of the developing brain of a mouse embryo inside the uterus by intraventricular injection of a vector and applying an electrical pulse. So far, each trainee uses 25 mice, until this complex procedure can be performed independently.

By using 3D-printing technology, we created a realistic embryo silicone model, on which new trainees will be able to practice the crucial steps of the IUE, such as the injection in the ventricle and the proper placement of the electrodes without using a live mouse.

This possibility will heavily reduce the number of mice used for learning the IUE and as it can be easily extended to other organ systems, it can effectively replace mice used in teaching for biomedical sciences and research, as Russell and Burch outlined in their work on the 3R-principles.

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220

Different mouse strains – different nesting materials. Searching for the perfect refinement in the housing of mice

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Introduction
The realization of the 3R concept is an important theme in every animal husbandry as well as for the department of biomedical research of the Medical University of Graz with major attention to refinement. Therefor it is essential to provide studies which demonstrate what is needed for animal well-being. In this study different nesting material is presented to different mouse strains, both in the conventional housing and under SPF conditions. The aim is to elucidate preferences for nesting material to identify an indicator for the well-being of different mouse strains. The findings of such studies could further positively influence the results of research projects and improve the comparability of research findings in animal experiments.

Methods
Three different nesting materials (either two Nestlets (Plexx), four Cocoons (Carfil) or one hand of wooden wool (JRS)) are presented to males or females of the strains C57B/l6 and NOD housed in pairs. The animals receive the materials randomized over night for the duration of 18 hours during the dark phase. The built nest is scored every morning at the same time by the same experimenter. The score started at 0 (did not interact with nesting material) up to 10 (built complete nest) possible points per day. The three materials were presented three days in a row as trial one and repeated as trial two to unmask a possible learning effect.

Results
We found differences in the preference of the three provided nesting materials as well as between the tested strains. Even varieties between male and female animals were discovered.

Discussion
Relatively simple measures like providing nesting material increase not only the well-being of our laboratory animals but also have a direct effect on the development and the behaviour as reported before [1]. The manner of keeping laboratory animals and optimizing the conditions for the kept strains under the 3R concept directly influences the scientific output of the used animals [2].

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AIVIS 1.1 – let's draw future!

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Toxicological science has been increasingly focused on mechanism-based testing strategies favoring animal-free methods. A number of such scientific strategies showed to be faster, more reliable and less expensive than animal testing, and/or provide additional information regarding affected molecular events [1]. Also society and European legislation explicitly demand support for the evolution towards the realization of animal-free safety assessment. During the "First Austrian Science Coordination Workshop for In Vitro & In Silico Approaches to Safety Assessment of Chemicals and Environmental Media", held in February 2017 in Linz, Austrian experts from science and public authorities discussed research interests as well as global needs. With this, the "Austrian In Vitro & In Silico safety science" (AIVIS) initiative was founded in the interest to support a broad set of short, medium and long term goals and coordinate important actions, all of which were outlined in the AIVIS workshop report [2]. However, in order to achieve the far-reaching goals, a democratic, broad multi-stakeholder motion is needed. This requires communicating needs and perspectives in a manner that is easy to understand, broadly attractive and convincing, but at the same time, scientifically correct, traceable and comprehensive. Therefore, we are now organizing available knowledge graphically into a hierarchical network [3]. We are inviting congress participants to improve this knowledge network by pinning further key knowledge to the poster or providing verbal contributions that will be recorded. The starting point for the highest-level conceptual message is: "Modern chemical management needs to comply with continuously increasing safety demands, economic sustainability and ethical responsibility. The current evolution of animal-free, mechanistic toxicology including systematic uncertainty analysis of existing animal-tests is starting to offer solutions to this demand. However, intensive and careful work at the regulatory-science interface is needed to achieve the desired break-through. Also Austrian scientists are ready to provide support for key research needs." This AIVIS 1.1. initiative shall be a next step for AIVIS as a longer-term science communication project. Ultimately it shall democratically reach out to all Austrian stakeholders and foster the new, highly desired climate of change and innovation.

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Comparison of breathing patterns for aerosol inhalation using an electro-mechanical lung simulator

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A EU wide health survey of the Organisation for Economic Co-operation and Development (OECD) [1] has shown that 6.1% of the population in Europe aged 15 years or older suffer from asthma. Additionally, 4.0% of the same population group were reported to suffer from COPD. These numbers implicate that overall more than 10% of the population in the EU with an age of 15 years or older suffers from severe respiratory diseases. Due to the increased utilization of aerosolized drugs in therapy of such diseases new research methods determining their impact and effectiveness have to be developed. One of the options of simulating such processes

Mechanical lung simulators are usually limited by the provided breathing waveforms (sinus, square) and signal periodicity [2]. All produced breathing cycles are therefore, almost identical, erasing individual deviation which naturally occur during human breathing. In order to obtain comprehensive information about particle deposition in the respiratory tract both, varying breathing patterns simulating physiological conditions of an individual and variable in-/ex-halation breathing pattern phases have to be taken into account.

For this purpose, the active electro-mechanical lung simulator (xPULM) was developed. The xPULM allows simulation of various breathing pattern under different conditions.

One of the focuses is to identify effect and evaluate the influence of three breathing waveforms on aerosol delivery: (1) sinusoidal breathing pattern, (2) human breathing at rest, (3) individually recorded human breathing pattern. Most commonly the aerosol deposition measurements are conducted under sinusoidal breathing simulation pattern. Such breathing pattern however differs from resting breathing of an individual in several parameters such as: I:E ratio, flow rate and overall shape of the breathing pattern. Studies, focused on aerosol delivery rate measurements [3], point out a potential benefit in increasing measurement accuracy and correspondence to real-live conditions when using individually recorded and digitized breathing patterns as a basis for breathing simulations. For this reason, comparison of sinusoidal breathing pattern with human breathing at rest and individually recorded human breathing pattern would bring further insight in this application. Additionally, different lung equivalents (i) latex bags, (ii) primed porcine lungs can be used during the simulations to provide even better similarities with human physiology and anatomy. In this context simulation with the porcine lungs provide good approximation to human's airways due to their complex inner structure and inner branching.

Innovative aspects influencing aerosol delivery modelling guided by the 3Rs [4] principles of alternatives to animal models are included. Specifically, porcine lungs obtained from the slaughter house process shall be included in the breathing simulation processes. As a result, the simulator allows to approximate realistic breathing simulation even on a higher level providing a better understanding of aerosol inhalation processes.

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50

Mimicking the initial phase of fracture healing *in vitro*

Moritz Pfeiffenberger [1], Alexandra Damerau [1], Igor Ponomarev [2], Frank Buttgereit [1], Timo Gaber [1] and Annemarie Lang [1]

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Fracture healing disorders occur in approximately 10% of human patients suffering from long bone fractures. As a result, immobility and pain that are accompanied by a tremendous loss of live quality lead to a financial burden on the society justifying the need to develop new therapeutic strategies. Until today, animal models are the "gold standard" to identify underlying mechanisms of impaired healing accepting the exposure of severe strain to involved animals although translation of results towards the human system is questionable. Thus, we developed a valid 3D bone healing model – combining the initial fracture hematoma (FH) and the cortical bone – to simulate the initial inflammatory phase of fracture healing *in vitro*, since particularly this phase is prone to negative influences.

As a first step, we established an *in vitro* FH model by coagulating human peripheral blood and mesenchymal stromal cells (MSCs). FH models were incubated in osteogenic medium for up to 48 h under hypoxic conditions (1% O_2) to mimic the restrictive microenvironment at the fracture site. We observed a continuous decline in the frequencies of leucocytes over time, whereas the MSCs population becomes the most prominent cell population as determined by flow cytometry. On transcriptional level, pro-inflammatory markers (IL6, IL8), representative for the initial inflammatory phase and matrix degrading markers for angiogenesis and migration (MMP2, MMP9) as well as osteogenic markers (RUNX2, SPP1, DLX5) were significantly upregulated after 48 h analyzed by quantitative PCR (qPCR). Furthermore, hypoxia enhanced the expression of genes associated with the adaptation towards hypoxia (HIF1A, LDHA, VEGFA). Accordingly, we observed an enhanced secretion of pro-inflammatory cytokines and chemokines (IL-6, IL-8, CCL2, CCL3) using multiplex ELISA. Secondly, we developed 3D scaffold-free bone constructs (SFBCs) based on the osteogenic differentiation of human MSCs to simulate the cortical end of the fracture gap. The osteogenic character of these SFBCs was verified by enhanced expression of osteogenic markers (RUNX2, SPP1, DLX5) as compared to the corresponding MSC-monolayer, massive mineralization, bone-like morphology, and the intense expression of osteopontin, osteocalcin, alkaline phosphatase, collagen 1 in the absence of collagen 2 as determined using qPCR, *in vitro* μCt, scanning electron microscopy, and histology, respectively. Thirdly, after co-cultivation of FH models with the SFBC for up to 72 h, we observed an up-regulation of osteogenic markers and an adaptation towards hypoxia within the FH model using qPCR. Again, the level of inflammatory cytokines (e.g. IL-6 and IL-8) was induced. Finally, to validate our model, we incubated the combined models under the influence of either dexamethasone (delay of fracture healing) or desferrioxamine (promotion of fracture healing) and found adaptive processes, which likewise result in alterations of gene expression (RUNX2, SPP1, LDHA, DLX5) as observed in the *in vivo* situation.

Conclusively, we here provide data which indicate that our established *in vitro* 3D bone healing model is at least in part able to mimic processes of the initial phase of fracture healing, which enables us to use our system for preclinical testing of therapeutic candidates potentially supporting bone regeneration by initiating the fracture healing process.

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122

Animal welfare right from the start: The 3Rs concept as a guiding principle in veterinary training

Birte Pfeiffer-Morhenn [1], Meike Merle Kuhlmann [2], Jessica Hornung [3] and Stephanie Krämer [3]

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The veterinary profession is associated with the responsibility to ensure protection of animals. Therby, it becomes a duty to take all acknowledged measures to eliminate or alleviate pain, damage, suffering and anxiety. At the Justus Liebig University (JLU) Giessen, awareness of this sense of responsibility begins with the first days of veterinary medicine studies. Since the amendment of the Animal Welfare Act in 2013, some teaching content, if accompanied by exercises on living animals, formally falls into the category of animal testing. This aspect is not only discussed and criticized by the veterinary profession itself, but also by the public. As early as 1959, Russel and Burch set the groundwork for dealing with the topic by the 3Rs concept. Currently, the following enrichments at JLU Giessen contribute to the implementation of veterinary teaching in the sense of the 3R concept:

Skills Lab

In the JLU Clinical Skills Lab basic veterinary skills as well as special skills can be learned and trained on models. This includes various injection techniques, intubation, resuscitation and auscultation, handling and marking, as well as the application of bandages and rectal and gynaecological examinations to the horse. Thus, the aspects of replacement and reduction in the sense of Russel and Burchs' 3Rs concept are taken into account. Thereby, a significant reduction in the number of applied propaedeutic animals can be achieved. By the time of the first contact with the living animal, the students have already experienced the necessary motor skills through training in the Skills Lab. Due to these measures strain and stress for the animals (and the students!) can be reduced to a minimum.

JLU's Skills Lab is already firmly integrated into the curriculum in the propaedeutics and practical semesters. Additionally, it can be used voluntarily at any time by students over all semesters.

Medical training

In Medical Training (positive Reinforcement), knowledge from the areas of learning behaviour and training (including classical and operant conditioning) is imparted and the transfer to the veterinary practice is created. Through targeted behavioural modification, patient cooperation can be achieved and the use of coercive measures can be significantly reduced. Animals used in veterinary training, e.g. to learn handling (incl. fixation) and diagnostic measures, can be manipulated more gently in the appropriate situations by means of medical training. This reduces stress and the resulting negative emotions (refinement). Patients therefore show significantly less excitement and defence reactions during the examination. This fear-free atmosphere significantly increases the learning success of the students and leads to fewer repetitions of the measures to be learned (refinement). Theoretical and practical courses of medical training have been offered at the JLU since 2016. and are now integrated within the framework of the propaedeutics lectures and implemented in the curriculum.

Conclusion

The projects presented here are now integrated within the framework of the propaedeutics lectures and successfully implemented in the curriculum. Thus, the JLU strengthens the awareness for animal welfare in the sense of the 3R concept already in the early phase of veterinary studies.

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Statement on ethics vote: No ethics vote is required.

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Retina-on-a-Chip: Merging organoid and Organ-on-a-Chip technology for complex multi-layer tissue models

Christopher Probst [1], Kevin Achberger [2], Jasmin Haderspeck [2], Julia Rogal [1], Johanna Chuchuy [1], Stefan Liebau [2] and Peter Loskill [3]

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Retinal diseases such as age-related macular degeneration or retinitis pigmentosa are the leading cause of blindness. However, there is often no cure or treatment available, which is partly due to a lack of suitable model systems. *In-vivo* animal-based models feature various similarities with the human retina but also significant differences e.g. not trichromatic or missing macula. *Ex-vivo* models based on human retinal tissue extracted post mortem are problematic due to their limited availability and cultivability. *In vitro* cell culture models mostly utilize cell lines and are not able to recapitulate the complex physiological structure and functionality of retinal tissue. Advances in stem cell biology and the possibility to generate 3D organ-like structures have opened up new possibilities for pharmaceutical research and disease modeling. Yet, those so-called organoids are still limited by shortcomings such as proper functional maturation and the physiological interplay of retinal cells. The Organ-on-a-Chip (OoC) technology has the potential to address those limitations by creating physiological accurate *in vitro* models of human tissues in a microfluidic environment. However, it is extremely difficult to recreate the complex, stratified 3D architecture of the human retina featuring a large number of cell types within an OoC in its entirety. By merging OoC and organoid technologies, we have developed a microphysiological Retina-on-a-Chip, which successfully recreates the tissue structure and recapitulates physiological cell-cell interactions. Through the combination of the biological self-assembly mechanisms in organoids with the precise micro engineering of OoCs, organoids and retinal pigmented epithelial (RPE) layers were cultured in a vascularized environment and a so-far unmatched functional interplay of photoreceptor segments and RPE cells observed, recapitulating the continuous lifecycle of photoreceptors. The developed 3D retina chip is extremely versatile and applicable for drug development, toxicity screening and disease modeling.

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Making drug screening visible: A human iPSC-derived biochip-based assay platform of the liver and kidney for real time assessment of drug toxicity

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Introduction

Within the last years, organ-on-chip (OoC) models emulating specific organ functions have been designed to improve basic research and drug screening. In pre-clinical studies complex human OoC models may possess the ability to streamline drug screening processes by use of patient specific human induced pluripotent stem cells (hiPSCs) that circumvent drawbacks of immortalized cell lines. In our experimental study, hiPSC-derived OoC models of the human liver and kidney will be established, validated and interconnected to a liver-kidney-axis. Furthermore, modification of reporter genes in the target cells of the liver and kidney will specifically allow the real time assessment of cell performance and drug-mediated toxicity based on an integrated luciferase assay.

OoC liver models benefit from iPSC-derived hepatocytes

The liver is the central organ in human drug metabolism being responsible for about 75% of drug metabolite formation by various CYP enzymes. So far, preclinical drug testing is dominated by animal experimentation associated with shortcomings in respect to data extrapolation to human applications. Thus, human OoC liver models are a promising alternative to screen for drug toxicity and undesired side-effects. So far, most biochip-based organ models are composed of immortalized cell lines. However, karyotypic variations, altered non-physiologic enzyme levels and the tendency of these cells to dedifferentiate upon prolonged passage limit its potential for drug screening studies. In contrast, hiPSCs possess stable karyotypes as well as the potential of unlimited availability. Furthermore, these cells are attractive tools for the identification of idiosyncratic drug reactions potentially resulting in organ dysfunction. IPSCs have already been used to generate organoids of the liver and kidney [1,2], but none of these models is currently able to reflect inter-organ cross communication for multi-organ drug screening. Here we present the establishment of a functional iPSC-derived OoC liver model that is expressing various CYP enzymes, stably secrets albumin and urea and possesses a physiological glycogen and triglyceride metabolism. These hiPSC-derived liver models will be combined with hiPSC-derived kidney models on a biochip-platform allowing efficient drug screening studies. Genetically engineered sentinel cells, which are embedded into the OoC models, will comprise reporter genes introduced by CRISPR/Cas-9. These sentinel cells will be able to detect toxic and unwanted drug side effects based on real-time detection of multicolorimetric luminescence emission.

Conclusion and outlook

We present microfluidic perfused iPSC-derived OoC models comprising all major cell types of the liver with improved functionality compared to conventional cell assays. In a next step, kidney modules of the glomerulus, the proximal and distal tubules will be established and connected to a *in vitro* liver-kidney axis. Organ-specific sentinel cells will allow for a systemic toxicity assessment under physiological conditions and in the context of functional immune cells to be circulated within the multi-organ model.

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The National Centre for the 3Rs

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This presentation will provide an update on the NC3Rs – the UK's national 3Rs centre. It will include its strategy and major activities, focusing on the Centre's role as a funder of 3R research and collaborative projects led by NC3Rs staff. It will mainly consider the UK environment for the 3Rs but will also cover the work the NC3Rs leads to champion the 3Rs internationally.

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Cytotoxic activity of the seaweed compound fucosterol, alone and in combination with 5-fluorouracil in colon cells

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Colorectal cancer (CRC) is one major cause of morbidity and mortality worldwide [1]. 5-fluorouracil (5-Fu), an antimetabolite, is one of the major cytotoxic drugs used to treat primary and metastatic CRC. However, resistance to 5-Fu and toxicity in normal cells reduce its effectiveness [2]. Safer and more effective therapies are needed to overcome shortcomings. Fucosterol is a sterol present in marine brown seaweeds with several biological activities [3], but its effect in CRC remains unknown. It already showed anticancer potential against HeLa cells [4]. Accordingly, the aim of this study was to assess the anticancer activity of fucosterol alone and in combination with 5-Fu on two human colorectal cancer cell lines (HCT116 and HT29), and the cytotoxicity in one normal colon cell line (CCD-18co). To achieve this aim, the cytotoxic effect of fucosterol alone and combined with 5-Fu were assessed in two cancer cell lines (HCT116 and HT29 cells) and in one normal colon cells (CCD-18Co) by MTT assay after 72 h of incubation. The results showed that fucosterol (at 10 µM) alone and combined with 5-Fu (at 10 µM) decreased viability in HT29 cells without cytotoxic effects in normal colon cells. To assess possible mechanisms involved in the anticancer action of fucosterol alone, and in combination with 5-Fu, effects on cell proliferation, cell death, clonogenic potential and cell migration were assessed in HT29 cells. The combination (fucosterol and 5-Fu) decreased cell proliferation assessed by BrdU assay, after 72 h of incubation. The combination also decreased the clonogenic potential of HT29 cells and cell migration, but without induction of cell death. These findings suggest that fucosterol seems very promising as an anticancer agent that could enhance the cytotoxic and anti-invasion effect of 5-Fu in resistant colon cancer cells. The results warrant further *in vitro* studies to clarify the molecular mechanisms behind the anticancer effect of the combination fucosterol plus 5-Fu. It would be relevant to test the combination in more complex *in vitro* systems.

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Cytotoxicity of secondary metabolites from the marine-derived fungus *Talaromyces stipitatus* KUFA 0207 in a melanoma cell line

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Melanoma is the most deadly skin cancer that develops from the malignant transformation of melanocytes. It is often characterized by its ability to metastasize and resist to cytotoxic drugs [1]. As such, more effective drugs and therapeutic strategies are needed, and the marine organisms, namely fungi, have been very promising sources of bioactive compounds [2]. In this study, *in vitro* anticancer activity of eight secondary metabolites, isolated from the marine-derived fungus *Talaromyces stipitatus* KUFA 0207, was tested in a human melanoma cell line (A375). Effects of these metabolites on cell viability were screened for the first time, using the MTT assay, after 48 h of incubation. The results showed that citreorosein (C4), secalonic acid A (C7), bis-hemin (C8) and 7,8-epoxyergosta-4,22-dien-3,6-dione (C9) significantly decreased A375 cell viability in a dose-dependent manner. C7 and C9 were the most effective compounds, showing low IC50 values, of 4 and 18 μM, respectively. To understand the possible mechanisms involved in the *in vitro* anticancer activity, the clonogenic potential, antiproliferative and cell death were also assessed in A375 cells. The results showed that C7 decreased the clonogenic potential and increased the percentage of cells with condensed nuclei, increasing also the percentage of cells double stained with annexin-V/PI. The results suggest that the metabolites C4, C7, C8 and C9, isolated from *T. stipitatus*, possess an *in vitro* anticancer effect in the studied human malignant melanoma cells, validating the interest for further studies on the cytotoxicity and molecular mechanisms of action of these metabolites.

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In vitro anticancer activity of preussin, a hydroxypyrrolidine alkaloid, isolated from the marine sponge-associated fungus *Aspergillus candidus* KUFA 0062 in breast cancer cell lines

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Marine microorganisms represent about 90% of the ocean's biomass and are relevant sources of bioactive compounds [1]. Some of the secondary metabolites isolated from marine-derived fungi have showed anticancer potential via different mechanisms of action [2]. Among several secondary metabolites we have recently isolated from the marine-derived fungus *Aspergillus candidus* KUFA 0062, the hydroxypyrrolidine alkaloid preussin showed cytotoxic effect against several cancer cell lines [3]. Breast cancer (BC) is a heterogeneous disease and can be divided in three major subtypes according to the expression of hormone receptors and the oncogene human epidermal growth factor receptor 2 (HER-2), that determine the therapeutic approach [4]. In this way, the use of a panel of cell lines covering the three subtypes of BC is the best approach in the initial steps of drug screening. Here we investigated the anticancer effect of preussin, isolated from the marine-derived fungi *Aspergillus candidus* KUFA 0062, against three breast cancer cells: MCF7, SKBR3 and MDA-MB-231 (representative of luminal A, HER2 and basal type, respectively). In addition, one normal breast cell line (MCF12A) was used. Cancer and normal cells were exposed to preussin at different concentrations for 48 h, and the cytotoxic and antiproliferative effects were assessed by MTT and BrdU assays, respectively. Effects on cell death were also evaluated by the nuclear condensation assay and annexin V/PI double staining. The results showed that preussin induced a dose-dependent decrease of cell viability in the three cancer cell lines. The antiproliferative effect was also observed in the three cell lines tested, but was greater in MCF7 cells, where preussin at 25 μM reduced around 50% of cell proliferation, without cytotoxic and antiproliferative effects in normal cells. Preussin also induced cell death as evidenced by the increase of nuclear condensation and number of cells double stained with annexin-V/PI in MCF7 cells.

The *in vitro* data support that preussin may have significant anticancer activity in breast cancer cells, revealing some differential effects according to the subtype. As such, further studies are warranted to clarify the mechanism(s) of action and in each subtype of BC.

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Utilization of *in vitro* and *ex vivo* wound healing models to address different aspects of cutaneous wound healing and drug testing

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Wound healing is a complex and well organized process. Its disorganization leads to delayed healing and can result in chronic wounds. Therefore, understanding of this process is of great importance and is the basis for the development of new therapeutics. We use different *ex vivo* wound healing models (basic, superficial, diabetic, infected) as well as *in vitro* wound healing assays (primary human keratinocytes and fibroblasts) to investigate different aspects of cutaneous wound healing as well as the effect of active ingredients, formulations and wound dressings. Here we describe our findings concerning wound healing outcome, barrier function, proliferation, migration and inflammation in basic research and by using a triterpene extract as an exemplary active ingredient.

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The RepRefRed Society

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Within the last years, animal welfare and animal ethics have risen to essential topics in the field of biomedical research. Since animal well-being is assumed to have major impact on research results, together with the revision of the law for animal experiments in Austria 2012 the 3Rs (replacement, reduction and refinement of animal experiments) became a crucial theme for any person involved in animal studies. To critically analyze animal experiments and husbandry, as well as to encourage alternative means, we founded the "RepRefRed Society" (*Verein zur Förderung von alternativen Biomodellen*) in 2016. Together with veterinarians, animal-anaesthesiologists, animal keepers and scientists the RepRefRed Society organizes congresses, meetings as well as workshops and offers a platform to facilitate knowledge transfer in the field of alternative methods. Thus, the RepRefRed Society contributes to enhance animal study protocols, allows scientists to learn innovative alternative techniques and promotes replacement, reduction and refinement of animal experiments.

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268

Establishment of a human alveolus-on-a-chip model to study microbial pathogenesis

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Introduction

To date, investigations concerning microbial infections of the lung are usually carried out in animal models, like rodents. However, lung anatomy and physiology as well as composition of the immune system differ significantly between rodents and men. Therefore, a microfluidically supported human alveolus model recreating a reactive tissue-tissue interphase between the vascular endothelium and the airway-facing epithelium is desirable. It could allow a much deeper insight into infection mechanisms of pathogens and their influence on various cell types in human lung than animal experiments and static, 2D monoculture.

Therefore, the aim of this experimental study was to establish a human *in vitro* alveolus model composed of vascular and epithelial cell structures with integrated macrophages resembling the human alveolus architecture and function for up to 14 days. Further, expression and localization of functional cell type-specific marker and surfactant production should be similar to human lung tissue. The model should be suitable for infection studies of microbial organisms like *Staphylococcus aureus* (*S. aureus*) and influenza virus (IV), that require interaction of immune and barrier cells to analyze their pathogenesis.

Methods

Biochips were seeded with human endothelial cells on the vascular site and with epithelial cells and macrophages on the airway site. This model was cultured for up to 14 days under dynamic flow conditions. Barrier integrity was proven by transepithelial electrical resistance (TEER) measurements and permeability assays. Expression and localization of cell-type specific markers and functional proteins was proven by immunofluorescence. Secretion of cytokines was analysed by cytometric bead array (CBA). Viral as well as bacterial infection occur through airway site with further designation of acute phase of invasion and early immune response thereafter for up to 8 hours.

Results

Dynamic culture conditions allow a stable barrier formation and an intact vascularity maintaining ALI for up to 14 days. Macrophage integration into the model resulted in a significant increase of barrier integrity proven by TEER measurement and permeability tests. Inflammation triggered by LPS lead to loss of vascular and epithelial cell integrity and increased cytokine secretion in the alveolus model with integrated macrophages. Epithelial cells in the alveolus model are able to produce surfactant, which is important for an adequate immune response to invading pathogens. Infection with microbial pathogens has been successfully established and pathogenicity factors can now be investigated.

Conclusions

We established a functional, biochip-based human *in vitro* alveolus model that is suitable for infection studies. Separated airway and vascular chambers allow an infection with a pathogen from the airway site. Thereby inducing an immune response it is possible to observe migration of immune cells from the vascular site into the infected sites to study species-specific mechanisms of pathogens.

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A human *in vitro* liver model for the investigation of inflammation and drug testing

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Introduction

The development of a drug until its approval and market entry takes up to 13 years and costs up to 2,8 billion Euro [1]. These expenses include all costs that arise by the development of the approved drug such as further drug candidates that fail during the process of drug development, due to toxicity or/and less or same efficacy of drugs already being on the market. The most critical selection phases during the drug development are the pre-clinical (cell culture and animal experimentation) and the clinical phases (human probands). Here, one of the highest obstacles is the restricted transferability of pre-clinical data derived from cell culture and animal experimentation to the human organism. In particular, absent efficacy and unforeseen toxicity in humans often result in expensive termination of clinical trials. Inflammation of the liver as an early onset and drug induced liver injury (DILI)/ hepatotoxicity are important reasons for the termination of clinical trials [2]. A human *in vitro* liver model that reflects the human physiology, complex tissue architecture, cellular interactions and that responds to agents is therefore desirable. It could allow a better prediction of hepatotoxicity of new drug candidates before starting animal experimentation. As a result, these models could streamline and reduce elaborate animal experiments in the drug development and, in the end, could lead to higher drug safety in humans.

Results

We established a human *in vitro* liver model in a microfluidically perfused biochip that emulates the correct anatomical structure and physiological function of a human liver. We are able to culture the model under dynamic conditions for up to seven days without loss of functionality. We can monitor oxygen levels inside of the chip by integrated sensors allowing a real-time examination of cell viability. The expression and localization of cell-type specific markers, functional proteins as well as the excretion via transporters was analysed without and after induction of inflammation. In addition, we investigated the infiltration of monocytes from the circulation into the inflamed liver model and their influence onto tissue regeneration. This was supported by measurement of central parameters like ASAT/ALAT, urea, LDH and albumin. The immune response was proven by analysis of secreted cytokines.

Conclusions

Performance and cellular setup of our biochip-based human *in vitro* liver model was proven to be suitable for the investigation of liver inflammation as well as drug testing studies. It is possible to investigate the immune response inside of the liver model after induction of inflammation as well as drug application. We can observe migration of immune cells from the vascular site into the inflamed/ injured tissue sites to study liver regeneration.

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Development of an *in vitro* potency assay for Zika Purified Inactivated Vaccine (ZPIV)

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Sanofi Pasteur, Marcy l'Etoile, France

Potency assays are part of the product specifications for biopharmaceutical product release. They are defined as a quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties. In the case of vaccines, the potency was previously evaluated by *in vivo* methods. However, since the European directive 2010/63 and in accordance with an internal 3R program, Sanofi Pasteur is willing to develop *in vitro* potency assays based on the mechanism of action of the vaccine antigen for all new vaccines. In this context, the development of a fully *in vitro* potency assay has been engaged for the development of the Zika Purified Inactivated Vaccine (ZPIV). In the case of Zika infection and more largely against Flavivirus family, the mechanism of action is based on a neutralizing humoral immune response targeting mainly the envelope protein (E). Thus, we have designed a quantitative immuno-assay based on highly neutralizing and protective human monoclonal antibodies targeting either E protein Dom III or quaternary epitope shared by E protein dimer (EDE). Such approach was proposed and endorsed by the FDA during the preliminary Investigational New Drug Application meeting (pre-IND) to initially promote relevant *in vitro* potency assay for ZPIV. Here, we present our assay development strategy resulting in its ability to be stability-indicating and to discriminate between potent and sub-potent batches.

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203

3D melanoma skin models: Unravelling the effects of the basal membrane and sub-populations of fibroblasts in tumour progression and invasion

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Leiden University Medical Centre (LUMC), Leiden, The Netherlands

Background
Cutaneous melanoma, the most malignant type of skin cancer, continues to rise in incidence and has become the most common cancer among young white adults [1]. Despite encouraging novel therapies, it remains a major health issue because of its propensity to metastasize. Crossing the basement membrane (BM) and invading the dermis are prerequisites to metastasize. Interactions between dermal fibroblast, the extracellular matrix (ECM) and cancer cells create either a tumour-promoting or -inhibiting microenvironment. How cancer associated fibroblasts (CAFs) render the stroma conductive to melanoma invasion is still obscure [2]. Appropriate models to study tumour-stroma interactions, and particularly the role of CAFs, are lacking.

Aim
To dissect melanoma invasion and progression by focussing a) on the ECM and overlying BM, and b) on subpopulations of dermal fibroblasts.

Methods
Sub a), we engineered multiple *in vitro* 3D-human cutaneous Melanoma-bearing Skin Equivalents (hMSE) using different dermal matrices, ((full-thickness collagen models (FTMs), fibroblast-derived matrix (FDM) and acellular or cellular de-epidermized dermis (DED)), sealed off or not by an intact BM, and investigated the invasive behaviour and growth of seeded melanoma cells in a top layer of keratinocytes forming a proper epidermis [3]. Sub b), we deployed the two main subtypes of fibroblast, papillary fibroblasts (Pfs) or reticular fibroblasts (Rfs), separately in hMSE to ascertain an optimum invasive milieu with, presumably, corresponding CAFs [4]. hMSE were established with melanoma cell lines WK and WM115 derived from primary tumours, and lines AN, RU and M14 derived from metastases. To study invasive behaviour, FTMs harbouring either Pfs or Rfs were first seeded with skin carcinoma cell lines (MET1, 2 and 4) representing different stages of malignancy [5]. Subsequently, models were studied in which AN, RU and M14 cell lines in combination with primary human keratinocytes were seeded onto Pf- or Rf-FTM. All models were analysed on morphology, mRNA and protein level.

Results
FDM melanoma models showed extensive invasion into the dermal matrix, most likely due to a BM that had not yet developed when epidermal cells are seeded. In contrast, acellular and newly developed cellular DED showed less invasion with an intact BM, making these dermal equivalents more suited to study invasive behaviour. SCC and metastatic cell lines of melanoma mixed with primary human keratinocytes showed more extensive invasion into a dermis composed of Rfs than Pfs. In addition, Rf-HSEs showed increased activation and stained positive for CAF biomarkers a-SMA and vimentin. Further analysis revealed that invasively growing cancer cells in Rf-HSEs express markers of epithelial to mesenchymal transition (EMT), N-cadherin and ZEB1.

Discussion
cDED-based models appear to be hold great promise for studying melanoma invasion, in contrast to FDM-based models. Furthermore, FTMs generated with Pfs contain cancer cells more within the epidermis, suggesting that Rfs are clearly the ones predisposed to differentiate into CAFs, assisting in invasion and EMT. These observations strongly suggest that Rfs-subtype could be a crucial factor in melanoma cancer initiation, progression and metastatic progression.

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Non-animal methods for skin sensitisation testing – is there really a need for integrated testing strategies?

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To meet regulatory requirements, and avoid or minimize animal testing, there is a need for non-animal methods to assess the potential of chemicals to cause skin sensitization. It is widely assumed that no one test will be sufficient and that combined data from several assays spanning key events from the adverse outcome pathway will be required. This presentation challenges that assumption. The predictive performance of a single assay, the Genomic Allergen Rapid Detection (GARD) assay, was compared with the performance, singly and in combination, of the three formally validated non-animal approaches that appear as OECD test guidelines: the direct peptide reactivity assay (DPRA), the ARE-Nrf2 luciferase test method, and the human cell line activation test (h-CLAT).

It is shown that GARD alone outperforms each of DPRA, ARE-Nrf2 luciferase or h-CLAT, alone or in any combination as a 2 out of 3 strategy, in terms of sensitivity, specificity and accuracy. Based on the datasets analysed here, the sensitivity and specificity of GARD alone are 90-92% and 79-84% ("2 out of 3", 86% and 76%). Thus, in any situation where the 2 out of 3 strategy is considered adequate, GARD alone could be used with equal or better performance.

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Alternative research methods for challenging disease: Human iPS derived Parkinson's-on-chip

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Neurodegenerative conditions including Parkinson's and Alzheimer's are debilitative, often intractable diseases often associated with age. Today, 16% of Europe's population is over 65, a number predicted to equal 25% in 2030. Current yearly estimates for dementia-related care in Europe reach €130 billion, establishing the predominance of age-associated neurodegenerative diseases among Europe's main medical and societal problems.

A major limiting factor in neurodegenerative disease modeling remains the paucity of sophisticated fundamental *in vitro* studies. Neuroscientific research in traditional, monolayer techniques suffers significant drawbacks due to the unrivaled complexity of the human brain as a three-dimensional (3D) structure [1]. Research in Alzheimer's and Parkinson's diseases has been conducted in animal models lacking naturally occurring pathology and rely upon traumatic or toxic induction. This may explain the confounding drug failure rate published by the FDA, 92 percent of drugs passed in preclinical tests, including required animal tests, never make it to the market [2]. The controversy of animal research exists not only because of ethical concerns but also due to untranslatable results and significant expense, spawning the desire for ethically sourced, economically efficient research techniques. In addition, animal research always includes further unquantifiable variables, including individual health status, diet, weight, and inherent genetic discrepancies [3].

Conversely, the integration of complex cell biology with microchip technology has the potential to generate new diagnostics and disease models to improve our understanding of the brain, while fulfilling the EU Directive to reduce, replace, and refine animal testing. Our research group is validating a midbrain-on-a-chip capable of evaluating new treatments in a reproducible, cost efficient model precluding animal trials. We integrate interdigitated electrodes to sense neurotransmitter release, offering quantification of regenerative capacities.

Confocal imaging reveals spontaneous asymmetric arrangement of dopaminergic neurons consistent with the human midbrain; dopamine secretion in medium can be detected at a sensitivity below 1 micromole using complementary redox cycling, cyclic voltammetry and chronoamperometry (quantified by HPLC). Serial oxygen concentration and consumption measurements have been conducted and are currently in data analysis. By comparing CRISPR gene controlled midbrain organoids to diseased organoids, our analyses aim to identify target treatments in personalized medicine as well as offer new insights into the biomolecular pathophysiology of Parkinson's. Microfluidic execution of this technology offer high throughput experimentation independent of expensive and ineffective animal trials and improves upon existing benchtop research utilizing medium perfusion to optimize nutrient diffusion, thereby minimizing current limitations in organoid engineering.

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Cartilage-on-a-Chip: The future of osteoarthritis modeling and drug screening?

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In this work, we hypothesized that equine cartilage microtissue developed inside a microchip would mimic the main characteristics of *in vivo* cartilage (relative acellularity, avascularity, low metabolic activity, differentiated cellular morphology) and exhibit similar protein expression patterns. Animal trials are expensive and controversial due to ethical considerations, creating the demand for ethically responsible and economic research methods. Thus, we pursued a promising research field addressing this need, called organ-on-a-chip technology, offering three-dimensional cell culture capabilities by placing cell lines normally situated within a functional extracellular matrix (ECM) into hydrogels in order to maintain their native phenotype, combined with inherent flexibility in design, material and functions of microdevices allowing feature characteristics of the biological niche such as relevant concentration gradients and shear force conditions [1].

Primary chondrocytes were aseptically isolated under informed consent from the knee joints of 3 equine patients euthanized for reasons unrelated to osteoarthritis. Microfluidic devices were fabricated as previously described. Fibrin hydrogel (20%) was seeded with CMFDA stained primary equine chondrocytes and loaded into the microchips prior to crosslinking; medium was exchanged every 48 hr. Cells were cultured up to 4 weeks under routine conditions with cellular morphology and viability monitored throughout the culture period. To simulate nutrient diffusion, fibrin hydrogel (20%) without cells was loaded into the device and FITC-BSA supplied via the medium channel under real time fluorescent microscopy. Metabolic activity of the cells was evaluated via TOX8 Assay. Microfluidic cultures were sacrificed qPCR and histology. All microfluidic cultures were compared to same-passage, same-donor monolayer chondrocyte cultures as controls.

Cell viability and morphology remained excellent in the microfluidic cells throughout the culture period in comparison to the monolayer cultured chondrocytes. Time lapsed FITC-BSA diffusion through the hydrogel appeared similar to nutrient delivery expected in native articular cartilage. TOX8 revealed decreased metabolic activity of microfluidic chondrocytes compared to monolayer cultured chondrocytes, consistent with a de-differentiated status in 2D, confirmed by end stage PCR, which revealed microfluidic upregulation in expression of SOX9, Collagen II, and Aggrecan and down regulation of Collagen I compared to monolayer cultivated chondrocytes. Histology revealed formation of a superficial like layer of chondrocyte migration and orientation distinct from the deeper layer of cells.

Results demonstrate 3D chondrocyte culture has high potential for establishment of a novel disease model for testing future therapies. The gradient formation is similar to physiologic conditions of cartilage and cells remained viable for extended culture time suggesting high potential for *in vivo*- like cultivation. Live cell fluorescent microscopy revealed spontaneous chondrocyte migration forming a superficial cartilage layer and linear chondron aggregations similar to the native deep layer of articular cartilage. A relevant tissue model with physiologic cellular behavior offers a new level of investigation into the behavior of healthy chondrocytes as well as a solid foundation for exploration into pathophysiology of osteoarthritis with direct significance not only in high throughput drug screening, but also offers an immediate avenue for patient customized diagnostics and therapeutic specificity.

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Microfluidic strategies for nanomaterial risk assessment at *in vitro* organ barrier models

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The application of nanomaterials (NMs) in technical products and biomedicine has become a highly expansive market. Since safety of novel NMs is of utmost interest, assessment of NM uptake and cytotoxicity on physiological human barriers is paramount to identify possible detrimental effects on human health. Here, we present microfluidic approaches with a variety of integrated biosensors to assess nanotoxicology at organ barriers including lung, placenta, and endothelial cell models. Also, briefly a combinatorial *in vitro* - *in silico* approach will be presented that may help to faster estimate cell-NM interactions.

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The European Consensus Platform for 3R Alternatives to Animal Experimentation (ecopa)

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The ecopa is a non-profit organization which explores all possible means to improve the exchange of information in the area of alternative methods. The primary mission of ecopa is to raise public, governmental and scientific awareness for a better use of alternative methods for scientific purposes with the final goal of fully replacing all *in vivo* tests while improving the quality of risk assessment in humans. Presently, ecopa is composed by 7 National Consensus Platforms (French, Finland, Norway, Spain, Italy, Germany and Switzerland) and has other members as individuals to include academic institutions, professional associations, companies, and any other organisation (or one of its divisions) which support ecopa's aims. National Consensus Platforms are usually structured with representatives from ecopa's four stakeholders: Government and regulatory authorities, academia, industry, and animal protection and welfare organisations.

The aims of ecopa are to facilitate the exchange of scientific information, expertise and experience between national consensus platforms to enhance implementation of refinement, reduction and replacement in the EU and worldwide. ecopa recommends the use of alternative strategies that may elucidate the real mechanism of actions of the different substances in humans, in particular when the animal models fail in predicting an effect. This is particularly true in the area of drug development where so many differences exist between humans and animals.

Adhering to the 3Rs, ecopa strives to raise public, governmental and scientific awareness for a better acceptance of alternatives both at member state and European levels. Members of ecopa are part of ESAC at EURL-ECVAM and it is constantly in contact with DG Environment and DG Health & Consumers. Additionally, many members are participating in European Integrated Projects. The ecopa is also an accredited stakeholder at ECHA. Some national consensus platforms also have strong interest in the food and feed sectors even though the main focus remains in the methodology for risk assessment.

New Approach Methodologies (NAMs) integrating advanced *in vitro*, *in silico* and *in chemico* tools should be developed and included in testing strategies to increase our understanding of toxicity mechanisms in humans and the risks to the environment. The same goal applies to biomedical research. ecopa's strategic goals apply to all areas where animals are used for scientific or regulatory purposes.

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163

In-silico tool to design CYP3A5 Silensomes™ using T-5 substrate and lapatinib as mechanism-based inhibitor

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Silensomes™ are human pooled liver microsomes (HLMs) irreversibly inactivated for one specific CYP450 using mechanism-based inhibitors (MBI) [1,2]. Intrinsic clearance of midazolam and testosterone measured with CYP3A4-Silensomes™ previously shown that testosterone is a specific substrate for CYP3A4, although midazolam found to be metabolized by both CYP3A4 and CYP3A5 [1,2]. Due to this cross reactivity between two CYPs, there is need to address contribution of CYP3A5 in the metabolism of test compounds. Therefore, it is crucial to find specific substrate and MBI of CYP3A5 for production of Silensomes™CYP3A5. Thanks to discovery of T-5 compound and its specific oxidation reaction to N-oxide by CYP3A5 offer an opportunity to access this aim and to identify the contribution of CYP3A5 during *in-vitro* drug metabolism by comparison to CYP3A4 [3]. In addition, discovery of lapatinib as MBI of CYP3A5 gave clue to design Silensomes™CYP3A5 using T-5 as substrate [4]. Here, we report *in-silico* Docking approach to find specificity of T-5 substrate for CYP3A5 as compare to testosterone and midazolam. We also evaluated the inhibition potency of inhibitors lapatinib towards CYP3A5 as inhibitor and potential MBI. Firstly, we discovered two binding pockets: P1/P2 in the CYP3A5 (protein data bank code 5VEU) and one binding pocket in CYP3A4. Therefore, all these pockets are considered separately for our protein-ligand docking analysis. CYP3A5 predicted higher specificity for substrate T-5 (T-5 [DE = -12.80 kcal/mole] > midazolam [DE = - 9.67 kcal/mole] > testosterone[DE =- 8.09 kcal/mole]) in the pocket P1. For CYP3A5 inhibitors, lapatinib (lapatinib [DE = - 16.67 kcal/mole] > LAP-OH [DE = - 14.51 kcal/mole] > azamuline [DE = -14.25 kcal/mole]) > ketoconazole [DE = - 13.52 kcal/mole] is predicted to has higher affinity for pocket P1. CYP3A4 also predicted specificity for substrate T-5 and testosterone (T-5 [DE = -9.80 kcal/mole] ~ testosterone [DE = -9.64 kcal/mole] > midazolam [DE = -8.49 kcal/mole]) in the pocket A1. However, CYP3A4 together with CYP3A5, the T-5 will not form T-5 N-oxide as metabolite (Specific reaction catalyzed by CYP3A5). Also, lapatinib (lapatinib [DE = -17.58 kcal/mole] > LAP-OH [DE = -14.81 kcal/mole] > azamuline [DE = -12.45 kcal/mole] > ketoconazole [DE = -11.30 kcal/mole]) is found to has affinity for pocket A1 of CYP3A4; however, it forms reversible complex with CYP3A4 and its reactive metabolite forms irreversible complex with CYP3A5 [4]. Therefore, our *in-silico* approach predicted that due to more specificity of T-5 for CYP3A5, T-5 could be use as substrate for CYP3A5. In addition, due to higher affinity of lapatinib for CYP3A5 predicted to be potential MBI for CYP3A5. In the poster, the detail analysis of binding energies score and interaction will be provided.

Our work conclude that Silensomes CYP3A5, with CYP3A5 irreversibly inactivated by MBI lapatinib, could be produced by using T-5 as substrate to understand the metabolism of given test compound via CYP3A5.

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Environmental pollution: Usefulness of different *in vitro* models to assess the effects of air and wastewaters on cell homeostasis

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Environment pollution is constantly increasing, mainly due to anthropogenic activities, and, consequently, the health risk, either for humans and ecosystems, is exponentially incrementing. International policies try to contain/reduce levels of pollution, in order to preserve life health and to reduce the social cost of this worldwide poisoning.

Whereas technical/engineering strategies are well established, the research of early biomarkers for toxicity assessment still needs effective strategies/models.

In order to identify, *in vitro*, significant toxicological endpoints, we used 4 different cell lines, chosen according to the aim of our investigation, and, mainly, for detecting the potential organotropism of the different mixtures of substances extracted from air or wastewaters samples. Endpoints were chosen in order to evaluate cell viability, oxidative stress, oestrogenic interference and tumour promotion. Cell lines were IAR203, MCF-7, BEAS-2B and A549: a) the IAR203, a rat liver-derived cell line, that represents the hepatic model was selected for its capacity to mimic hepatic precursors and to be highly communicative by means of Gap Junctions, b) the transgenic MCF-7, mammary-derived cell line, was set for EDCs evaluation, because of its high responsiveness to oestrogens, and, by end, c) the BEAS-2B e A549 cell lines were preferred as models for pulmonary system (bronchial and alveolar tissues, respectively).

Results showed that the choice of multiple cell lines is required and fundamental to optimize the accuracy of the *in vitro* tests since organotropism results in different cell responses to toxicants, thus confirming the pending problems for human health. In addition to the most suitable setting of cell lines, the choice of specific and suitable biological endpoints is important as much (beyond the difficulty to foresee the behaviour of substance's mixtures). The advantage of *in vitro* models is based on the possibility to perform screening (contained cost and high versatility).

This research is the result of an interdisciplinary approach (Chemistry, Environmental Engineering, Public Health and Toxicology) and demonstrates the importance of distinct and complementary professional competences to assess toxicity from bench (mechanistic studies) to clinical/preventive medicine for adequate toxicological studies and their application in the assessment of impact on environment and human health.

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The human hepatic HepaRG cells combined with PBPK modellings as first practical efforts towards modern toxicology and the 3Rs in Tunisia

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An initiative was launched in 2015 at the Veterinary school of Tunis about the implementation of the 3Rs in the country [1]. After a latency period, things are moving forward and decisions have been made.

Inspired by the works led at the Joint Research Center (JRC) at Ispra, we retained as a starter pack a hepatic *in vitro* cell culture system tightly combined with software modelling for addressing toxicology issues in an up to date manner. Contrarily to what is done classically in developing countries, we chose for the cell system the HepaRG cells, instead of the HepG2 or the primary rat hepatocytes.

HepG2 line is very widely used, with thousands of papers relying on those cells. In addition to that large historical background, the cells are easy to grow, they are not demanding nor in time of culture nor in specific culture media.

But the cells are notoriously of low performance in the field of toxicity, the cells have an unstable karyotype and phenotype, the phenotype is fetal, and many hepatic functions are not or badly expressed. E.g., the cells have no functional CYP levels what prevents studying the metabolite effects of a test compound.

The primary rat hepatocytes are relevant in matter of functions but remain rat cells. And nowadays in toxicology, human data are a must.

The ready to use and commercially available HepaRG cells seemed to us offering a good compromise in matter of cost and quality of data and scientific recognition.

Then, to make the most of the data generated in each HepaRG cell experiment, and for the training of our students, since the use of software is of a lower cost compared to the *in vitro* bench works, we decided to make use of modern bioinformatic tools and to perform modelling. We chose the pbpk domain for the modelling, obviously because of its relevance in toxicology. Pbpk considers i) the binding of test compounds to the plastic ware and to the protein present in the culture media, ii) the intracellular concentrations of a test compound under the control of the influx and efflux pumps and of the biotransformation enzymes.

We will rely on PKsim, reputable Pbpk software and on VCBA. Both give an affordable access to the up-to-date technology in the field.

Only a few more decisions need to be made about the cases to study and we will take advices at the next EUSAAT meeting at Linz.

The training periods for using the HepaRG cells and the softwares are scheduled and the first outcomes will be shown in the poster.

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114

Tracking of small aquatic organisms with custom-made tracking plates

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Over the past decades researchers tried to qualify and quantify the complex animal behaviour, in order to understand and predict it. The radical advances in this field lead to the formation of a new scientific domain called "computational ethology". However, a major gap in this field is that most studies focus on terrestrial and aerial species in comparison to aquatic organisms. The major reason for this discrepancy is that compared to terrestrial species, additional technical challenges need to be overcome when studying aquatic species e.g. light refraction and reflection interferences at the air/water boundary, positioning of the light source and suitable body marking techniques. Nevertheless, a deeper understanding of the movement patterns of small-sized aquatic invertebrates and planktonic organisms is urgently needed, as locomotion and/or swimming behavior can be used as stress sensitive indicators for a wide range of environmental contaminants. Furthermore, changes in their locomotion could be used as an endpoint when studying neurotoxic effects. As a result, the aim of the present study was to improve the current tracking techniques of *Daphnia magna*, *Artemia franciscana* and *Paramecium caudatum*. In order to do so, custom-made, polydimethylsiloxane/glass or polymethylmethacrylate plates were constructed. The tested organisms were placed in those costume-made plates and recorded under the microscope or in a commercially available observation chamber, respectively. Horizontal or vertical tracking of the tested species was performed with a tracking software. The results of the present study showed that our custom-made plates had a higher tracking efficacy compared to commercially available 96-well plates. Therefore, these easy to fabricate and cost-effective plates can be implemented on behavioral and ecotoxicological studies on small-sized aquatic invertebrates and planktonic organisms, which is especially interesting for sub-lethal concentrations.

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Evaluation of a novel hydrostatic presure bioreactor on bovine cartilage chips

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Cartilage diseases affect millions of people worldwide, with osteoarthritis alone being in the top ten of disabling diseases in developed countries. Cartilage injury, inflammation or damage result in pain, joint deformations and restriction of movement. Articular cartilage is avascular, alymphatic, aneural and has a low metabolic rate with very poor regenerative abilities. Therefore, a misguided orchestration of extracellular matrix degradation and renewal can have tremendous effects on individuals. Consequently, tissue homeostasis is of highest priority for cartilage functionality. Under physiological conditions, cartilage tissue experiences mechanical stimuli including hydrostatic pressure that are crucial for tissue homeostasis. Therefore, our aim is to develop a system to study chondrogenesis, tissue homeostasis and cartilage diseases by applying hydrostatic pressure to bovine cartilage chips as an alternative to animal experiments. Those chips were sampled from waste material of the metatarsophalangeal joints from freshly slaughtered animals (local butcher). After extraction, the chips were cultured *ex vivo* in medium with or without chondrogenic differentiation factors. Using our novel hydrostatic pressure bioreactor, cartilage chips were dynamically loaded at a pressure of up to 10 MPa and compared to non-loaded chips. The results indicate various effects and differences on tissue homeostasis and cell viability, especially differences in the glycosaminoglycan content and – potentially correlated to that – the construct size. Furthermore, gene and protein expression are affected, shown by qPCR, Western Blot and histology. This novel culture system might be used as a high-throughput test to study chondrogenesis, cartilage diseases and to evaluate cartilage tissue engineering strategies in an animal-free model.

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In vitro fluid-dynamic intestinal device as fast and reliable approach for food nutrients permeation tests

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Intestinal permeability is a crucial factor that determines the bioavailability and consecutively the biological activity of micronutrients or nutraceuticals. The absorption of bioactive compounds is mainly due by their solubility and dissolution in intestinal fluid and by the permeability across the intestinal membrane [1]. Therefore, the evaluation of intestinal permeability of food compound becomes an indirect approach to assess and quantify its *in-vivo* functionalities. Due to the complexities associated with studying the intestinal permeability in humans, alternatives models have been developed such as (i) *in-vitro* membrane based approaches, (ii) *ex-vivo* tissue based analysis and (iii) *in situ* intestinal perfusion systems.

However, these techniques are time consuming and not adopted for high throughput screening.

In particular, *in-vitro* techniques like Caco-2 model and Ussing chamber lack of biological factors (such as the mucosal layer) making the intestinal absorption prediction less accurate [2]. *In situ* and *ex vivo* models, widely used to replicate the *in vivo* situation with a more accurate prediction than *in vitro* models, require sophisticated surgical procedures and instrumentation.

To avoid these limitations, we here developed and validated a fluid flow Multi In Vitro Organ (MIVO) device as alternative intestinal system to evaluate the permeability kinetics of bioactive compounds in a fast, reproducible and accurate way. As proof of concept, we have evaluated the intestinal permeability of lactulose, a non-absorbable sugar used in the treatment of constipation and hepatic encephalopathy.

The dynamic MIVO device consisted of peristaltic pump, a tissue chamber divided in donor and receiver compartments, tubes and connectors for sampling the media over time. The EpiIntestinal™ by MatTek Corporation was adopted to recapitulate the human small intestine structure *in vitro*. Permeation tests have been performed by (i) placing of the EpiIntestinal™ tissue within MIVO device, (ii) application of 0.2 ml of lactulose at different concentrations, ranging from 0.07 g/ml to 0.035 g/ml, in the donor chamber, (iii) application of saline solution with a blood vessel capillary velocity in the circuit below the tissue, (iv) sampling of the media of the receiver chamber each 30 minutes up to 2.5 hours, (v) determination of the percentage of lactulose permeation over time, expressed as permeation percentage respect to the applied quantity. The static condition has been adopted as control.

In vitro results highlight that the permeation of lactulose reaches a plateau behavior at 1% and 0.5% of the applied lactulose quantity, respectively under dynamic and static conditions, independently by the lactulose concentration. The dynamic condition triggers a higher diffusion of lactulose, if compared with the static one, suggesting an active role of the physiological environment reproduced within MIVO, such as peristalsis motion and improved mass transport from the absorption site. Interestingly, these values observed *in vitro* are in line with those obtained *in vivo* in patients, validating this approach as a fast, easy and reliable platform for mimicking the intestinal epithelium for *in vitro* nutraceuticals permeation tests.

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Transition to animal-free research – on possibilities for phasing out animal experiments and stimulating animal-free innovation

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Netherlands National Committee for the protection of animals used for scientific purposes (NCad), Den Haag, The Netherlands

Although there is scientific, economic and societal potential for innovations without laboratory animals, according to NCad, these are currently not being sufficiently exploited to promote and accelerate the transition towards animal-free research. "Only with a broad-ranging and coordinated effort by the ministries involved and other stakeholders can significant progress be made in reducing the use of laboratory animals in research." This is stated in the advisory report "Transition to animal-free research – on possibilities for phasing out animal experiments and stimulating animal-free innovation" by the Netherlands National Committee for the Protection of Animals Used for Scientific Purposes (NCad). The Dutch Minister of Agriculture requested NCad to draw up a reduction plan for animal testing and to map out the possibilities for being a frontrunner in animal-free innovations by 2025.

If we are to make the transition to animal-free research, a paradigm shift is needed away from existing mindsets and practices. That way, according to the NCad, we can focus on animal-free innovations in a number of fields. Laboratory animal use in regulatory safety testing of chemicals, food ingredients, pesticides and (veterinary) medicines can be phased out by 2025, whilst maintaining the existing safety level. The same applies to the use of laboratory animals for the release of biological products, such as vaccines. In the field of fundamental scientific research, the opportunities for a substantial reduction in the use of laboratory animals vary from one field to another. The NCad recommends that 10-year visions are created for each area of fundamental scientific research (or for each cluster of disciplines) in consultation with the public and the scientific community. These visions must include clear transition objectives that are linked to the core focus of the area of research concerned. They must also give an insight into the potential of animal-free innovations in these areas.

The transition to non-animal research does not happen automatically and requires strong (international) management and focus. The NCad advised the Dutch minister of Agriculture to manage the transition and also involve other ministries for a policy that will be widely accepted. In this way the animal testing policy is linked to other policy issues, such as innovation and sustainability. In its report the NCad makes recommendations under three different themes: Clear transition objectives, Transition strategy and Management of the transition.

In order to speed up the transition, the NCad advised the minister, among other things, to make international efforts to review the current regulatory risk assessment process. A more chain-oriented approach to innovation policy (whereby not only the development of innovative methods will be stimulated, but also the further development towards validation, acceptance and implementation) will stimulate multidisciplinary collaboration. This will in turn allow animal-free innovations to move more easily from development to actual application. Monitoring, evaluation and creating available knowledge about animal-free innovation and the 3Rs play a central role in gaining better understanding of progress in this area.

This presentation will include the recommendations made by NCad, the approach taken to draw these up, and actions taken since publication in the Netherlands and internationally.

Reference

https://www.ncadierproevenbeleid.nl/documenten/rapport/2016/12/15/ncad-opinion-transition-to-non-animal-research

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Motivation by restriction? – Starting points for controlled fluid and food intake in neurocognitive research from a 3Rs perspective

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Netherlands National Committee for the protection of animals used for scientific purposes (NCad), Den Haag, The Netherlands

For neurocognitive research, experiments are performed, whereby animals must perform activities in order to obtain an insight into the activity in the functioning brain. To motivate animals to perform these activities, their fluid or food intake is restricted to such an extent that they become thirsty or hungry. In its most recent opinion report "Motivation by restriction?", the Netherlands National Committee for the protection of animals used for scientific purposes (NCad: Nationaal Comité advies dierproevenbeleid) provides criteria for controlled fluid and food intake during neurocognitive research for rodents and non-human primates (NHPs) from the perspective of the 3Rs (Replacement, Reduction and Refinement).

In the scientific community, the harm that animals experience related to fluid or food restriction is generally ranked as mild or moderate. There are societal and political concerns regarding this method of research, referring to the intrinsic value of the animal, which is also the key principle on which legislation governing animal procedures is based.

The NCad concludes that a Synthesis of Evidence (SoE) conducted prior to the research must be used to ascertain whether the research can be performed without the use of animals. Should this be impossible, it must be established whether the behavioural tasks can be taught without fluid or food restrictions. The motivation method least harmful to the animal must always be chosen. The NCad has prepared a decision tree, which will help researchers to choose the most refined research design. Should restriction be unavoidable, the general framework and the Codes of Practice for rodents and non-human primates apply, which are enclosed with the opinion as a guideline. Also, absolute lower daily intake limits have been defined that apply to animals (adult, not pregnant) subject to restriction.

Recommendations by the NCad include the following:

1. Use the "no, unless"-principle to begin with and do not apply any restrictions unless there are well-substantiated scientific arguments and/or compelling public interests.
2. Morally unacceptable are situations such as: simultaneously applying fluid and food restrictions, "push days", withdrawal of fluid or food for more than 24 hours, restriction of pregnant animals, and restriction protocols that could cause severe discomfort.
3. Promote the development and application of replacement alternatives for fluid or food restrictions by promoting the acquisition of more knowledge and understanding of possible replacements in the field of neurocognitive research, and by investing in technological developments and innovations that involve no laboratory animals in this field.
4. If restrictions are convincingly unavoidable, use the framework and Code of Practice (CoP) as reference documents and monitor compliance.
5. Promote and guarantee the knowledge of different motivation methods among the parties involved. Have the Central Authority for Scientific Procedures on Animals (CCD: Centrale Commissie Dierproeven) assess whether this knowledge is sufficiently guaranteed in the project application and have the Netherlands Food and Consumer Product Safety Authority (NVWA: Nederlandse Voedsel- en Warenautoriteit) ensure that this knowledge is guaranteed in the workplace, e.g. by using training records.

Reference

https://english.ncadierproevenbeleid.nl/advice/documents/publications/18/6/11/advisory-report-motivation-by-restriction

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81

Rehoming of former laboratory animals

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The advisory report "Rehoming of former laboratory animals" by the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) is aimed to guarantee the quality of life of non-human primates (NHPs), dogs and cats that remain alive at the end of an animal procedure. "Putting up for adoption" and "retiring" are terms that are often used to describe such situations, but in its advisory report, the NCad chose to use the term "rehoming". This term applies to situations where an animal remains alive at the end of an animal procedure, and is able to spend the rest of its life at a location suitable for its needs without being subjected to any further animal procedure.

Based on the viewpoint that these animals have intrinsic value and should therefore always be treated as sentient beings, one should assume that all dogs, cats and NHPs kept alive (the "yes, unless" principle) are being rehomed.

Rehoming is not always in the best interest of the animal involved. There are situations in which, for good reasons, rehoming is not an option. These include situations where: 1) the experiment requires that the animals are killed because, for example, an autopsy provides essential information; 2) Reuse is possible, compatible with legal guidelines, and ethically acceptable, with consideration having been given to cumulative distress; 3) Laws and regulations prohibit rehoming, for reasons such as risk to public health; or 4) The Animal Welfare Body (IvD) and designated veterinary physician have good reason to believe that the quality of life and life expectancy of the animal following rehoming will be too low.

In the Netherlands, various options currently exist for rehoming a former laboratory animal. Yet, the research establishments that offer these opportunities have each developed their own guidelines and procedures.

To establish a more coordinated and transparent rehoming process, NCad was commissioned by the Dutch Minister for Agriculture to draw up a framework that provides a general description of the rehoming process applicable to several different types of animals. In addition, species-specific Codes of Practice have been established for dogs, cats and NHPs to encourage the rehoming of eligible cats, dogs and NHPs. Additionally, the NCad recommends: 1) the encouragement of the implementation and application of the CoP's for cats, dogs and NHPs in everyday research practice; 2) the encouragement of a change of attitude within the field of animal experimentation and training, whereby at the end of an experiment animals do not need to be euthanized and can in principle be rehomed, beginning with dogs, cats and NHPs; and 3) the creation of an environment where various parties endeavour to facilitate rehoming, including a balanced division of the attendant costs.

With this presentation we will give an overview of the advisory report and CoP by the NCad.

Reference

https://english.ncadierproevenbeleid.nl/advice/documents/publications/16/7/19/adoption-of-former-laboratory-animals

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3Rs Centre in Sri Lanka to promote "Replacement" concept

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Attendance at EUSAAT conferences paved the way for fruitful scientific collaborations. Following introduction of "Replacement" concept of the 3Rs principle of Russell and Burch to Sri Lankan scientists in January 2014, EUSAAT made the foundation for the initiatives taken to establish the "Centre for 3Rs (C3Rs)" with the financial support of "Animalfree Research (AfR)" organization in Switzerland.

The principal objective of this Centre is to maximize replacement models (Replacement) for research and education while reducing the number of animals (Reduction) breed and use for research purposes leading to reduction of animal suffering (Refinement).

Following acceptance of the application submitted to AfR, the Contract was signed between University of Colombo and the AfR in July 2017 and the By-Laws were drafted. Under this agreement, the first project that is planned to be implemented is the use of Fluorescent Antibody Virus Neutralization (FAVN) test in replacing "Mouse Inoculation Test (MIT)" to test the potency and efficacy of canine anti-rabies vaccines.

Furthermore, the curriculum of the postgraduate Certificate and Diploma courses on laboratory animal science which includes a teaching/learning module on alternatives, will enable the course organizer to disseminate knowledge and skills on the Replacement concept. Skills training on animal handling, feeding and blood drawing will be performed using artificial animal models "Natsume Rats and Mice" of Natsume Seisakusho Co, Ltd, Japan which were purchased under the financial assistance from the American Physiological Society. Our aim is to develop this Centre as a resource providing Centre in the Asian region while establishing collaborations with few other Centres exist at global level currently.

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187

Ability of three rainbow trout (*Onchorhynchus mykiss*) cell lines to eliminate benzo(a)pyrene and extrapolation to derive a fish bioconcentration factor

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Permanent fish cell lines constitute a promising complement or substitute for fish in the environmental risk assessment of chemicals. One potential area of application is in biotransformation assessment. To test the feasibility of this application, we initiated a pilot study using benzo(a)pyrene (BaP) as model chemical. We moreover selected three cell lines from different tissues to more fully account for whole body biotransformation *in vivo*: the RTL-W1 cell line representing the liver as major site of biotransformation; and the RTgill-W1 (gill) and RTgutGC (intestine) cell lines as important environment-organism interfaces, which likely influence chemical uptake. All three cell lines were found to effectively biotransform BaP. However, rates of *in vitro* clearance differed, with the RTL-W1 cell line being most efficient, followed by RTgutGC. Co-exposures with alpha-naphthoflavone as potent inhibitor of biotransformation, assessment of CYP1A catalytic activity, as well as the progression of cellular toxicity upon prolonged BaP exposure revealed that BaP is handled differently in the RTgill-W1 compared to the other two cell lines. Application of the cell line derived *in vitro* clearance rates into a physiology-based toxicokinetic model predicted a BaP bioconcentration factor (BCF) of 909-1057 compared to 920 reported *in vivo* in rainbow trout [1].

Based on these promising results, we are currently exploring additional chemicals in an effort supported by CEFIC-LRI_Eco34. Here, the cell-line based approach is compared to other *in vitro* models, namely a primary gill cell system and S9 fractions of rainbow trout liver and intestinal cells. Chemicals were chosen, among other criteria, based on their uncertain predictability in terms of bioaccumulation using available one- and multi-compartment models. Combined with the newly accepted OECD guidelines for bioaccumulation predictions with incorporating of biotransformation rates obtained with primary rainbow trout hepatocytes or liver S9 fractions, these efforts may pave the way for a much-reduced need in fish for bioaccumulation predictions.

Reference

[1] Stadnicka-Michalak, J., Weiss, F. T., Fischer, M. et al. (2018). *Environ Sci Technol 52*, 3091-3100.

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186

Predicting chemical impact on fish growth by combining computational modelling with *in vitro* fish cell line responses

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A paradigm shift in chemical risk assessment is required in order to comply with the testing needs of the 21st century, which call for less resource-consuming and ethically controversial schemes than currently accepted ones using animal tests. Fish are the most extensively used vertebrates in environmental risk assessment, including assessment of a chemical's acute toxicity, bioaccumulation potential and impact on fish growth. Each single test on fish growth requires at least 400 fish and lasts, depending on the fish species used, between several weeks to months. To pave the way for an alternative testing strategy, we hypothesized that a fish that grows less due to chemical impact must have fewer cells and that, therefore, monitoring cell population growth *in vitro* can be used as proxy for chemical impact on fish growth. To pursue this idea, we developed a procedure that links information on the proliferation and survival of a fish gill cell line (*in vitro*) to the effect of chemicals on fish growth (*in vivo*). Research was divided into two phases. In the first phase, we have tested *in vitro* several chemical concentrations that correspond to those used in *in vivo* experiments. In the second phase we have upgraded our approach so that no prior knowledge about chemical concentrations tested *in vivo* was required: *in vivo* data were needed only to validate the model but not to decide which chemical concentrations should be tested *in vitro*. Ten organic chemicals (including fungicides, herbicides, insecticides, industrial compounds and pharmaceuticals) were tested for different fish species (rainbow trout, fathead minnow and zebrafish). The results indicate a very good agreement between measurements and predictions determined for different species of fish, being exposed *in vivo* from 7 to 62 days, depending on the species and test design. Results moreover confirm that it is possible to predict chemical impact on fish growth without prior knowledge on concentrations that are used in *in vivo* studies for chemicals that do cause an effect on fish weight as well as for those that do not. Therefore, in spite of several assumptions and simplifications, combining *in vitro* experiments with computational modelling can result in a powerful strategy for screening chemicals to determine their effects on fish. In addition, considering the simplicity, rapidity and low costs of this approach, it is an encouraging step toward alternatives to long-term whole organism toxicity testing.

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184

A biotechnological metabolic activation system – potential for replacing rat liver S9 fractions

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The *in vitro* investigation of substances that become toxic after metabolic activation employ rat (i.e. *Rattus norvegicus*) liver S9 fractions as the source for liver enzymes. According to standard protocols, isolation of S9 fraction with enhanced enzymatic content requires treatment of animals prior to liver extraction, for instance by treatment with Aroclor-1254 or with a combination of sodium phenobarbital and beta-naphthoflavone. As a consequence, large numbers of animals suffer for the production of a supplement for *in vitro* tests. This is controversial in terms of the ethical use of animals in testing (3Rs). Moreover, S9 fraction isolation constitutes a very time consuming and costly process, since rats have to be maintained under GLP/GMP conditions until reaching maturity and considering that the poisoning-extraction-purification process requires trained personnel. Thus, replacement of S9 fraction for an *in vitro* system capable of metabolic activation would be extremely beneficial in toxicological testing, both in terms of animal welfare and with regards to production quality.

We hereby show the results of an assessment of S9, the animal derived products, and the newly developed non-animal-derived biotechnological product called ewoS9. ewoS9 is aimed to directly substitute the rat liver-derived S9 fractions.

We have investigated both products in biochemical assays with resorufin and coumarin based substrates that are conversed by cytochrome enzymes to fluorescent products. The conversion rates can be correlated to enzyme abundance and kinetics. A shotgun proteomics analysis has been conducted to elucidate the total protein composition as well as the relative cytochrome abundance in the metabolisation products. Furthermore, several biological *in vitro* tests used in the genotoxicity/mutagenicity assessment of sewage effluents and chemicals were conducted. The test battery consists of the AMES fluctuation test, micronucleus test and the UMU-chromotest. The comparison of S9 with ewoS9 show very promising results and bear the promise that S9 can be substituted with the non-animal-derived product ewoS9.

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23

A human epidermal burn wound model as alternative for animal testing

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Introduction

According to the world health organization (WHO), about 180,000 people die due to severe burn injuries every year. Moreover, burn injuries are a leading cause for morbidity, especially in low- and middle-income countries. For the treatment of severe burn wounds numerous products are available, which still show severe disadvantages e.g. in aspects of efficacy. Until now, the efficacy of a new product is tested with two-dimensional (2D) cell culture or animal test models. These models show only limited comparability to the *in vivo* situation in humans and, in case of animal models, involve painful treatments for plenty animals. The development of a three-dimensional (3D) test system based on human primary cells could represent an improvement for the preclinical testing of new applications and wound dressings. Therefore, a human burn wound model, which is based on a reconstructed human epidermis, was developed that holds the potential to replace, or at least reduce, animal-based test procedures.

Experimental methods

Reconstructed human epidermis (RHE) models were generated, using primary juvenile keratinocytes cultured at the air liquid interface according to the opensource reconstructed human epidermis protocol initially published by Pumay et al. and refined by Groeber et al. By contact with an 83°C heated metal rod for 5, 10 and 15 seconds burn injuries were introduced. Models were examined 24 hours, 48 hours and 6 days after injuring. The properties of models were characterized by histological examination, electrical impedance measurement, viability testing, secretion of ß-lactate and lactate dehydrogenase (LDH) and glucose consumption.

Results and discussion

Burnt areas in wound models showed no viability that was surrounded by a ring of vital keratinocytes at the wound edges, while untreated models showed homogenous staining in MTT tests. Burning of the models led to an increased production and secretion of LDH, while glucose consumption was not altered. Impedance spectroscopy showed only little influence of burning, since the stratum corneum, which is mainly responsible for skin barrier characteristics, was not damaged by the burning process. Excessive damage of basal and suprabasal cells in the burn area, and first ingrowing cells from the wound edges six days after injurie were showed by histological staining.

Conclusion

By burning RHE models with a heated metal rod a reproducible *in vitro* test model for burn wounds could be created. The models showed symptoms of injury in histological examination, as well as in molecular and physical measurements.

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168

Human cell- and tissue-based models of the lungs in state of health and disease

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Pulmonary drug delivery has gained attention by offering the possibility to administer drugs in a non-invasive way, as well as avoiding digestive enzymes and first-pass metabolism. In order to reduce animal testing, our lab has worked on novel *in vitro* approaches to mimic the human air-blood barrier to study the interaction of novel pulmonary drug carriers, as well as the biocompatibility of novel anti-infective drugs [1]. We were the first to publish a primary culture of human alveolar type 1-like cells (hAEpC) with tight intercellular junctions, allowing the performance of meaningful transport studies across the human air-blood barrier. The method to isolate and grow such monolayers has been described almost 20 years ago and since then continuously been refined [2]. Based on this, a new human alveolar epithelial cell line (hAELVi) with similar barrier properties could recently be created based on a mild lentiviral immortalization and is meanwhile commercially available [3]. This approach has been expanded to cocultures of human primary epithelial cells and macrophages, the two main players of the air-blood barrier. In order to make the model better reproducible human primary cells were replaced by the cell lines hAELVi and THP-1 [4].

Elaborating these co-culture models that represent the lung in healthy state, focus has also been laid on *in vitro* systems mimicking the state of disease, e.g. inflammation and/or infection. Thereby, the diseased state of the cocultures was simulated by inflammatory stimuli, as for example lipopolysaccharides [5]. To study the infected lung *in vitro*, the implementation of bacterial biofilms in a model of bronchial epithelial cells is being realized, as it occurs in cystic fibrosis patients during infection with *Pseudomonas aeruginosa*.

The here described human-based cell culture models not only allow the investigation of cellular and immune responses. Furthermore, they can be used for the evaluation of safety and efficacy of novel (anti-infective) drugs and delivery systems. With respect to animal experiments, such systems allow the precise investigation of (patho-)physiological factors with human relevance, and avoid the problem of species differences, when it comes to facilitate the translation of experimental data into the clinic.

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150

The CAM assay as alternative *in vivo* model system to study tumor aggressiveness

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Metastases are the leading cause of cancer-related deaths worldwide. Thus, translational cancer research has to focus on a better understanding of molecular mechanisms associated with tumor aggressiveness. The epithelial-to-mesenchymal transition (EMT) represents a fundamental process for tumor cell spread into surrounding tissues as well as to distant sites of the body. During EMT disseminating cancer cells acquire a mesenchymal phenotype, which is required for migration of tumor cells through the extracellular matrix, survival in the circulatory system and invading distant tissues. Thus, EMT seems to be also closely associated with angiogenesis. Since there is a lack of suitable *in vivo* models to study the single steps of tumor progression the molecular basis of metastasis is still poorly understood.

In our study we utilized a highly reliable alternative model for animal experiments, namely the *in vivo* chorioallantoic membrane (CAM) assay (Ribatti 2017), to mirror-image major hallmarks of cancer such as 1) proliferation/cell death, 2) invasiveness/EMT, 3) tumor angiogenesis, and 4) the ability to metastasize.

Exemplarily we used different tumor cell lines and CRISPR/Cas9 mediated, gene specific knockout clones of the colon carcinoma cell line HCT-116, engrafted them onto the CAM and analyzed various tumor characteristics. CAM tumors were analyzed for gene and protein expression pattern as well as by immunohistochemistry in order to evaluate hallmarks of cancer and hence the aggressiveness of the formed micro-tumors. We evaluated the invasive margins at the tumor/CAM border to correlate metastasis formation with tumor budding characteristics. Using *in vivo* optical imaging system (IVIS Spectrum, Perkin Elmer) and an Alu-specific qPCR of different chicken embryo organs we were able to quantify metastasis formation. The ability of tumor cells to interact and remodel the ECM was investigated by two-photon fluorescence imaging and collagen composition, amount, fiber size and spatial orientation was examined.

We present valuable data that the CAM-assay which allows for time-dependent molecular, biochemical, immunohistochemical and optical imaging analyses of fresh or FFPE tumor tissues is a suitable model system to study tumor aggressiveness. Its ease of use without the requirement for an animal protocol approval, the high reproducibility and cost effectiveness make the CAM assay an attractive alternative *in vivo* model for researchers at Universities.

Reference

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56

Development of an advanced primary *in vitro* model of the human small intestine

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Introduction

The human small intestine represents the organ with the largest contact surface to the environment. Its main function is the absorption of essential nutrients, water and vitamins, and it is a barrier protecting us from toxic xenobiotics and pathogens. The intestinal mucosa provides an elegant system to study stem cells as well as aspects of transport mechanisms and barrier functions.

Objectives

In our study, we applied epithelial cells in a 3D *in vitro* culture system in order to mimic the microenvironment of the gut *in vivo*.

Materials and methods

Intestinal crypts including stem cells were isolated from human small intestinal tissue samples and cultured on a decellularized porcine gut matrix together with intestinal fibroblasts. *In vitro* models were maintained under static and dynamic conditions for 7 days. Epithelial integrity was tested by FITC-dextran (4kDa) and TEER-measurement. Models were further characterized by qPCR, immunohistochemistry, electron microscopy and transport assays.

Results

Intestinal cells have formed a monolayer including all the differentiated cell types shown by, Mucin2, Villin, Chromogranin A, and Lysozyme immunohistochemistry. Electron microscopy depicted essential functional units of an intact epithelium such as microvilli and tight junctions. FITC-dextran and TEER-measurement proved tightness of the cell layer. Models showed characteristic transport activity for several reference substances.

Conclusion

As previously demonstrated for several culture systems, the development of intestinal *in vitro* systems based on human primary cells instead of using cell lines more closely reflects the *in vivo* situation. Therefore, our small intestinal model provides a very promising tool not only for the significant reduction of animal experiments, but also as a more reliable preclinical test platform to study drug absorption, cytotoxicity or the bacteria-host interactions.

Reference

Schweinlin, M. et al. (2016). *Tissue Eng Part C Methods 22*, 873-883.

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216

Berlin – bundling 3R expertise in the German capital

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Berlin, more and more becoming a leading city for science and business of international renown, shall develop into a leading center for the development of animal-friendly research methods according to the 3R principle of reduction, replacement and refinement.

Established in 2014 by funding of the Federal Ministry for Education and Research, the Berlin-Brandenburg research platform BB3R pools research activities of Freie Universität Berlin, Charité – Universitätsmedizin Berlin, Federal Institute of Risk Assessment, Technische Universität Berlin, Zuse Institute Berlin and University of Potsdam to replace, reduce, and refine animal tests. From start on, BB3R trained the next generation of scientists by introducing the first graduate school worldwide on 3R education. The established graduate program qualifies students in the recently introduced techniques in biomedical sciences, pharmacology and toxicology in particular, for a subsequent career in the field of 3R-related life science or scientific administration.

Started in 2018, Charité 3R aims to bundle and coordinate the interdisciplinary research at the Charité – Universitätsmedizin Berlin with emphasis on 3R and better translation in biomedicine: finding the best therapies by using animal-free methods whenever possible, establishing meaningful human disease models, increasing animal welfare. Giving a discipline and departments overarching organizing structure, Charité 3R will promote researchers with their 3R projects, implement 3R in education in cooperation with BB3R and communicate the challenges and needs of 3R research to the public.

Together, BB3R and Charité 3R will be the nucleus to create a 3R network in Berlin. To reach our goal to foster vigorously 3R research and better translation, we will join forces and consequently collaborate with local, national and international partners from public research institutions, and pharmaceutical and biomedical companies.

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Normal human skin and atopic dermatitis models – penetration and efficacy of topical glucocorticoids

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In skin diseases, topical treatment is preferred over systemic routes of administration because of less systemic adverse effects. A limited dermal penetration, however, can exclude topical therapy which can be overcome by innovative nanocarriers.
Insights into the pharmacokinetics and pharmacodynamics of drugs contacting the skin are possible by human-based 3D skin disease models. Atopic dermatitis models generated by exposure of the reconstructed human skin (RHS) to pro-inflammatory cytokines and/or filaggrin knock-down [1] allow to estimate drug penetration as well as wanted and adverse effects. Cutting-edge technology, such as X-ray stimulated microscopy (STXM), even allows mapping a glucocorticoid [2] and nanocarriers [3] within the human skin with high spatial resolution. Thus, dexamethasone passage into the epidermis and dermis can be investigated label-free.

Here, we compared data generated by STXM and HPLC-MS/MS and proofed dexamethasone penetration in human skin *ex vivo* being well in accordance. Applying dexamethasone in a gel with high ethanol content strongly enhanced drug penetration into RHS compared to human and mouse skin *ex vivo*. This is due to a rapid lipid elution from the thin RHS by ethanol as derived by stimulated RAMAN spectroscopy.

Dendritic core-multishell nanotransporters as well as a cyclodextrin coated thermoresponsive nanogel improve the penetration and efficacy of loaded dexamethasone in the atopic dermatitis model [4,5].

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Using a concerted analysis of corresponding cellular assays to create a complete *in vitro* bone culture and implant testing system

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A range of cellular assays is available for testing bone implant materials and resorbable bone substitute materials; however, animal testing is still used during material development even in early research stages, when consequences for the animals can be devastating. This is considered the easiest way to account for complex interactions between bone formation, degradation and vascular invasion. We have combined and advanced several *in vitro* assay procedures to improve prediction of clinical behavior and scientific precision for osteoconductivity and osteoinduction analysis. After standard 3h adhesion and 48 h proliferation testing, both osteoblast-like and osteoclast-like cells, reproducibly induced from cell lines, are cultivated for 28 h in perfusion systems with growth factors. The model cell line ST-2 possesses stromal cell properties, giving the necessary plasticity to enable induction of fibrous instead of mineral tissue formation, if the material has respective tendencies. Analysis is concluded with a cellular coculture of both cell types and a vascular invasion assay using a modified HET-CAM procedure. This way, a true *in vitro* bone culture system can be realized with bioreactor system that allow for both individual sample geometry adaptation and integration of sensory devices. The results are evaluated using a schematic system of comparative interrelation of the different assay outcomes, which is demonstrated for both an established and a non-suitable implant material.

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The photostabilizer ethylhexyl methoxycrylene (EHMCR) affects the photostability and phototoxicity of sunscreens containing retinyl palmitate

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Nowadays, there is a tendency to combine antioxidants to conventional sunscreens in order to increase their photoprotective effects. However, the use of photounstable combinations can compromise the efficacy and also leads to formation of reactive intermediates which can cause contact dermatitis and phototoxic skin reactions [1]. Vitamin A derivatives, such as retinyl palmitate, have been used in many daily use products containing UV filters, since they can promote recovery and protection of photodamaged skin [2]; however, they can undergo degradation under UV radiation. Thus, the aim of this study was to evaluate the effects of the photostabilizer ethylhexyl methoxycrylene (EHMCR) on the photostability and phototoxicity of a sunscreen containing UV filters and retinyl palmitate (RP). Photoprotective formulations/combinations containing avobenzone (AVO), octyl methoxycinnamate (OMC) and retinyl palmitate (RP) were prepared and supplemented or not (F1/C1) with EHMCR ($F1_{EHMCR}$/$C1_{EHMCR}$). These formulations were applied on glass plates and exposed to UVA radiation (18.7 J/cm^2), and then were extracted with dimethylformamide and subjected to HPLC and spectrophotometry analysis. The phototoxicity potential was evaluated by using 3T3 monolayer fibroblast culture, which was submitted to UVA radiation (9 J/cm^2) for the determination of cell viability in the presence and absence of radiation, according to OECD TG 432 [3]. Photostability studies demonstrated that AVO and RP showed the highest photodegradation when present in formulation F1 (AVO remaining concentration: 77% and RP remaining concentration: 44%). The addition of EHMCR to this formulation ($F1_{EHMCR}$) resulted in an improved photostability of both substances compared to F1 (AVO remaining concentration: 99% and RP remaining concentration: 70%). Furthermore, the spectrophotometry analysis showed that the formulation F1, which did not contain EHMCR, showed a significant photodegradation (reduction of UV absorption: 22%), when compared to formulation $F1_{EHMCR}$ (reduction of UV absorption: 1%). The 3T3 NRU phototoxicity showed that combination C1 was considered probably phototoxic since it presented MPEs values between 0.1 and 0.15 (MPE = 0.107; 0.112); however, this effect was reduced by the addition of $_{EHMCR}$ ($C1_{EHMCR}$) (MPE = 0.05; -0.002). The photodecomposition of AVO and RP in formulation F1 probably resulted in the formation of ROS and other reactive/toxic intermediates, which may have caused damage to the fibroblasts exposed to UVA radiation. The results observed in this study are very promising because it was demonstrated that the photostabilizer EHMCR presented in formulation F1EHMCR can increase the photostability of AVO and RP and consequently, reduce the phototoxic potential of the combination C1.

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Evaluation of *in vitro* models of the rat and human airway epithelium for assessment of acute airway toxicity

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Introduction

Acute airway toxicity is an important consideration for development of pharmaceuticals, chemicals, cosmetics and agrochemicals. Acute toxicity information is used to inform safe use of products, procedures for accidental exposure and dose range selection for *in vivo* studies. Currently validated methods require the use of animals. However, as part of a 3R's strategy (refine, reduce, replace), *in vitro* models are being developed as an alternative safety testing system. One such commercially available airway model is EpiAirway™ (MatTek Corp., Ashland, MA, USA) constructed from primary human airway epithelial cells. At present there is no means to correlate the responses of these tissues to human outcomes as available *in vivo* data has been conducted in rodents. To address this gap in information, an *in vitro* airway model was generated similarly to the human EpiAirway™ model using cells collected from the airway epithelium of Charles River rats.

Methods

The rat EpiAirway™ model was prepared by MatTek using epithelial cells isolated from Charles River rats. The cells were cultured at the air-liquid interface for a maximum of 27 days forming a functional model of the rat airway epithelium. Both models showed a high level of differentiation including features such as cilia and a pseudostratified epithelium. Using 14 test chemicals, each prepared at 4 different concentrations, the responses by the human and rat EpiAirway™ models were compared by MatTek and Charles River Laboratories Edinburgh Ltd in at least three independent experiments. Tissues were exposed to the test chemicals for three hours, rinsed and allowed to recover overnight before assessing viability (MTT assay) and barrier function (transepithelial electrical resistance, TEER). IC75 values (the concentration required to reduce viability or TEER to 75% of the vehicle control) for each test item were calculated

Results

For both models, pre-exposure TEER measurements were high confirming that a robust barrier had developed. A clear dose response in MTT and TEER data was observed for each test chemical in each species. TEER and MTT assay data were broadly in agreement and consistent between experiments. Both species responded similarly to most test items with IC75 values being of the same order of magnitude in each species. The exceptions to this were methyl methacrylate and ethyl alcohol which rat EpiAirway™ was more sensitive to than human EpiAirway™. The rank order of chemicals was generally the same between laboratories, indicating the consistency and transferability of the EpiAirway™ models and testing techniques.

Conclusions

The human and rat EpiAirway™ models were largely consistent with each other and between laboratories, suggesting that they will provide a valuable platform for toxicity testing. Correlation of these results with *in vivo* results will be important for showing the predictivity of these models for toxicity screening and safety assessments.

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Bioinformatics analysis of IPS cell data to elucidate mechanisms of actions of nephrotoxins

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The introduction of new *in vitro* based methods in risk assessment leads to the generation of a large amount of novel heterogeneous data that needs to be mined and interpreted to elucidate the mode of action and to bring it into relation with adverse outcome pathways (AOPs). Within in3, a Marie-Curie network program dedicated to advancing 3Rs, the data and knowledge generated by different partners needs to be shared, harmonized, and combined with data coming from publicly-available sources and from literature. It will, therefore make it available for use in modelling and bioinformatics workflows. Bioinformatics workflows can be used to mine this data to uncover enriched pathways providing information on possible mode of actions, specific biomarkers for the toxicity and common toxicity-inducing key events like oxidative stress or DNA damage. As a starting point, a list of nephrotoxins and non-nephrotoxins for the target organ kidney from the AOP wiki and literature mining are retrieved and related transcriptomics data (*in vivo* and *in vitro*) is extracted from databases such as GEO. These compounds and data are used, after a proper normalization and preparation, to investigate 1) differences in species, *in vivo* and *in vitro* responses, 2) possible biomarker genes leading to pathway enrichment, and 3) the possibility of relating these biological findings to key events (KEs) of existing AOPs or use them to propose additional KEs for AOP development. These workflows can then be used for other target organs which are being used within the in 3 project activities.

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Importance of computational modelling in the design of organ on a chip devices

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Organ on a chip devices are becoming increasingly complex and given the high cost of tooling and manufacturing, it is essential that design iterations are minimised. Computer modelling can be used to predict some of the key performance parameters prior to manufacturing and hence increase the chances of success [1]. Cells in culture require close control of oxygen levels in the media and other factors such as flow stress can also have an important influence on cell viability, differentiation and metabolic function [2,3]. For the recreation of organ like behaviour, the physiological relevance of the cell environment is critical [4].

This paper illustrates the value of computer modelling in the design of a particular family of cell culture devices (Quasi Vivo®) which incorporate flow of media between multiple chambers, a key requirement for future organ on chip devices.

We review the recent research findings from four academic groups that illustrate the influence of chamber geometry and flow rate of media on the achievement of oxygen levels and flow stress over or around the cells under culture.

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Development of a multi-organ *in vitro* model of the interactions between liver and cardiac cells under uni-directional dynamic flow

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Improved *in vitro* models are required for accurate prediction of drug pharmacology and toxicity in the early stages of drug discovery process. Organ-on-a-chip or microphysiological systems based *in-vitro* models are currently being evaluated for their utility for this purpose. In the present paper, we report a milli-fluidic system utilising Quasi Vivo® multi-chamber dynamic flow technology that supports the connection of multiple components representing different organs and simulates the interactions between them, thus representing a complex multi-organ *in vitro* model. This meso-scale *in vitro* platform offers many advantages: 1) it allows the culture and analysis of large populations of cells, 2) both cells and culture fluids could be harvested for multiple or single endpoint investigation, 3) it allows flexibility to connect multiple chambers in different configurations, on different growth surfaces, 4) precise control of dynamic flow rate of culture media mimicking physiological conditions.

Our previous studies on individually cultured cells under dynamic flow condition have shown improvement in cell viability and functionality when compared to cells grown under static condition. For example, the gene expression of Phase I and Phase II detoxification genes in primary human hepatocytes when cultured under dynamic flow as compared to static cultures was increased. The basal activity of drug metabolising enzymes including CYP3A4, CYP2B6, and CYP1A2 was also increased under flow conditions [1,3]. Similarly, viability was increased in the case of primary cardiac myocytes when cultured under flow. Advancing the *in-vitro* approach to enable the complex multi-organ set-up, we tested the Quasi Vivo® system to mimic the interactions between human liver cells and cardiac cells [2]. We optimised cell number and media conditions in order to have equal or enhanced viability of this multi-organ system under flow conditions when compared to cells under standard static conditions. This allows us to not only monitor toxicity in two of the most prone organs to drug-induced injury, but also provides us with a metabolically competent system that allows the monitoring of parent compounds as well as any potential metabolites that are generated in the liver and then flow via the circulation into the heart as is often the case *in vivo*. This meso-scale model system will help develop greater understanding of both cardio- and hepato-toxicity in a systems biology manner and to test potential interventional agents to prevent drug-induced toxicity.

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S. aureus-infection-model at human liver-on-chip

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Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that can colonize the epithelial surface of many healthy individuals, but is also one of the most frequent causes of sepsis. To invade human tissues *S. aureus* could rely on a wide spectrum of different virulence factors and possess also the ability to dynamically react to environmental changes. In order to adapt to the intracellular milieu by *S. aureus* is able to switch its phenotype to the "small colony variant" (SCVs) which is associated with a reduced metabolic activity, a higher resistance to antibiotic treatment and the ability to circumvent detection by immune cells.
Tissue macrophages (MΦ) of the liver, termed Kupffer cells (KC), are critical regulators of host defense against systemic *S. aureus* infections by sequestering the majority of bacteria from the blood stream.

To analyze the role of different KC activation pattern on *S. aureus* clearance by liver, we aim to establish a microfluidic human liver-on-chip infection model for *S. aureus* comprising tissue macrophages. We focus on the immune response to the pathogen and analyze how different KC activation pattern influence SCV formation and bacterial persistence during the course of infection.

Preliminary results 48 h post infection show that a confrontation with the pathogen leads to a strong acute immune response associated with a significant tissue damage in presence of non-activated (M0) / pre-activated macrophages (M1), but to a lesser extend in regenerative macrophages involved in tissue remodeling (M2). The amount of SCVs formation however was higher in M2 than in M0 macrophages, whereas no SCV formation was observed in M1-macrophages. Cytokine-profiling shows an acute increase in pro-inflammatory cytokines and a delayed, dampened pro-inflammatory cytokine release in M2 and M0 macrophages (M0 > M1 >> M2).

Our preliminary data point to a role of M2-polarized macrophages that act as an initial reservoir for *S. aureus* during infection to avoid efficient bacterial clearance. M2 macrophages persistence thereby favors formation of SCV, chronic infections and sequelae such as osteomyelitis.

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1

The elephant in the room: PREPARE before you ARRIVE

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Despite widespread journal endorsement of reporting guidelines, preclinical *in vivo* research is still being criticised for inadequacies that include poor experimental design and statistical analysis, underuse of analgesics and poor reproducibility, even within the same laboratory. This is a major threat to the reputation of preclinical research. In order to improve the situation, pre-registration, greater use of systematic reviews and better reporting are frequently proposed as the major drivers for change.

We propose that there is an elephant in the room that has been ignored for too long. Reporting cannot improve the intrinsic quality of an experiment which has already been performed. Improvement of animal research must begin at the beginning, with guidelines for planning experiments. These guidelines will include a number of topics which are seldom reported, but which can have dramatic effects on not only the scientific validity of the research but also on animal welfare and the safety of all those involved. Such an approach is in keeping with ethical thinking and the 3R's principle of Replacement, Reduction and Refinement.

With this in mind, we have constructed a set of reporting guidelines called PREPARE, based upon our experiences over the last 30 years in planning and supervising animal experiments. PREPARE contains a "checklist" as do the ARRIVE guidelines for reporting, but it is designed to be more of an *aide memoire* than yet another form to complete. PREPARE provides a gentle reminder to all scientists of the items which should be addressed pre-study, much in the same way as pilots, however experienced, work their way through a checklist before take-off. Importantly, PREPARE has its own website which expands upon the topics in the checklist. PREPARE can thus offer scientists links to specific guidelines for each topic, adding new ones as they are published. An intelligent search engine on the 6,000-page Norecopa website where PREPARE resides (https://norecopa.no/PREPARE) facilitates the location of additional resources.

It is our hope that the debate on poor reproducibility will swing towards planning, rather than reporting, of animal experiments. We are in danger of wasting time discussing the quality of the lock on the door of the stable from which the horse has already bolted.

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Human primary fibroblasts *versus* immortalized Balb/c mouse 3T3 fibroblasts for cytotoxicity and LD50 prediction

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There is a high percentage of new drugs that fails in clinical studies, besides successfully tested in animals during preclinical research, this indicates that animal testing has difficulties correlating with results in humans [1]. Thus, it is mandatory that *in vitro* test replace animal experimentation presenting high correlation with humans. Consequently, the aim of this study was to analyze the validated *in vitro* cytotoxicity method (OECD 129) [2] by using immortalized Balb 3t3 fibroblasts and primary human fibroblasts (HF). Besides the positive control SDS, the antifungal terbinafine (TB), caffeine (CF) and the UVB filter benzophenone-3 (BZF-3) were used to compare the assays due to some reports of topical permeability/toxicity [3-5]. All of the substances were considered cytotoxic when tested in 3T3 and HF, with exception to CF that was not cytotoxic in HF. SDS, TB and BZF-3 IC50 results obtained for both cell types were considered statistically equivalents by ANOVA, once no analyzed substance presented statistical difference. SDS presented a standard deviation (SD) of ±2.30 µg/mL, in accordance with the GD, only for the 3T3. TB on the other hand, had an ideal SD only for HF demonstrating to be more reproducible than SDS for HF. CF showed no cytotoxicity in HF at the concentrations tested and presented high SD in 3T3; also, BZF-3 presented high SD in both cell types. This BZF-3 and CF results may be justified by the low solubility at the medium on higher concentrations changing the bioavailability for both cell types. It is important to mention that different sources on the literature present different IC50 for these substances, which can be related to a different cells/ substances incubation periods (24/ 48 h). This study presented estimative starting doses for acute oral systemic toxicity tests (LD50) in rodents of 590.90/706.44 mg/kg for SDS in 3t3/ HF cells, respectively; 615.46/544.74 mg/kg for TB; 648.05/939.17 mg/kg for BZF-3, showing to be lower than LD50 values found in the literature. However, these assays fulfill their function in reducing the doses tested in *in vivo* LD50 assays. For CF the LD50 in 3T3 of 1154,83 mg/kg was very different from literature (192 mg/kg for rats) (TOXicology Data NETwork), indicating a limitation of the test for this lipid soluble substance. Thus, the present study demonstrates a correlation between immortalized cell line 3T3 with the HF for cytotoxicity evaluation but also a limitation for caffeine LD50 prediction.

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Patient-specific, additively manufactured vascular models enabling an animal-free neurointerventional training setup

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Aim

Physicians performing neurointerventional treatment of cerebral aneurysms are frequently require training due to the ongoing progress in endovascular techniques. Animal models in rabbits or pigs are utilized for this purpose. The aim of the research project ELBE-NTM is to replicate patient-specific characteristics of endovascular aneurysm treatment in an *in-vitro* training setup replacing animal tests in further education.

Methods

Hollow vascular models of intracranial and cervical vessel segments are designed based on original medical scan data e.g. by adding connectors and defining the wall thickness [1,2]. A portfolio of over 25 patient-specific aneurysm models is elaborated, which is going to be continuously expanded. A standardized individualization process for vascular models is implemented reducing the process complexity and effort for the design and fabrication of new patient-specific models [2]. The additively manufactured models [3] are integrated in a neurointerventional simulator ("HANNES"; Hamburg ANatomical NEurointerventional Simulator). HANNES is evaluated by physicians with different levels of experience regarding the potential to replace animal models in trainings.

Results

Additive manufacturing of vascular models enables high geometrical freedom for complex anatomies combined with the production flexibility for low quantities of patient-specific models. Elastic vessel walls are feasible with such additive manufacturing technologies as Stereolithography or Multijet Modeling. Due to the standardized individualization process with additive manufacturing a complexity-reduced product and process structure could be realized while offering a high variety of vascular anatomies.

Multiple neurointerventional training courses could be performed with HANNES replacing animal models. HANNES was evaluated as highly valuable for training of neurointerventional procedures. This is supported by the implementation of the entire vascular tree as hollow vascular models starting from entrance in femoral artery up to the cerebral area with the aneurysm. The implementation of patient-specific cerebral models combined with standard vessel segments of the cranial base and aorta without any inner edges leads to a highly realistic vascular anatomy which is evaluated as a significant advantage compared to animal models. The intracranial and cervical vessel models can be easily exchanged with quick-change adapters, greatly facilitating the workflow during training sessions.

Conclusion

An animal-free training setup is feasible by additively manufactured vascular models for an individualized integrated neurovascular training environment of aneurysm embolization. Original patient data and real instruments create a highly realistic training environment. The faithful replication of the vessel tree and the anatomical variety based on real patient-specific vascular diseases are significant advantages over animal-based training models and will facilitate their replacement.

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Understanding and implementation of the 3Rs of animal welfare bodies at German research facilities and universities

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According to the EU Directive 2010/63 the Member States were required to establish animal welfare bodies (AWB) in order to promote the principles of the 3Rs in research facilities using animals [1]. Members of AWBs in Germany are to date tasked with a multitude of responsibilities, which are mostly disproportional to the actual resources provided. To shed light on this problem we aimed to assess the current understanding of the 3Rs in research and their implementation in compliance with the directive by designing a questionnaire for members of AWBs at German research facilities (public and private). The 29 questions were widely consistent with a similar survey conducted at Swedish Universities [2] and aim at gathering comparable information to the Swedish project as well as similar projects from other Member States [3,4]. The response of Animal Welfare Officers was much higher than other Members of AWB (AWO = 63.41%, Members of AWB = 27.64%, no assignment = 8.95%).

The correct understanding of the 3R definitions was quite high but the survey revealed some misconceptions concerning all three Rs (> 17% for Replacement, > 20% for Reduction and > 27% for Refinement).

About 40% considered all 3Rs equally important in research and education at their institution while others stated that for their institutions Refinement and Reduction have a higher priority than Replacement. Concerning the tasks of advising researchers on the application of the 3Rs in their projects or informing members of staff about scientific developments within the field of the 3Rs, the survey showed that some members of AWBs either don't know about the 3R-status at their institutions (> 10%) or did not answer the questions (> 10%). Around 20% stated, not to follow up on the 3R in projects progress and outcome. According to the survey, raising the awareness for the 3Rs could be achieved through a variety of measurements including better training, financial encouragement and mandatory regulations considering the 3Rs.

Thus far the survey identified knowledge gaps concerning the 3Rs, different perceptions of responsibilities at research facilities and starting points to advance the acceptance and implementation of the 3Rs. Keys for improvement of the current status quo would be improved frameworks throughout research facilities, more resources for 3R projects and AWBs, better training/education of staff and enhanced knowledge exchange.

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252

Examples of applying of human 3D models and human multi-organ-chips (hMOCs) in industry

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Participants of the round table will give examples of their experience with human 3D models and human multi-organ-chips (hMOCs) in their specific sector of industry, e.g. in the drug, cosmetics, chemicals and food industry. In addition, representatives of producers and/or developers of human 3D models and multi-organ-chips will join the panel.

It is the aim of this Plenary Round Table Discussion to encourage colleagues in the audience to start establishing human 3D models and MOCs in their laboratories.

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258

Unusual, innovative, and close to *in-vivo*: MUG-Mel1 and MUG-Mel2 melanoma cell lines for drug development

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Melanoma is a leading cause of death in developed countries. The tumor frequently spreads to the brain and is associated with deterioration in quality of life. Due to the fact that melanoma is one of the most aggressive and therapy-resistant human cancers, resulting in poor prognosis with a median survival of less than six months, more effective therapies and innovative *in-vitro* models are urgently needed [1]. Within melanoma, NRAS mutation has been associated with aggressive tumor biology and poor prognosis. While plenty of cell lines exist, only few melanogenic cell lines retain their *in vivo* characteristics.

Our group presents an intensively pigmented and well-characterized cell line derived from a highly aggressive NRAS p.Q61R mutated cutaneous melanoma, named MUG-Mel2, including the clinical course, unique morphology, angiogenic properties, growth characteristics, 3D cell culture, and exome gene sequencing. Amongst several genetic alterations, mutations in GRIN2A, CREBP, PIK3C2G, ATM, and ATR were present. These mutations, known to reinforce DNA repair problems in melanoma, might serve as potential treatment targets [2].

Furthermore, we succeeded to establish a brain melanoma metastasis cell line, named MUG-Mel1 and two resulting clones D5 and C8 with morphological variety, differences in lipidome, growth behavior, surface and stem cell markers. Mutation analysis by next-generation sequencing, copy number profiling and cytogenetics demonstrated the different genetic profiles of MUG-Mel1 and its clones. Tumorigenicity was finally established in zebra fish.

As innovative treatment option with high potential to pass the blood-brain barrier, a peptide isolated from lactoferricin was studied for its potential toxicity. MUG-Mel1, MUG-Mel2 and non-tumorigenic cells were used in different 3D models to compare treatment effects of various peptide concentrations.
Advanced melanoma and brain metastases are a major clinical challenge; therefore, the development of relevant *in-vitro* models provides valuable information about tumor biology and offers great potential to screen for innovative therapies.

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205

Elucidation of cytotoxic effects of β-β-dimethylacrylshikonin in melanoma cells

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Background and aim

Cancer is one of the most common causes of death in developed countries. Among skin cancers, malignant melanomas are responsible for 79% of all deaths. Therefore, new approaches and therapeutics for melanoma treatment are needed [1]. A possible new drug to treat melanoma is β-β-dimethylacrylshikonin (DMAS), which has shown promising effects in preliminary cell culture experiments [2]. Based on these experiments, aims of this project are the elucidation of specific cytotoxic effects of DMAS in melanoma cells, especially the determination of affected pathways. The effects will be compared between various melanoma cell lines with different mutational profiles to determine the suitability of DMAS as a possible anti-cancer drug.

Methods

To determine the cytotoxicity of DMAS, viability tests were performed on melanoma cell lines with various mutations, fibroblasts and melanocytes. Additionally, Annexin-Sytox staining as well as Caspase 3 and 9 assays were performed to elucidate whether the cells undergo apoptosis or necrosis. To show potential cell cycle arrests, cell cycle analysis was performed. Furthermore, possible affected pathways were elucidated with mRNA sequencing and specific intracellular molecular targets of DMAS were investigated via RT-qPCR and Western Blots.

Results and outlook

DMAS effectively influenced the viability of various melanoma cell lines in a time and dose dependent manner. The cells primarily undergo apoptosis, as shown by Annexin-Sytox and Caspase assays. Before undergoing apoptosis, melanoma cell lines face a G2 cell cycle arrest. qPCR and western blot results show overexpression of NOXA and PUMA which indicates induction of cell death via the mitochondrial apoptosis pathway. Furthermore, a decrease in pAKT could be observed, suggesting that DMAS inhibits cell survival pathways. The apoptotic effect was significantly stronger for melanoma cell lines in comparison to fibroblasts in preliminary experiments.

As melanoma mortality rates are still increasing, the development of new therapeutic anti-cancer strategies is a very important research objective. The high efficiency, combined with low application concentrations needed to kill melanoma cells, makes DMAS a promising new anti-cancer drug.

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250

Strategy for the development of an advanced *in vitro* chordoma tumor model

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Chordoma tumors are rare types of slowly growing bone tumors, which were first described by Luschka et al. in 1856. The primary tumor originates along the axial spine and they are believed to arise of remnants of the notochord. Over the past few years, it was a challenge to establish cell lines due to the very slow-growing of this tumor entity, finally some groups were successful and many adequate tumor cell lines were established. However, the most common cultivation is two-dimensional (2D) and innovative three-dimensional (3D) models to express the physiological behavior of chordoma are still needed.

Therefore, the aim of this study was to design and develop a novel approach for an advanced 3D *in vitro* disease model using the two patient-derived chordoma cell lines, MUG-CC1 (clival chordoma) and MUG-Chor1 (sacral chordoma), and the promising 3D bone matrix OSTEOpure™.

For the cultivation of chordoma cells on the bone matrix a tailor-made perfusion reactor system was used. The total circulation volume of each perfusion reactor chamber was 10 mL and the flow rate was consistently set to 0.6 mL/min. Additionally, cells of both cell lines were cultivated in 2D and in 3D under static conditions as controls. Every second day a partial medium change of one mL for the dynamic 3D samples and a total medium change for the static samples was performed. Finally, the morphological characteristics and the viability of all samples (static 2D, static 3D and dynamic 3D) were assessed by 4 DAPI/AM/PI staining. Moreover, after embedding the samples in paraffin a hematoxylin eosin staining to confirm the morphology of typical chordoma cells, as well as brachyury staining to further characterize the obtained samples, was performed.

MUG-CC1 and MUG-Chor1 chordoma cell lines are able to grow and proliferate in complex 3D environment, utilizing OSTEOpure™ as a scaffold, under static and dynamic conditions. This finding potentially provides the basis for future research towards a more physiological *in vitro* chordoma disease model.

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182

Spatial defined cell characterization of a miniaturized heart tissue model

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Microfluidic technology has enabled the development of small-scaled, highly sophisticated tissue- as well as organ-like *in vitro* systems. Such miniaturized models are useful tools to study mechanisms of healthy as well as diseased tissue while enabling the reduction of animal experiments [1]. Microfluidic systems provide defined spatial and temporal control over the cultivated cells while still being easily adjustable. However, it remains challenging to monitor and analyse the cellular response to a treatment within a microfluidic device.

This project aims to investigate the applicability of a microfluidic system with three distinct laminar fluid streams. Mouse cardiovascular progenitor cells will be used to form cardiac bodies which will be transferred into microfluidic devices and treated with different media in three parallel laminar streams. Due to the distinct media components, different cardiac cell populations will be spatially favoured [2]. Moreover, spatial defined immunofluorescence-based cell characterization and RNA isolation for gene expression analysis will be performed.

The described model system will be used to characterize cardiac cell populations by spatial specific cell lysis, RNA isolation and investigation of the expression of cardiomyocyte and smooth muscle markers. The implementation of the proposed analysis technique is the next step in the development of an easy-to-use miniaturized cardiac model for drug screening purposes.

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285

Lung-on-a-Chip: The interplay of primary human epithelial and endothelial cells improves the alveolar barrier function

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Introduction

The pulmonary alveolar barrier is one of the largest entry ports for xenobiotics into the body and consists of a thin barrier of epithelial and endothelial cells. Yet, establishing a biologically relevant model of the pulmonary alveolar-capillary barrier is challenging, due to poor availability of human primary alveolar epithelial cells. In addition, the effect of mechanical stretch on the cells due to breathing is thought to be important in regulating lung functions but has so far not thoroughly been considered due to the lack of appropriate tools.

Aims

The goal of our work is to establish a stable pulmonary alveolar-capillary barrier and understand the interplay between lung alveolar epithelial and endothelial cells in an *in vivo*-like environment.

Methods: Primary human pulmonary alveolar epithelial cells (pHPAEC) from patients undergoing lung resections were cultured on inserts (0.4 µm pore size) or lung-on-a-chips (3.5 µm pore size) with and without four different endothelial cell types. Medium was exchanged every other day, following which trans-epithelial electrical resistance (TEER) was measured for up to 14 days.

Results

The TEER of pHPAEC monoculture rises above 1000 Ωcm^2 until day 4-6 and then begins to drop reaching values below 500 Ωcm^2 at days 8 to 14. Co-culturing pHPAEC with human VeraVec endothelial cells increased TEER values significantly and lead to a more stable culture (values above 4000 Ωcm^2 at day 12). Culturing pHPAEC together with primary human pulmonary microvascular (HPMEC) or umbilical vein (HUVEC) endothelial cells, or with immortalized HPMEC ST1.6R resulted in slightly lower TEER values compared to VeraVec cultures but significantly higher compared to monoculture of pHPAEC.

Conclusion

The integrity of the alveolar-capillary barrier is a key parameter that prevents any unwanted leakage from either side of the barrier. TEER is often used to assess the quality of the barrier integrity. Here, we show that a co-culture of primary human alveolar epithelial cells together with endothelial cells does not only significantly increase the barrier integrity but is also a factor of a long-term stability.

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152

A decision tree – how are the 3Rs with algorithms applied in animal experiments?

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It is a general problem to judge an experiment in correct way from the ethical aspect, especially for the young generation who are just engaging in scientific work. The rules and principles are changing but basic requirements are the same. Semmelweis University runs a course for researchers' assistants, titled "Experimental animals-animal experiments" in which the participants' number is about 100 in every year. We would like to assist these young researchers in planning experiments in legally and ethically acceptable manner.

In our study the aim is to summarize the requirements of 3Rs and 5Fs in science-based and animal-welfare based algorithms that help to make the right decision. Our idea is based on questions and answers that form algorithms. In this system the basic principles of 3Rs and the animal welfare is placed in the foreground.

Our algorithm forms a tree from the roots to the branches highlighting the meaning of the 3Rs and 5Fs and scientifical and ethical connection between them. As the root the possibility of replacement is determined. If a validated *ex-vivo* method is available, the procedure must not be carried out on animals (Directive 2010/63/EU). Then harm/benefit evaluation is an important step. If the pain, suffering, distress or lasting harm take too long and they cannot be attenuated, the experiment ethically is unacceptable. According to 5Fs for finding the best model species practical (e.g. lifespan, size, number of offspring, cost), ethical and scientific (e.g. anatomical and physiological similarities, suitable disease model) aspects need to be taken into consideration. Re-using animals could reduce the number of animals in the experiment. Reliable hygienic status and proper circumstances promote the reduction and essential for refinement. Homogeneous animal groups, that involves similar genetic and hygienic status, same macro- and microenvironmental effects, adaptation, same age, and gender, support the reduction as well because it reduces the standard deviation in a group so the result data will be more reliable. After all, at the highest decision level the refinement is one of the most important factors where we can interact in the most effective way. If there are several options the species should be chosen that has the lowest capacity to experience pain, suffering, distress or lasting harm. The following questions could arise that are connected to the basic principles of refinement. Do we need to terminate the animal or can we apply any picture imaging methods (e.g. PET-CT) that allow longer life and re-use of the animals? How can the sampling and treatment frequency and volume be reduced? What is the part of the body which is less sensitive for the treatment? How can we reduce the pain? What are the most modern ways of applying anesthesia and analgesia? Can we adopt the animals to the circumstances of the experiment that reduce the stress, anxious behavior and fear feeling?

The decision tree has many "branches" depending on the type of experiment.

This decision tree helps not only the researchers' planning of the experiment but also the ethical decisions of the IACUC.

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183

"Everyone is responsible" – animal welfare in the education

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Animal welfare is the well-being of the animals. Laboratory animal welfare is determined by external and internal factors and mainly depends on the persons working with them (technicians, assistants and researchers). The proper care and accommodation gives the reliable background of an experiment and ensures appropriate health and well-being. Well trained, so called sufficient staff is essential to maintain the proper circumstances. The choice of the most "refined" method helps to reduce the pain, suffer or stress. Monitoring the genetical background can reduce the disadvantageous consequences of a harmful phenotype.

Animal Welfare is a priority for the European Union. The aim is to improve the quality of animals' lives. That can be realized by the well-organized education that involves theoretical background, practical demonstrations and chance to practice as well. It has ethical and legislation aspects that should be taken into consideration. Teaching the application of EU rules on animal welfare helps to reach the aim of 5Fs and 3Rs. The guiding principle of EU strategy on animal welfare is: "Everyone is responsible".

During the educational phase our main pursuit is to assess the animal welfare in many aspects. The education is species specific which means that after general basic information we teach the claims the different species. This involves the differences in macro- and microenvironment, in social contacts and in the proper food and water supply. We draw the attention to the consequences of the inappropriate circumstances e.g. lonely keeping instead of social contacts, poor environment instead of environmental enrichment, feeding with unsuitable food. We teach how to handle the animals in different hygienic status and how to disturb least their well-structured environment. Harmful phenotype, as a consequence of the genetic modification is a special problem. To evaluate the phenotype status and the well-being of the animals we can use different guidelines.

One of the well-known methods is the "Grimace scale" for rats, mice and rabbits. For well-being evaluation "General distress scoring sheet" based on Morton and Griffiths can be used.

Acclimatization, adaptation, condition to experimental circumstances and handling are all important factors that can reduce the anxious, fear feeling behaviour and promote the animal welfare. We have pictures, videos, *in situ* demonstrations to teach the proper handling, sampling and treatment. These procedures could be practices more times. A well-trained, expert staff could result in more peaceful animals and more reliable data.

"Everyone is responsible" means that the European Union Commission, the Member States and Research Institutes have important responsibility to transposition the animal welfare into legislation and to control it regularly. The main responsibility remains on researchers who need to keep in mind the legislation, ethical aspects, the animal welfare because he/she is the person who has personal contact with the animals. He/she should be the best-trained who need to know what to do in different situations. That is why we work on to have better-and-better education to reflect different aspects of animal welfare.

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121

The added value of the 90-day repeated dose test for substances of low (sub) acute toxicity for REACH purposes

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One of the key principles in REACH is the promotion of alternatives to animal testing (Article 1) and the intention that animal testing should be "a last resort" (Article 25). With these principles in mind it is therefore important to periodically review the necessity of the standard information requirements under the REACH regulation.

Since 2011 there have been a series of independent projects looking at the added value of the conduct of the 90-day repeated dose test for substances that have a low toxicity profile in all (sub) acute tests. There is now a consistent pattern arising, whereby if a substance meets the criteria for having a "low toxicity profile in a high-quality dataset", the results of the 90-day test would not lead to classification or alter the management of the substance. It appears that 10 to 15% of industrial chemicals could fit the profile for which there appears to be little added value to the conduct of the 90-day test in addition to the 28-day test for the determination of (sub) chronic toxicity, at least via the oral route. The evidence includes four retrospective analyses of registered industrial chemicals in the EU and a prospective study in which predictions were made prior to the conduct of the 90-day study.

In this presentation we provide an update on these studies, two of which are our own projects and open for discussion what can be done to facilitate greater uptake of this important waiver.

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123

A review of REACH from the animal perspective

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It is now 12 years since the EU's new chemicals legislation (Regulation No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)) was adopted. Two important statements in the Regulation in relation to animal testing and alternatives are; Article 1(1), which states that one of the purposes of the Regulation is to promote alternative methods and Article 25(1), which states that animal testing should be a last resort. We have been heavily involved in the implementation of the REACH regulation since its adoption with a view to ensuring that these principles are upheld. Our review looks at the mechanisms within REACH that were put in place to achieve these aims and asks not only if they are being implemented properly but if they are sufficient.

Whilst the industry has employed data sharing and read across to maximum effect, we estimate that over 2.2 million animals have already been used for REACH registrations and that the Commission's worst case estimate of 4 million by the end of the registration process is very likely. The use of *in vitro* methods as complete replacements remains at relatively low levels. There are issues with lack of balance in the views and engagement of member states and a failure to recognise that animal testing as a last resort applies to them. There are also issues with the low funding of alternative methods and slow formal adoption of those that have already been validated.

Our review recommends that renewed emphasis is placed on increasing the availability of alternatives and enforcing their use, although this will come too late to prevent unnecessary animal testing for the final registration deadline of June 2018.

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Measuring fecal testosterone metabolites in house mice: Replacement of invasive techniques

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Testosterone plays a key role in male fertility as it influences sexual behavior and affects gametogenesis. Testosterone is the main reproductive hormone in male mice and studies assessing testosterone levels require collecting blood samples. Such invasive procedures induce pain and distress to the sampled animals and can influence the parameters investigated. Here, we aimed to establish a non-invasive alternative to blood sampling in house mice in assessing testosterone metabolites in fecal and urinary samples. We started to determine the route of excretion and assessed the effect of the circadian rhythm and sex on the metabolism and excretion patterns of radiolabeled testosterone metabolites. Experimental mice (C57BL/6J, n = 32) were divided in two groups containing 8 males and 8 females each. Group one received an IP injection of radiolabeled ^{3}H-testosterone at 7:00 a.m., i.e. at the beginning of their inactive phase, whereas the second group received the injection at 7:00 p.m., i.e. at the beginning of their active phase. Subsequently, feces and urine were collected 0, 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 h post injection and thereafter in 12 h intervals for 5 days. We found that males secreted significantly more ^{3}H-testosterone metabolites via the feces than females (males: 59.0 ±7.3%, females: 49.5 ±5.5%; $p < 0.001$), whereas females showed higher proportions of radioactivity in the urine compared to males (males: 41.0 ±3.7%, females: 50.3 ±5.5%; $p < 0.001$). The recovery rate of the radiolabeled metabolites did not differ between sexes ($p = 0.52$). Overall, the time course of ^{3}H-testosterone excretion via feces and urine was comparable between sexes. Similarly, the time of peak excretion did not differ between males and females ($p = 0.356$). However, peak excretion in urinary samples occurred significantly faster than in fecal samples and was reached after 2 hours ($p < 0.001$). In fecal samples, time of peak excretion was significantly dependent on the time of ^{3}H-testosterone administration ($p < 0.001$). Mice that received the injection at the beginning of their active period reached peak excretion on average after 4 hours, compared to mice that received the injection at the beginning of their inactive period, which reached peak excretions after 8 hours. Again, we found no difference in the recovery rate of radiolabeled metabolites between treatment groups ($p = 0.94$). In summary, we were able to identify excretion patterns and peak excretion times in urinary and fecal samples of house mice in relation to their activity pattern and sex. Currently, we characterize the type and relative abundance of ^{3}H-testosterone metabolites, so that unlabeled testosterone metabolites can be identified in future studies. These results help to optimize sample collection times for testosterone assessment and are a first step towards replacing invasive blood sampling techniques and contributing to the 3Rs.

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Development of advancing human 3D trabecular meshwork models to assess onset of glaucoma

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Glaucoma is a progressive optic neuropathy that leads to irreversible blindness. As known, the main causes of glaucoma onset are oxidative stress and vascular alteration, which damage trabecular meshwork (TM) status, impairing the eye's drainage system which in turn, increase intraocular pressure (IOP). Nowadays IOP is the only hallmark of disease and therapies can slow down it, without prevent blindness [1,2]. In addition, the glaucoma diagnosis occurs too late because most of screening tests are not predictive. The lack of glaucoma models human-based is the mainly limitation for drug discovery.

Therefore, the aim of this study was to develop an *in vitro* human-based model to define the key events involved in the onset of glaucoma, and its long-term complications. The model could be a useful tool to select and check targets for therapeutic drugs to prevent or treat glaucoma disease.

Firstly three different *in vitro* models of human TM cells, conventional 2D and innovative 3D-static and –biodynamic models, were assessed, to verify which better mimics the glaucoma onset.

For 3D models, TM cells were embedded into Matrigel, and dynamic condition was set up using a fluidic multi-chamber device, connected with a peristaltic pump (LiveBox1 and Live Flow, IV-Tech srl). While 2D-TM, after 7 days of standard culture conditions, showed a marked suffering, the 3D-TMs were cultured up to 15 days. The metabolic state, performed by Alamar blue assay, pointed out a better healthy state of 3Ds vs 2D. This effect was confirmed also by confocal imaging that evidenced a cellular morphology and organization more similar to physiological environment in 3D-TM.

Starting by these observations, we analyzed if the different TM-models could response otherwise to a chronic stress, mimicked by daily exposure for 2 h to 500 μM H_2O_2, with 22 h recovery phases in between. The repetitive injury was carried out for 7 and 15 days, in 2D- and 3D-TMs, respectively. At each check point time, cell metabolic activity was assessed by Alamar blue test in real time, revealing that cell-flattening of 2D-TM showed a different sensitivity to H_2O_2 chronic exposure compared to 3Ds. Indeed, during chronic oxidative stress, metabolic index resulted in a time-dependent increase in 3D-TMs, while did not vary in 2D culture. Furthermore, at the end of experimental treatments, cleaved PARP-1 was found in both static 2D- and 3D-TM, instead biodynamic 3D-TMs shown an increase of full-length PARP-1 only. To investigate the co-factor role of PARP-1 in inflammation pathway via NF-kB, the activation of this latter marker was analysed, under chronic oxidative stress [3]. Activation of the transcription factor was observed only in both 3D-TMs, but mostly in biodynamic models. Further analysis were performed to verify apoptosis in biodynamic 3D-TM, during the first 72 h exposure to chronic oxidative stress, by a dedicated protein array. The observed increase of several proapoptotic markers at 48 h only, would suggest a progressive cellular adaptation to pro-oxidant stimulus. Taken together these findings, dynamic 3D-TM showed features more closely to *in vivo* pathophysiology.

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292

Application of impedance spectroscopy for analysis of BeWo clone b30 human choriocarcinoma cell line as model of placenta-on-chip

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BeWo cells are used for construction of *in vitro* human placental barrier models [1]. Only the early placenta possesses multiple trophoblast layers whereas third trimester placenta consists of a single layer [2]. BeWo cells don't undergo contact growth inhibition and form multilayer structures. This makes important controlling monolayer state for transport experiments. Impedance spectroscopy was applied for studying of electrical characteristics of BeWo clone b30 human choriocarcinoma cell line during cell growth and in response to HIF-1 activator (model of hypoxia).

Methods

Cells were cultured as described previously [3]. Individual inserts of a 96-well Corning Transwell plate (1 μm pore size) were cut from the plate and inserted into Homunculus microfluidic system (SRC Bioclinicum, Russia). BeWo b30 cells were seeded 3×10^4 per one insert. After 48 h when the confluency was achieved a potent activator of HIF-1 named "D014-0021" was added to several inserts (final concentration 10 μM). This compound is one of oxyquinoline derivatives which previously have been found to activate HIF-1 [4]. Impedance spectra were acquired with impedance spectroscopy system (SRC Bioclinicum). For extraction of electrical parameters, we used the equivalent electrical circuits based on the previous studies [5]. CPE is a constant phase element (represents capacitance of the cell monolayer), R (or TEER) is resistance of the cell monolayer, $R_{interlayer}$ is resistance of the interlayer (due to extracellular matrix or cell-cell junctions), R_{med} is integral resistance of culture medium and transwell membrane. Student's t-test was used to calculate the statistical significance of difference, p-value less than 0.05 were considered statistically significant.

Results

We have predicted from the mathematical model that R_{med} and the radius of the impedance hodograph (and hence R) will rise as the number of layers N increases (linear dependency). In contrast, in case of one layer the radius can rise due to formation of tight junctions but the R_{med} should remain stable. It was obtained that R as well as R_{med}increases. Moreover, we have found statistically significant difference of R_{med} between 96 and 48 h. It can be concluded that after 48 h BeWo cells form multilayer structures and should not be used for transport studies. The impact of HIF-1 activator D014-0021 on impedance spectra was also investigated. The addition of HIF-1 activator leads to an increase in R after 6 h and to a decrease after 24 h (these results are in a good agreement with previous studies where conventional TEER measurement was used). In addition, a significant decrease of capacitance after 24 h incubation with D014-0021 was detected.

Conclusion

In this work we applied impedance spectroscopy for monitoring of BeWo clone b30 human choriocarcinoma cell line growth. We have shown that formation of multilayer structures can be readily detected with impedance spectroscopy and hence it can be used for quality control of the *in vitro* placental models based on these cells. We have also shown that it is possible to use impedance spectroscopy for detection of changes of additional parameters such as electrical capacitance in different experiments including hypoxia modelling with chemical activator of HIF-1.

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Refinement by systematic severity scorings in animal experiments

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Pain, harm and suffering caused to the animals in animal experiments have to be restricted to the indispensable minimum [1]. A prospective assessment of burden is obligatory due to European and national legal provisions 1. Additionally, abortion criteria have to be defined [1]. During the experiment all procedures and burdens have to be reviewed and documented.

Working with Score Sheets is a suitable tool for this obligation. In Score Sheets expected symptoms are listed and rated. Interventions and abortion criteria are defined. Thereby suffering of laboratory animals is securely limited and reduced. Thus, Score Sheets serve as an applied tool for refinement.

The Working Group of Berlin Animal Welfare Officers has developed a guideline on Score Sheets in order to support scientists, animal caretakers and competent authorities [2]. It focusses on tabular listed symptoms which are specific for the experiment. According to legal requirements each symptom is assessed as mild, moderate or severe. Symptoms and severity levels are related to obligatory instructions that define interventions or abortion criteria for staff members. Consideration of symptoms that exceed the officially approved degree of burden is recommended. This ensures that staff members are able to react immediately complying with determined interventions in the interests of animal protection.

The prospective definition and assessment of specific symptoms that are expected in the animal experiment leads to a sensitization of project leaders and staff members for the condition of the animals in the projects. It is obligatory to educate and train all personnel concerning signs of illness and application of treatments to guarantee reliable and correct use of Score Sheets. Taking this into account Score Sheets are effective tools for refinement in the sense of 3R.

The Working Group of Berlin Animal Welfare Officers provides recommendations on Score Sheets, categories of distress and abortion criteria on the homepage [3]. They might be applicable for animal welfare officers, leaders and assistants of animal projects and representatives of competent authorities [3]. Their consequent application and development are an applied tool for refinement [2].

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260

Establishment of a model for equine grass sickness: Extracorporeal blood perfusion of equine ileum

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Equine grass sickness (EGS), also known as equine dysautonomia, is a degenerative disease of the neuronal system in equidae which has been first described in 1909. There are reported cases all over the world, though the disease primarily occurs in the UK. The disease almost exclusively appears in grazing horses and although many agents are being discussed the causative factor remains to be found. Clinical signs and severity of the disease are largely defined by degeneration of the interstitial cells of Cajal in the enteric nervous system. Neuronal loss is found in the entire gastrointestinal tract, with the most evident pathology present in the ileum. Acute and subacute forms of EGS are invariably fatal, with a reported survival rate of about 40% for chronic cases, nevertheless these cases need extensive nursing care and have a long recovery period. [1]

Due to the severity of the disease it would be ethically favourable to study EGS without having to harm live horses, fulfilling the criteria for the 3R, especially replacement of animal experiments [2]. The extracorporeal perfusion of organs from horses slaughtered for meat production offers an ethical method which allows studying a system very similar to the living animal [3]. Patan et al. developed a very successful model using isolated equine limbs to investigate equine laminar tissue [4]. Based on this model we established extracorporeal perfusion of the equine ileum. Normothermic perfusion is performed for 4 hours using an oxygenated mixture of autologous blood and plasma as perfusate. Collection and preparation of blood and ileum as well as perfusion set up and procedure are being presented.

This model closes a gap between *in vitro* cell cultures and experiments in live animals and could be further used to investigate reperfusion injury, to model organ transplantation in human medicine, as well as in hormonal studies, as was successfully done in rats [5].

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Toxicogenomics reveals neuronal specific and non-specific responses to cadmium possibly involved in neurodegeneration

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting the voluntary motor nervous system. Genetic factors play a major role in the familial form, represented by 10% of cases. Whereas, in the remaining 90% sporadic cases a multifactorial origin is supposed, in which environmental factors may be involved. Particularly, the involvement of metal toxicity is proposed, based on epidemiologic data and on the increasing number of case reports of ALS-like syndromes associated to metal exposure. Among metals, cadmium is of particular concern for its heavy anthropogenic release (~30,000 tons/year) and for its chemical properties. Cd exposure can primarily occur through food, drinking water, air particles, cosmetics and cigarette smoking. Once assumed, even at very low concentrations, Cd is accumulated with a long biological half-life (> 26 years). Cd accumulation contributes to oxidative stress and alteration of divalent ions homeostasis, primarily Zn^{2+} and Ca^{2+}, both crucial for neuronal signaling.

Studies of neurodegenerative diseases mainly rely on the use of animal models and on stem cells. Already ten years ago in a Nature Report the validity of neurodegenerative disease animal models was questioned, and direction towards the reduction of transgenic models is mandatory.

Human neuroblastoma SH-SY5Y is a dopaminergic cell line that reproduces biochemical and morphological properties of neurons, thus being often used as an *in vitro* model for human neurons in neurotoxicity or neuroprotection studies in experimental disease research.

We have applied a toxicogenomic approach to evidence deregulated pathways in SH-SY5Y cells exposed to cadmium to unravel neuronal specific and non-specific responses to the toxic metal.

A whole genome analysis by cDNA microarray was performed in SH-SY5Y cells exposed to non-cytotoxic concentrations of cadmium (10 and 20 mM for 48 h).

Very interestingly, microarray analysis evidenced that the top up-regulated genes were enriched in pathways related to the mineral absorption biological process in which metallothioneins (MT), and heme oxygenase-1 (HMOX-1) are among the highest up-regulated genes. MT are low molecular weight proteins that bind essential metal ions and sequester toxic metals, thus representing an important broad mechanisms of metal homeostasis. MT expression was visualized in SH-SY5Y cells by Western blot analysis revealing a dose-dependent trend of the protein, validating microarray data.

The increased HMOX-1 expression has just been related [1] to the progression of several diseases including neurodegeneration.

Another remarkable result concerns NEU4 that was found in our cell samples as the top down-regulated gene upon cadmium treatment. This gene encodes for a neuraminidase specifically expressed in brain that regulates neuronal function by catabolizing brain gangliosides. Sialidase NEU4 has been shown to be localized in the outer mitochondrial membrane and involved in different cellular processes, such as apoptosis, neuronal differentiation and tumorigenesis [2]. A dramatic decrease of NEU4 expression level was previously observed prior to apoptotic neurodegeneration.

Overall our results evidence specific neuronal responses to cadmium and suggest the possible mechanisms triggered by this toxic metal in neurodegeneration.

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Promoting transparency in preclinical research: Preregistration of preclinical studies on www.preclinicaltrials.eu

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Background and objectives

The validity and reproducibility of animal studies are limited by publication bias, selective outcome reporting and risks of bias. This threatens their translational value [1,2]. Preregistration of animal study protocols, just like preregistration of clinical trials, is a promising method to create transparency and reduce these limitations [3,4]. We have developed an online platform to preregister animal study protocols on www.preclinicaltrials.eu.

Methods and results

The registration form is designed by an expert group on preclinical animal studies and preclinical evidence synthesis and comprises 34 fields. It contains details of the study's hypothesis, design, primary and secondary outcome measures, measures to reduce bias and sample size rationale. Authors need to indicate whether their study is exploratory or confirmatory. Reference to publication(s) or data repositories can be provided. Protocols can be made publicly directly after submission, or after an embargo period. Preclinicaltrials.eu aims to provide an overview of all executed animal studies, including those that remain unpublished, and thus contribute to a reduction of publication bias and unnecessary duplication. It allows fellow researchers and reviewers to access additional information on the study design. This platform increases awareness and transparency concerning risks of bias and selective outcome reporting and potentially reduces these biases.

Future perspectives

We encourage all researchers to preregister their confirmatory animal studies on preclinicaltrials.eu. We believe all stakeholders involved in animal research should encourage preregistration and call upon researchers, institutes, medical journals, funding bodies, policy and law makers, scientific societies and others involved parties to make prospective registration the standard and mandatory [5].

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The UK must adopt the EU's final goal of full replacement of procedures on live animals for scientific and educational purposes

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In the 60 years since Russell and Burch established the 3Rs, significant efforts have been made in the EU to replace the use of animals in scientific procedures. Public interest in animal protection and animal-free science has also continued to gain momentum. Now, as the UK approaches the final Brexit deadline, the spotlight has turned to the ways in which national animal-protection legislation may change, and as such, People for the Ethical Treatment of Animals (PETA) is urging the UK government to develop bold policy initiatives to eliminate the use of animals for scientific purposes that are fully reflected in national legislation.

Directive 2010/63/EU on the protection of animals used for scientific purposes lays out the rules and principles regarding the use of animals whilst they continue to be used in experiments. Importantly, though, in addition to securing the principles of the 3Rs in EU legislation, the Directive is set in the context that the ultimate goal is to replace all experiments on animals. In particular, Recital 10 states that "this Directive represents an important step towards achieving *the final goal of full replacement of procedures on live animals*" [emphasis added]. Although the UK's Animals (Scientific Procedures) Act 1986 was amended to transpose the mandatory Articles of the Directive, transposition of the Recitals within the preamble was not required, and consequently, they have not been included in the UK legislation. It is also clear from the UK annual statistics on the use of animals in scientific procedures that the present national efforts to reduce the number of experiments on animals are inadequate.

To ensure that the UK commits to a renewed focus on animal-free science in this politically turbulent era, PETA UK is urging the government to adopt the EU's final goal of completely replacing the use of animals for scientific or educational purposes with non-animal approaches. The government must establish a clear policy within a legislative framework seeking to end experimentation on animals and provide a clear strategy and timeline for achieving this goal. This poster examines the background of the current situation and the potential consequences for the future of science and innovation in the UK with and without this vital step.

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Practical guidance in LAS-continuing professional development

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The EU directive 2010/63 – and subsequently national Experiments on Animals Acts – clearly set new requirements for the training of people conducting animal experimental work. In the Dutch situation, for instance, this involved, among other revisions, that more species-specific knowledge is required, and that scientist, animal caretakers and animal technicians must be demonstrably competent to be allowed to perform activities related to animal experiments.

In the new situation, like in many health care professions, the "lifelong learning" (or Continuing Professional Development: CPD) concept must also become the standard in animal research. CPD is compulsory for all employees who take care of experimental animals, and/or perform or design animal experiments. Depending on the function, the training could involve 3Rs topics like, animal welfare, experimental design and scientific validity. The designated competency officer (art 24.1c of Dir. 2010/63) is responsible for the training of employees according to set requirements, both theoretically to retain qualification (continuing education) and on a practical level to retain or acquire skills (practical competences). He/she should be aware of the opportunities to take appropriate training and additional training and distributes these. CPD can consist of a wide range of activities; from conference participation, attending symposia and lectures, workshops, literature studies, and educational activities, to e-learning activities. For practical skills, CPD can consist of practical training of techniques within or outside the institution and where possible in combination with e-learning modules.

Each organisation could set requirements for the number of hours that have to be spent on CPD, but there is a reasonable consensus about the average number of hours that have to be spent per year (see FELASA guidelines for instance: http://www.felasa.eu/recommendations/guidelines/guidelines-for-continuing-education-for-persons-involved-in-animal-experime/). The completed training courses should be documented in a CPD training record, which can be reviewed annually by the line manager, and can also be presented to the competency officer or authorities for inspection.

The 3Rs-Centre Utrecht Life Sciences and a working group of different animal welfare bodies produced a guidance document including a manual with guidance with respect to the requirements for 1) further education, and, 2) demonstrable competence in the practical performance of procedures on animals. We are happy to share this CPD guidance document with you: https://www.uu.nl/en/organisation/3rs-centre-uls/education-and-training

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A kinomics approach to safety testing: Towards an animal-free safety test for whooping cough vaccines

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As part of the release strategy, whooping cough vaccines are routinely tested for the absence of residual pertussis toxin (PTx) before their release on the market. This is done using the mandatory lethal mouse histamine sensitization test. The *in vitro* alternative CHO cell clustering assay is a highly sensitive assay that is already used by industry to test detoxification further upstream in the manufacturing process. The CHO cell clustering assay is quantified by counting the number of clusters induced by PTx. Here, we aim to develop a method able to quantify residual PTx based on kinase activity.

This research is part of the VAC2VAC project, which aims to demonstrate proof-of-concept of the "consistency approach" by using sets of *in vitro* assays to ensure that each vaccine batch from an individual manufacturer is consistent with a batch proven to be safe and efficacious.

We have previously demonstrated that human A549 cells, like CHO cells, show a similar clustered morphology in response to PTx. In previous work, we have shown that exposure to PTx does not elicit changes in gene expression in five out of six human cell lines tested. We hypothesize that PTx-induced clustering is caused by changes at the protein level and we aim to identify molecular markers in both cell types that can be used to detect the presence of PTx in whooping cough vaccines and products further upstream in the manufacturing process. Because kinase activation and subsequent target protein phosphorylation are central to the cellular signaling cascades underlying morphological changes, we make use of kinomics to search for these molecular markers. PamGene's microarray assay for kinase activity profiling is based on measuring peptide phosphorylation by protein kinases in a sample. Arrays consist of up to 144 peptide sequences that harbor phosphorylation sites correlated with one or multiple upstream kinases (protein tyrosine kinases or serine/threonine kinases). Fluorescently labeled anti-phospho antibodies are used to detect real-time phosphorylation activity of kinases present in the sample. Our proof-of-concept data show that treatment of A549 cells with PTx for 10 or 15 minutes already increases the tyrosine kinase activity. Currently, we are analyzing the data and repeating experiments to determine the variability.

The data from the kinase activity profiling assays will be used to unravel the molecular pathways involved in the response of CHO and A549 cells to PTx exposure. This will greatly contribute to the mechanistic understanding of PTx mode of action. Possibly, we can also establish links between the *in vitro* cellular response and disease pathology. Ultimately, we wish to contribute to the development of an improved *in vitro* assay able to quantify the presence of PTx in upstream products or final vaccine batches in a simple and robust way, either in the sensitive CHO or the relevant human A549 cells, leading to a large reduction of animal use in vaccine safety testing.

The project is supported by the EU/ European Federation of Pharmaceutical Industries and Associations (EFPIA)/Innovative Medicines Initiative (IMI2) Joint Undertaking (VAC2VAC grant n° 115924).

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Establishment of an *in silico* model to understand adrenocortical carcinoma and preclinical drug screening

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Adrenocortical Carcinoma (ACC) is a very rare but aggressive type of cancer. The survival prognosis with 6%-13% for stage IV patients is very poor [1-4]. Current standard therapies are chemotherapy, radiotherapy, and the adrenolytic agent mitotane, but these are palliative [4]. Knowledge about the pathogenesis of ACC is still limited and new therapeutic options are needed [1]. Thus, animal models of ACC are on the way, for instance a mouse xenograft model was developed in 2017 [4]. As an alternative, we established an *in silico* model to understand the molecular mechanism of ACC and to develop new treatment strategies from clinical data. Our model characterizes tumor drivers and identifies molecular subgroups. A machine learning approach predicts risk models with valuable clinical outcomes, enabling the identification of drug targets and preclinical screening of patient-tailored treatment strategies. For instance, exemplified for ACC patients, our *in silico* model predicts treatment responses for a mitotane and an immune therapy. In conclusion, our *in silico* model of ACC represents an alternative to preclinical animal models as it directly uses clinical sequencing data from patient. It allows to study the molecular mechanism and to develop new therapeutic options for ACC. We expect that our *in silico* model will reduce animal experiments for preclinical testing of new treatments and improve clinical therapy decisions.

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Implementation of 3R in veterinary education through virtual patients

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Education of veterinary students includes clinical training using animals. This training involves the demonstration of certain examinations through teaching staff, repetition of those techniques by students under supervision and the refinement of the procedure. Since the amendment of the German Animal Welfare Act in 2013 the use of animals in education must be registered as animal experiments.

In July 2016 the eLearning project "QuerVet" started at Freie Universität Berlin not only to create virtual patients respectively virtual cases for the obvious benefits of eLearning such as flexibility and interactive teaching but also to be able to better prepare students for the future contact with animals and to reduce the numbers of live animals for education.

Training modules developed within "QuerVet" are implemented as interdisciplinary courses in 6th, 7th and 8th semester of the veterinary curriculum. Virtual patients within these modules have to be interdisciplinary, practically oriented and case-based; requirements that the previously offered lecture-based formats did not adequately fulfil.

Successively the newly developed modules are introduced using a modular blended learning concept. They are grouped by themes and aligned with topics of the remaining curriculum. Each module is created with an average of three virtual patients and one on-site activity in the lecture hall; the latter to present additional information on the topic and provide an opportunity for discussions.

In cooperation with the Centre for Digital Systems (CeDiS) of the Freie Universität Berlin, patient examinations and relevant procedures were video-taped and added to the virtual patients as background information. These recordings are constantly accessible for students and available to the teaching staff as well to be used for other occasions. In addition to the recordings, interactive elements were implemented into the virtual patients so that the students are able to practice skills such as a sample taking or sonographic examination.

The enrichment of virtual patients not only makes the online appearance more authentic but also reduces the number of demonstrations needed in clinical training, prepares the student much better since it can be repeated and watched at any time and therefore refines the further educational practical training.

The students were asked to anonymously participate in concurrent surveys after each online case and each module. The acceptance of the new concept, case-based structure and authenticity as well as achievement of educational objectives and learning motivation were assessed. Furthermore, each digital item such as the videos or interactive element were evaluated by the students for their educational benefit. So far, more than 400 students have participated in the various surveys.

Results show a high acceptance of this blended learning concept, as well as a good achievement of their subjective educational objectives. The educational implementation of patient examination recordings and interactive elements were graded as very useful and the virtual patients as authentic.

Blended learning concepts including virtual patients are well accepted by students, provide another format for veterinary clinical training and help to reduce and refine the use of animals and therefore the number of animal experiments in veterinary education.

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Protein-to-lipid ratios determine dexamethasone penetration into skin and reconstructed human skin

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Scanning transmission X-ray microscopy (STXM) detects drugs in a label-free manner at an unprecedented spatial resolution in excised human skin [1,2]. However, the method has not been compared to other analytical approaches regularly used for dermal absorption studies. Here we relate dexamethasone (DXM) penetration profiles following STXM and high performance liquid chromatography mass spectrometry (HPLC-MS/MS) in epidermal and dermal layers of reconstructed human skin (RHS) and human as well as mouse skin *ex vivo*. As human skin explants are limited in supply, RHS serves as an internationally accepted alternative for penetration studies [3].

100, 300, and 600 µg/cm² DXM in an ethanolic hydroxyethylcellulose (HEC) gel is applied onto the tissues for 10-300 min. After 30 min exposure to 600 µg/cm² DXM, we are able to detect DXM penetration via STXM. Lower DXM concentrations do not yield a sufficient STXM signal. HPLC-MS/MS shows that after 300 min less than 10% of total DXM penetrates the epidermal RHS layer. According to STXM, DXM accumulates in intercellular spaces of human stratum corneum (SC) [2], yet quickly reaches deeper RHS layers. This is in accordance with a less developed barrier function of RHS [4].

The penetration enhancer ethanol disrupts SC barrier integrity and dissolves SC lipids [5]. In order to visualize the solvent effect – DXM and ethanol amounts increase in parallel – we employ stimulated Raman spectroscopy (SRS) and track the ethanol-induced modification of corneocytes and lipids by detecting protein and lipid structures at their respective wavenumbers. With increasing ethanol amounts, protein-to-lipid ratios in RHS and skin change, which may explain the higher DXM amount detected in the epidermal RHS layer. Whereas SC proteins are strongly affected in RHS, the predominant effect in excised human and mouse skin remains lipid dissolution, which suggests the protective function of such lipids against protein denaturation.

In conclusion, by combining STXM, HPLC-MS/MS, and SRS, we are able to understand drug penetration and the effect of a penetration enhancer in different skin matrices more thoroughly. Further studies with topical drug formulations should consider the differences.

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The hen's egg test on the chick area vasculosa (HET-CAV) as a test system to evaluate the antimicrobial activity of drugs and formulations

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Introduction

Due to the steadily growing rate of antibiotic resistances, the screening of new antibiotic drugs or the development of new formulations is a major concern in pharmaceutics. To test the antimicrobial activity and the biocompatibility of new drug candidates or novel formulations, more sophisticated alternative test systems are necessary. In contrast to the commonly used *in vitro* tests, such as disk-diffusion or broth and agar dilution tests, the modified hen's egg test on the chick area vasculosa (HET-CAV) offers the possibility to evaluate the antimicrobial activity and biocompatibility concurrently *ex ovo*.

Methods

Fertilized hen's eggs were incubated for 72 h and transferred into petri dishes for further incubation. Simultaneously, different microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*) were cultured in appropriate broth media. Aliquots of diluted bacteria suspensions were pipetted into an O-ring placed on the CAV. As model drugs tobramycin, meropenem and ciprofloxacin were dissolved and additionally applied on the CAV at a distant spot using different concentrations. Evaluation of the antibiotic concentration dependent survival of the chicken embryo (dose-response relationship) and observation of local toxic reactions caused by the substances or microorganisms were performed over 68 h. Additionally, *in vitro* microplate laser nephelometry (MLN) and CellTiter-Blue® (CTB) assay were performed to determine the efficacy of the antibiotics (IC50) against the planktonic and the biofilm form of *P. aeruginosa*, respectively. To confirm the applicability of the test system for the concurrent efficacy and biocompatibility evaluation for formulations, commercially available antibiotic eye drops (Tobradex® and CILOXAN®) were investigated for irritative effects (hemorrhage, vascular lysis, thrombosis) and the antibiotic activity (survival rate) after local administration.

Results

The cultivation of different microorganisms on the CAV caused dose and strain dependent local infections and toxic reactions. Whereas the cultivation of *C. albicans* caused no lethality for up to 48 h, cultivation of *P. aeruginosa* revealed strong hemorrhagic reactions followed by embryonic death. The gram-negative *P. aeruginosa* was found to induce more severe toxic reactions on the CAV compared to the gram-positive *S. aureus ex ovo*. The treatment of *P. aeruginosa* by local application of ciprofloxacin, tobramycin or meropenem decreased the lethality dose-dependent. The observations indicated the antibiotic susceptibility of the bacteria and allowed the calculation of effective concentrations for each antibiotic drug. Moreover, antibiotic drugs showed almost similar effects against the planktonic forms of *P. aeruginosa* strains (MLN), whereas up to ten-times higher concentrations of the antibiotics were needed for killing the biofilm forms (CTB assay) of the strain. *In vitro* (CTB-Assay) and *ex ovo* (HET-CAV) tests identified ciprofloxacin as most active drug, followed by meropenem and tobramycin which indicates a correlation of these test systems. The antibiotic activity of the antibiotic eye drop formulations was confirmed by an increase of the survival time of the *P. aeruginosa* infected hen's egg accompanied by a good biocompatibility.

Conclusion

In conclusion, the modified hen's egg model allowed the determination of the antimicrobial activity and biocompatibility of drugs and antibiotic drug formulations in a complex biological environment according to the 3R concept.

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More animal testing for less human safety? How animals continue to suffer for our (novel) food and why oral toxicity tests should be replaced

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Despite common commitments to protect animals used for scientific purposes, there are still new obligatory animal tests demanded in EU regulation. Since 2018, a sub-chronic toxicity study on rodents (repeated dose 90-day oral toxicity) should be conducted during the authorisation process for novel foods in the EU [1]. However, 90-day feeding studies, especially when applied for whole foods, are criticised for causing nutritional imbalances [2] and a poor transferability to the human metabolism. Furthermore, the transmission of test substances via oral gavage causes even more uncertainties. Gavage feeding bypasses the oral mucosa and thereby shifts the absorption and bioavailability of the transferred substance [3]. Additionally, gavage handling causes potential pain, suffering, distress, lasting harm and even premature death to the involved animals [4].

Thus, testing on animals in the authorisation and risk assessment of (novel) foods does not provide further safety for humans and should be replaced by human-relevant, modern animal-free testing methods [5]. This exemplifies the need for replacing animal testing. Alternative methods account for a better and scientifically more reliable human health and consumer safety assessment.

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Recommendations to improve non-technical summaries of animal experiments

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To increase transparency towards animal experiments conducted in the EU, member states have to publish non-technical summaries (NTS) on authorised projects. NTS shall provide information on the objectives of the project, including the predicted harm, benefits, the number and types of animals to be used, as well as a demonstration of compliance with the requirement of replacement, reduction and refinement (3Rs) [1]. We have analysed the quality of a selection of NTS from Germany and the UK, including the status of the publication of NTS from further EU member states [2]. Here we are representing the results for Germany. We observed a constant lack of information, even of subjects particular demanded in the Directive 2010/63/EU, as well as deficiency in the description of the expected adverse effects on the animals and what procedures are performed on them. Specific good and bad examples are highlighted.

The ideal content of NTS should contain information that is neutral, not biased and comprehensible for laypersons. Therefore, we compile recommendations to support scientists and regulatory authorities to improve NTS and to comply with their duty to improve transparency on animal testing.

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Development of an additive manufactured mouse phantom for quality assurance of a small animal irradiation device

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Introduction

In radiation oncology, experiments are often carried out using mice as a model for *in-vivo* research studies. Due to recent technological advances in the development of high-precision small animal irradiation facilities, the importance of quality assurance is increasing. However, the typical phantoms used to this purpose have a simple geometry (e.g. cylinder or cube) and consist of a single material (e.g. PMMA or water), thus not coping with the complexity of mouse anatomy. Additive manufacturing (AM), on the other hand, provides the possibility to produce such complex models from a three-dimensional data set. The aim of this work is therefore to develop an anatomically correct mouse model for quality assurance of a small animal irradiation device by means of AM.

Materials and methods

For developing a model mimicking the x-ray absorption properties of biological tissues, five different AM-processes with sixteen different print materials were analysed. In particular, the Hounsfield Units (HU) of such materials were evaluated with a computer tomographer (CT). In a second step, a solid model of the mouse body with the main organs was constructed using CT data of a real mouse and the digital mouse-atlas "Digimouse" [1]. The model includes cavities corresponding to the most important organs, to be filled with tissue-equivalent material as well as a hollow to integrate an Optically Stimulated Luminescence (OSL) radiation dosimeter after printing. In a first step, air was chosen for mimicking the lungs and an agarose mixture for brain, gastrointestinal tract, and kidneys. Finally, plaster was chosen as a bone substitute.

Results

The analysed AM print materials resulted to be suitable for mimicking x-ray absorption properties of bones (130-490 HU), soft tissue (50-79 HU), adipose tissue (-187- -104 HU), and water (5-10 HU). Therefore, they can be used for printing the mouse phantom. The mouse phantom prototype was produced with a Formlabs Stereolithography (SLA)-printer "Form2+" using clear resin as a material. The final solid model was constructed in two parts that can be attached with a plug connection after filling the cavities corresponding to the relevant organs and bones. Micro-CT scans showed an excellent quality and adequate contrast. First radiation dose measurements with OSL dosimeters and radiochromic films were in agreement with calculations within a few percent.

Conclusion

AM shows great potential for the development of mouse models that are inexpensive, easy to adapt and accurate, thus enabling their use for quality assurance in radiotherapy. A printable mouse phantom was developed and produced, which enables verifications of small animal irradiation devices and could significantly reduce the use of living animals to this purpose.

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Comparative analysis of the difference of toxicity test between Chinese and European Pharmacopoeia

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Background

The legislative is one of the key roles to promote the practice on animal alternatives in biomedical research.

Objective

To compare the differences on animal used requirements between the China and European pharmacopoeia in biomedical research.

Methods

Two authors independently reviewed The European Pharmacopoeia 8th edition issued in 2013 and The Pharmacopoeia of the People's Republic of China 10th issued in 2015 and analyzed the requirements on new medicine and biomedical products on animal use requirements, including first testing, the second testing and results classification, etc. If any differences about the judgment were solved by discussion. The main findings were summarized in a descriptive way.

Results

There is a similar testing methods and process required for animal used in drug testing and biological products to detect toxicity test in China and European pharmacopoeia.

The toxicity test for medicines and biomedical products were similar in first animal testing, e.g. 5 healthy rats 18-22 g in China and 17-24 g in Europe; and for those toxicity test for biomedical products required two species of animals: 5 healthy rats and 2 guinea pigs. There is a little difference on the weights of the animal use, e.g. guinea pig 250- 350 g in China and 250-400 g in Europe, etc.

The main differences between the two pharmacopoeias were as follows.

The preparation of sample to be tested were described in details, the top dose of injection on biological products was no more than the dosage of one person used, which should be followed by the product label and instruction manual, and the speed of injection was also slower in European Pharmacopoeia, e.g. the injection completed in 15-30 minutes in Europe, while finished in 4-5 minutes in general, except those required slowly injection allowed finished in 30 minutes in China.

The number of animal used in the second testing required double number than its first testing in China Pharmacopoeia. E.g. 10 rats and 2 guinea pigs were required in the second testing in China, etc.

The results of the tested animal observation time longer in China than its in Europe, e.g. The judgment of medicine harm on animal testing should be waiting 48 hours in China, while 24 hours in Europe, etc.

The animal's blank control was also required for biomedical products in China pharmacopoeia, while no requirement in Europe pharmacopoeia.

Conclusion

The requirements for animal use for toxicity test between Chinese and European Pharmacopoeia were similar. However, there are some differences on the products testing; animals use details and observation time between the two pharmacopoeias. It seems more consideration of science and human benefit while increase the number of animal in China. It is necessary to better balance on the legislative, scientific and ethical of animal use in biomedical research in future.

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Microelectrode array (MEA) recordings of neuronal activity as *in vitro* tool to assess acute and developmental neurotoxicity

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Neurotoxicity testing still heavily relies on ethically debated, expensive and time-consuming animal experiments, that are not always predictive for human risk and are unsuitable for safety screening of large numbers of chemicals or environmental monitoring. There is thus a clear need for the development of innovative, predictive and high-throughput *in vitro* testing strategies. To ease acceptation by regulatory authorities, *in vitro* models should represent the *in vivo* situation as closely as possible.

To capture the complexity of the *in vivo* brain and cover a multitude of potential targets, an *in vitro* model forming neuronal networks consisting of a mixed population of multiple types of neurons (excitatory and inhibitory) and supportive cells (e.g., astrocytes) is adequate for neurotoxicity testing. These complex, developing networks have been shown to be highly predictive of (developmental) neurotoxicity when used for functional measurements, such as microelectrode array (MEA) recordings.

We therefore used rat primary cortical cultures grown on MEAs to assess effects of environmental pollutants, such as insecticides, on neuronal activity. Our data show that different insecticides induce distinct effects; organophosphates and carbamates reduce neuronal activity, whereas organochlorines increase neuronal activity. Due to the non-invasive nature of MEA recordings, this technique also allows for assessing effects of chronic and developmental exposure. Although acute exposures generally predict neurotoxicity accurately, developmental exposure often lowers the effective concentration 3- to 10-fold.

To further extend the applicability domain of MEA recordings, we also performed a hazard characterization for a diverse set of recreational drugs. Our data show that amphetamine-like stimulants, like MDMA, PMMA, 4-FA and amphetamine, inhibit neuronal activity with a remarkable comparable potency, whereas the new psychoactive compound methoxetamine is over 100 times more potent. The remarkable potency of methoxetamine prompted further research to test for interspecies differences.

The recent availability of human induced pluripotent stem cell (hiPSC)-derived neurons allowed us to compare the sensitivity of rat primary cultures to hiPSC models. In hiPSC-derived neuronal cultures, the potency of amphetamine is unaltered, whereas methoxetamine is ~20-fold less potent. This interspecies difference clearly highlights the need for human models in *in vitro* (developmental) neurotoxicity testing.

Importantly, care should be taken when implementing such human models, as these are often far less characterized and our recent results demonstrate that changing the ratio of excitatory/inhibitory cells or adding astrocytes can alter the chemical sensitivity of these models. Nevertheless, these models hold great promises as they eliminate the need for interspecies translation and may ease translatability to the human *in vivo* situation.

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Highly relevant human *in vitro* cell models grown under serum-free culture conditions

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Cultivation of human cells *in vitro* is of ever increasing importance in various aspects of biotechnology, biomedicine, as well as basic research. Especially, the need for improved hazard and risk assessment in pharmaceutical, cosmetic and chemical industries, for reduction, replacement and refinement of animal trials ("3R" principle) and for identification of new therapeutic drugs or drug targets requires the use of human standardizable and relevant cell cultures.

Since currently used *in vitro* models are afflicted with significant drawbacks such as limited replicative life span in case of primary somatic cells or loss of the differentiated phenotype in case of tumor-derived cells, we aimed at developing cell cultures characterized by an optimal balance between growth and differentiation. Therefore, we ectopically expressed the catalytic subunit of human telomerase in various human primary cells followed by a detailed characterization of the cellular phenotype. Additionally, cell culture media were developed that allow cell cultivation without the need for fetal calf serum as medium additive.

Data on human telomerized epithelial cells as well as mesenchymal stem cells grown under serum-free cell culture conditions are presented. These cell cultures can be propagated without limitations albeit expressing typical cell type specific markers and functions and thus allow the establishment of standardizable and relevant *in vitro* bioassays.

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EpiIntestinal on a chip: Label-free microphysiometry

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Microphysiometry showed to be a useful tool to monitor the energy metabolism of living cells and its interaction with living cells. In the past the technique was mainly used for monitoring of 2D monolayers of living cells [1]. Recently, our group showed that it is also possible to monitor the extracellular acidification rate (EAR) and transepithelial electrical resistance (TEER) of 3D skin constructs in an automated assay maintaining an air liquid interface (ALI) with the IMOLA-IVD technology.

In this work we present an Intestine-on-a-Chip by monitoring EAR and TEER of the MatTek 3D-small intestinal tissue model (EpiIntestinal) for 12 h. A periodic cycle of 96 min ALI, 10 min TEER measurement and 15 min washing step was used. The test substance (0.2 % sodium dodecyl sulfate) was applied after 8 h of measurement. After application of the tests substance a reduction of the EAR and the change in TEER could be monitored. To be able to monitor the EAR a low buffered basal medium was used. The presented work shows a proof of principle of automated monitoring of EAR and TEER at a 3D intestine model maintaining an automated ALI. The EpiIntestinal model on the IMOLA-IVD chip is a promising research tool for the use in the field of toxicology, cellular metabolism studies or drug absorption research.

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MUI animalFree Research Cluster

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The MUI animalFree Research Cluster' was launched on June 14th, 2018, at the Medical University of Innsbruck (MUI). 2017 the government granted an infrastructure proposal "Austrian Biomimetic Center 3Rs", which aims in developing more sophisticated human models and animal-free research compounds. The project unites the Medical University of Innsbruck as lead university with the Medical University of Graz and the University of Natural Resources and Life Sciences, Vienna (BOKU).

Arising from this initiative, the MUI animalFree Research Cluster was initiated by the lead scientists of ABC3Rs, Prof. Doris Wilflingseder and Prof. Gerhard Gstraunthaler, at the Medical University of Innsbruck. They assembled an interdisciplinary speaker's team consisting of basic and translational researchers, physicians and ethics representatives and with the help of the rectorate of the Medical University of Innsbruck, the cluster was launched this year at the Biocenter Innsbruck by organizing a symposium. The symposium was opened by a keynote lecture held by Prof. Thomas Hartung, who greatly supports the cluster, followed by flashtalks from MUI researchers working on alternatives to animal testing. The MUI animalFree Research cluster shall bundle this expertise in Tyrol and shall be expanded to other universities and federal states. The cluster will contribute to significant progression with respect to replacement and reduction of animal experiments and aims in joining all activities in this area. In the near future, following cluster activities are planned at the MUI:

- Establish 3R seminars with national and international experts.
- Make topics regarding alternatives to animal testing at the MUI more visible.
- Set up an interactive homepage about activities going on in Innsbruck respecting alternatives to animal testing.
- Teach junior scientists about alternatives to animal testing.

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iPSC-derived renal proximal tubular cells: Development and evaluation for their application in nephrotoxicity studies

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The kidney plays a primary role in elimination and metabolism of xenobiotics and is thus susceptible to injury by these compounds. Within the nephron, proximal tubular (PT) cells are among the most frequently affected cell types, hence the development of human PT cell culture models is of great importance towards the goal of replacement of animal models. Here, we describe a method to differentiate human induced pluripotent stem cells (iPSC) into proximal tubule-like cells (PTL) for applications in chemical safety assessment. The advantages of iPSC derived cells include: (1) the ability to account for donor specific genetic background, (2) to have an unlimited source of material (unlike primary cells), (3) the cells are not immortalized or transformed (unlike cell lines), and (4) the unique ability to create different tissues from the same donor.

iPSCs generated within the StemBANCC consortium, were differentiated into PTL using a 2-step protocol. In a first step intermediate mesoderm was induced with small molecules including CHIR99021 and TTNBP, followed by proximal tubule differentiation using additional growth factors. Barrier formation was examined by measuring TEER, expression of tight junction proteins and water transport.

PTL show a typical polarized phenotype and express the tight junction proteins occludin und ZO3. They form a relatively high TEER when cultured on microporous growth supports. In addition, PTL express markers of the proximal tubulus, including megalin and respond to parathyroid hormone by increasing cyclic AMP levels. Initial toxicity experiments have been performed with cisplatin, cyclosporine A, CDDO, paclitaxel and tolcapone.

In summary we developed promising renal cell models from iPSC that are currently further characterized for the usefulness in toxicity studies.

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High-throughput, perfused intestinal tubules for real-time assessment of drug-induced barrier disruption

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In vitro models that better reflect *in vivo* epithelial barrier (patho-) physiology are urgently required to better predict adverse drug effects ahead of clinical trials. Here, we describe extracellular matrix-supported intestinal tubules, continuously-perfused in a high-throughput microfluidic system. These tubules exhibit key tissue polarization markers (ErB1, ErBb2, Ezrin, ZO-1), relevant transporters (Glut-2, MRP2), and crypt-like morphology. Leak-tight polarization of these gut-on-a-chip barrier tubules was achieved after just 4 days in culture, and high-content characterization of the tubules was made possible due to the absence of any artificial membranes in the microfluidics.

Recently, functional compound-induced toxicity was assessed by apical exposure of model compounds to these *in vitro* intestinal barrier tissues. Within each microfluidic system, forty leak-tight intestinal tubules were cultured in parallel, and their response to pharmacological stimuli was recorded over 125 hours of exposure. Overall, this study comprised 357 gut-on-a-chip tubes, of which 93% were confirmed to be leak tight before exposure. Kaplan-Meier plots and EC50-time curves could be extracted from the leakage datasets, providing insight into both concentration and exposure time response.

Full compatibility with standard equipment and user-friendly operation make this gut-on-a-chip platform readily applicable in routine laboratories and high-throughput screening facilities, allowing prediction of intestinal toxicity far ahead of costly animal and clinical trials.

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Microfluidic-embedded 3D networks of iPSC-derived neurons and glia, for highly-predictive mechanistic neurotoxicity screening

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The reliable prediction of neurotoxicity remains a major scientific challenge in drug development, due to the complexity of the central nervous system. Current strategies to evaluate toxicity of drugs and chemicals are predominantly based on *ex vivo* or *in vivo* animal studies, which have shown limited predictability for neurotoxicity in humans and are not amenable to high-throughput testing. Here, we describe the development of a 3D neurotoxicity model based on iPSC-derived neurons cultured within in a high-throughput microfluidic platform, as well as the initial results from a recent screen of neurotoxic compounds. The stratified microfluidic design allows for fine control of the extracellular environment, facilitating highly-reproducible screening on miniaturized CNS models, comprised of neurons, astrocytes, and supporting glia in co-culture.

In recent work, a mixed population of human iPSC-derived neurons consisting of GABAergic and glutamatergic neurons, with supporting astrocytes, was cultured in 3D to closely represent the physiology of the human brain. As a part of the validation, proper network formation was observed by neuron-specific immunostainings (β3-tubulin, vGLUT, vGAT), and neuronal electrophysiology was analyzed by a calcium sensitive dye, indicating spontaneous neuronal firing. Notably, these 3D CNS mimics showed significantly less non-physiological synchronization of electrophysiological firing compared to 2D systems, increasing the sensitivity for predicting the seizurogenic potential of test compounds. Additionally, dose-dependent neurotoxic effects of methylmercury and endosulfan were measured using multiplexed mechanistic viability assays, including standard live/dead staining, mitochondrial depolarization assays, and phenotypic quantification of inhibited neurite outgrowth.

The 3D iPSC-derived CNS mimics described can be applied to refine animal experiments, and hold the potential to better predict adverse effect in humans and hence improve clinical development success.

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179

3D NephroScreen: High throughput drug-induced nephrotoxicity screening on a proximal tubule-on-a-chip model

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Renal toxicity remains a major issue in clinical trials, and stresses the need for more predictive models fit for implementation in early drug development [1]. Here, we describe a perfused, leak-tight renal proximal tubule cell (RPTEC) model cultured within a high throughput microfluidic platform (Mimetas' OrganoPlate®) [2], along with recent results from a 12-compound nephrotoxicity screen performed within the "NephroTube" CRACK IT consortium in collaboration with sponsors and the NC3Rs.

Human RPTEC (SA7K clone, Sigma) were grown against a collagen I ECM in a 3-channel OrganoPlate®, yielding access to both the apical and basal side. Drug-induced toxicity was assessed by exposing kidney tubules to 4 benchmark and 8 blinded compounds with known clinical effects supplied by the sponsors for 24 and 48 h. The tightness of the barrier was evaluated by diffusion of a dextran dye from apical to basal compartment. Parallel to this, cell viability with a WST-8 assay and the presence of LDH in the supernatant were assessed. Finally, kidney tubules were lysed, and RNA was extracted for gene expression analysis of acute kidney injury markers.

Upon perfusion flow, RPTEC form leak-tight confluent tubular structures against the collagen I ECM in the OrganoPlate®. The NephroScreen revealed significant decreased barrier tightness and cell viability in 7 out of 12 compounds. Furthermore, the release of LDH was significantly increased in 9 out of 12 compounds. An increased expression in HMOX1, TNFα and NGAL was observed in 9, 5 and 7 out of 12 compounds, respectively whereas claudin-2 showed a decrease in 6 out the 12. Overall, more effects were observed after 48 h in comparison to 24 h exposure.

The kidney-on-a-chip model in the OrganoPlate® provides a promising *in vitro* renal toxicity tool to answer the desire to provide a better alternative to animal studies in terms of throughput, costs and predictivity and ultimately will be commercialised after further validation.

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176

BBB on-a-chip: A 3D *in vitro* model of the human blood brain barrier (BBB)

Karlijn Wilschut
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The BBB ensures a homeostatic environment for the brain and is made up of specialized endothelial cells and supporting astrocytes and pericytes. The BBB protects the brain from harmful substances. It also prevents large lipophilic compounds, including most therapeutic drugs, from entering the brain. This makes it difficult to treat brain diseases1.

While recent developments in microfluidic engineering have resulted in promising *in vitro* models of the BBB, the throughput and ease of use of these systems is low. This makes these models not suited for regular academic research and drug development. Here we present a novel BBB model using the 3-lane OrganoPlate®. This platform is based on a 384-well microtiter plate and allows for parallel culture of 40 perfused miniaturized tissues, making it fully compatible with standard lab procedures and equipment.

The BBB-on-a-chip model comprises a perfused 3D microvessel of human brain microvascular endothelial cells. Perfusion through the lumen of the vessel is induced without pumps and can be controlled to model mechanical cues. In addition, the microvessel is supported by human astrocytes and pericytes that interact and support the endothelial vessel. The phenotype of the BBB-on-a-chip was characterized using immunofluorescent staining and showed presence of junctional markers VE-cadherin, PECAM-1, Claudin-5, and ZO-1. In addition, we have confirmed barrier function and adopted transporter assays to show functionality of two major BBB transporters, Pgp and GLUT1.

In conclusion, we present a novel human BBB model in an easy to use microfluidic platform. This model can be used for fundamental BBB research, drug development or studying neurological disorders.

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Predicting human lung toxicity induced by aerosols released from thermal utilization of polymer nanocomposites using human alveolar epithelial A549 cells and the human 3D airway model MucilAir™

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Objectives

End-of-life disposal of nanocomposite products has led to increased environmental, health and safety concerns, due to the potential release of nanoparticles during the disposal process. The aim of this study was to investigate the toxicological relevance of dusts and aerosols released during thermal utilization of nanocomposites (NCs) to the human respiratory tract using human alveolar epithelial A549 cells and the human 3D airway model MucilAir™.

Material and methods

NCs were combusted in a small lab furnace. The released aerosols were guided directly into a VITROCELL® Automated Exposure Station for toxicological analysis. With this system, it was possible to assess native emissions from combustion of nanocomposites online using cell-based test systems under air-liquid interface (ALI) conditions. Human alveolar epithelial A549 cells, a cell line representative of the alveolar respiratory region, and a human 3D airway epithelium model (MucilAir™) mimicking the upper airway epithelium, were used. The test systems were exposed both to native aerosols and the particle-depleted (filtered) gas phase (VOCs). Investigated toxicological endpoints were cytotoxicity, determined by the lactate dehydrogenase (LDH) assay, and inflammation, measured by release of the pro-inflammatory cytokine IL-8. The investigated thermoplastic nanocomposites consisted of a polyethylene matrix (PE) filled with 10% (w/w) nano-CuO, 10% (w/w) nano-TiO_2 or carbon nanotubes (CNTs).

Results

The results show that exposure to native combustion products from thermoplastics lead to a significant induction of toxicity in A549 cells but not in MucilAir™, independently of the presence of nanofillers. We assume that the observed cytotoxicity in human lung A549 cells, or loss of membrane integrity, is induced primarily by the volatile emission components rather than by the particulate matter emitted. Moreover the data show that A549 cells are more sensitive to combustion products from thermoplastics than MucilAir™. Although emissions resulting from combustion of pure nanofillers (nano-CuO and CNTs) do not induce cytotoxicity in A549 cells, they do provoke a significant release of IL-8. Moreover, the combustion of nano-TiO_2 and PE leads to an increase of IL-8 in MucilAir™.

Conclusion

These findings are important in understanding the hazard potential of the aerosols formed during thermal utilization and decomposition of thermoplastic nanocomposites. Furthermore, the used human alveolar epithelial A549 cells and the human 3D airway model MucilAir™ provide a robust test system to determine inhalation toxicology endpoints and it is useful for risk assessment of aerosol toxicity. In terms of relevance MucilAir™ can be classified between classic submerged *in vitro* models and animal-based models.

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Detection of antibodies in animal serum samples using by surface plasmon resonance biosensor

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The main obstacle to the well-being of the livestock industry is viral etiology diseases. At the present stage, there are many methods for detecting pathogens of viral infectious diseases, which are recognized as a standard of the laboratory diagnosis. The main method of lifetime diagnosis today is the serology, that is, the detection of antibodies in blood serum of animals [1]. The most common method is ELISA, in some cases, the reaction of immunodiffusion in agar gel (RID) [2]. However, these methods have several limitations, for instance, a high cost of kits and low sensitivity, long-term expectation of the result, and inability to use for a field diagnosis. An alternative method of detecting antigens of viral diseases can be an optical method, based on the surface plasmon resonance (SPR). Well-known the analytical SPR devices have high sensitivity to small concentrations of investigating agent in liquid and gaseous substances [3,4].

The purpose of this work was to investigate a possibility of detecting antibodies to viral infectious diseases of domestic animals using of SPR-method. To do this, the receptor-analyte method was used, that is the selective covalent binding of the analyte (antibodies) to a complementary virus (receptor antigen) which is immobilized on the surface of a sensing element of the biosensor. The three types of blood serum were tested in a buffer solution (saline, pH 7.24): semi-positive (10% in saline), diluted semi-positive (1% in saline) and negative. The research was carried out on the "Plasmon" biosensor developed by the V.Ye. Lashkaryov Institute of Semiconductor Physics, NAS of Ukraine. This device allows us to measure with high accuracy the kinetics of physical and biochemical processes on the surface of its sensing element by changing the angular position of the minimum of reflection coefficient. All processes of interaction were monitored in real-time by measurements of kinetics. First, the receptor surface was formed by immobilizing the antigen on the sensitive biosensor element. For this purpose, the antigen was pumped through the measuring cuvette of the device for 12 minutes. Then the cuvette was washed with a buffer solution (saline). After washing, a subsequent replacement of serum and buffer was carried out.

In the results, diluted serum with antibody concentrations of 1% and 10% interact with the antigen, as evidenced by the corresponding sensor responses. In this case, the duration of the analysis is 70 times less than the duration of the analysis by the method of RID.

The possibility of detecting antibodies in blood serum of domestic animals using by SPR-method is shown, which allows to make an express analysis (less than an hour) and has a higher sensitivity, compared with standard methods. Moreover, SPR allows to determine with the high reliability the concentration of antibodies lower than detection minimum of ELISA and RID, which makes it possible to confirming a diagnosis in laboratory at the early stages of the disease.

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140

Optimizing animal-free culture of primary human respiratory cells for live cell analyses

Viktoria Zaderer

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To study respiratory challenges with upper and lower respiratory tract cells in an animal-free *in vitro* environment over time and for repeated exposure, we set up an innovative up-side-down three-dimensional (3D) epithelial cell culture system.

This approach of cell seeding offers advantages regarding differentiation analyses of the cultured epithelium. Usually when cells seeded on Transwells are prepared for live cell imaging Transwell membranes have to be cut out and turned upside down due to the high density of the porous membrane. This makes it impossible to visualize cells grown normally on Transwell membranes – thus for every live cell image one membrane has to be "sacrificed". To monitor the progress of differentiation over time by using the same well, we established the up side down 3D model. For this, the animal-free GrowDex™ cellulose hydrogel was coated on the basal side of the Transwell membrane before seeding normal human bronchial or small airway epithelial (NHBE, SAE) cells. Therefore, the apical airside of the epithelium containing ciliated cells and mucus grows in the lower chamber of the Transwell. This inverted growth allows to simply transfer the Transwells into glass bottom dishes for live cell imaging without any damage to the membrane. By this method we could illustrate using the same well for live cell imaging that the animal-free GrowDex™ cellulose hydrogel exerted several advantages compared to the commonly used rat-tail collagen, such as improved growth and faster differentiation of the epithelium. Not only a faster proliferation of the normal human bronchial epithelial cells was observed but also the fully differentiated ciliated and mucus-producing cells were earlier detected.

In summary here we provide a novel innovative tool to study respiratory challenges in repeated dose experiments on the same 3D layer of cells within a completely animal-free setting.

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Systems toxicology assessment of a 72-hour repeated exposure to Swedish snus extract and total particulate matter from 3R4F cigarette smoke on gingival organotypic cultures

Filippo Zanetti, Alain Sewer, Bjorn Titz, Anita R. Iskandar, Athanasios Kondylis, Laura Ortega Torres, Claudius Pak, Florian Martin, Emmanuel Guedj, Ashraf Elamin, Keyur Trivedi, Stefan Frentzel, Nikolai V. Ivanov, Julia Hoeng and Manuel C. Peitsch

Philip Morris Products S.A., Neuchâtel, Switzerland

The "tobacco harm reduction" strategy framework is aimed at decreasing smoking and smoking-related population harm without the complete elimination of tobacco and nicotine use. Swedish snus is a smokeless tobacco product that contains reduced levels of harmful compounds due to its manufacturing procedures and therefore could be a less harmful alternative to smoking. In Sweden, where snus use exceeds smoking among men, relatively low rates of major smoking-related diseases were recorded. Identifying the mechanisms by which Swedish snus can reduce the impact of smoking use on biological systems is of great importance to understand the molecular basis of the "tobacco harm reduction" paradigm.

In recent years, we have used human oral organotypic cultures successfully as *in vitro* models to examine the biological responses to tobacco product exposure. The present study was aimed at determining the biological impact of a repeated 72-hour exposure of human gingival epithelial organotypic cultures to extracts from a commercial snus and a reference snus (CRP1.1, Coresta) in comparison with the total particulate matter (TPM) from conventional 3R4F cigarette smoke. The snus extracts and 3R4F TPM were diluted and tested at comparable nicotine concentrations. A higher concentration of the snus extracts, reflecting nicotine levels observed in the saliva of snus users, was also tested. To assess the exposure effects, we employed a systems toxicology approach that complements classic toxicological assays with transcriptomics analysis. While 3R4F TPM treatment induced substantial morphological and inflammatory alterations, cultures treated with snus extract, even at a nicotine concentration 25 times higher than that of 3R4F TPM, induced lower biological changes. Network enrichment analysis of the mRNA profiles indicated a greater impact on cellular fate, cellular proliferation, cellular stress, and inflammatory processes following treatment with 3R4F TPM than following snus extract treatment – which was particular pertinent for genes involved in xenobiotic metabolism. Integrative analysis of the global mRNA and miRNA expression profiles suggested a regulatory role of miRNAs in smoking-relevant biological processes following TPM treatment but a reduced effect following snus exposure. These results showed that the Swedish snus extract has a reduced biological impact on oral organotypic cultures compared with that of TPM from cigarette smoke.

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Monitoring neurotransmitter release from human Midbrain Organoids (hMOs) supernatant using an electrochemical redox cycling microsensor

Cristian Zanetti [1], Julie Rosser [1], Jens Schwamborn [2] and Peter Ertl [1]

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Parkinson Disease (PD) is the second most common neurodegenerative disease; its prevalence is predicted to increase due to the human population growing older. To date, PD is incurable and its origins are still unclear; for this reason, advanced technologies are needed in order to progress in PD research [1].

Dopamine (DA) is a neurotransmitter released by dopaminergic neurons in the midbrain and, among other functions, ensures controlled muscular responses. PD causes a deficiency in DA levels in the striatum, this is correlated to the typical motor dysfunctions of PD. Consequently, information on DA levels in the midbrain is an important parameter in understanding neurodegenerative processes that take place in PD [2].

DA is electroactive and for this reason it can be readily detected using microelectrodes. Electrooxidation of DA is a reversible process, allowing its sensitive detection using redox cycling. However, DA electrochemical detection poses a range of obstacles including interferences, sensitivity loss, and electrode fouling. Despite these problems, electrochemical detection is a cost-effective, straightforward, and implementable way to detect dopamine in *in vitro* neural cell culture systems.

We are using interdigitated microelectrodes to monitor the electrochemical signal of supernatants of hMOs differentiated from human neuroepithelial stem cells [3]. The electrochemical assay will be later complemented by an HPLC analysis to quantify the release of DA and other biomolecules associated to PD, such as serotonin [4], throughout the duration of the culture.

The final goals of this work are: (i) the development of an advanced *in vitro* disease model of PD using organ-on-a-chip technologies with microelectrodes for on-chip monitoring of DA levels, and (ii) implementation of HPLC to analyze supernatants collected from hMOs; permitting the profiling of several biomolecules associated to PD. This would help gain a deeper insight into the role of neurotransmitter release, including DA, during the onset and progression of PD.

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Implementing the 3Rs into ecological research

Miriam A. Zemanova
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Conservation of ecosystems and species gives rise to a paradox in which effective measures often involve very adverse (even lethal) interactions with several animal individuals on behalf of the good for the whole population. Marking and sampling practices include taking blood samples, toe-clipping of amphibians and reptiles, or using implants and subcutaneous dyes. At the moment, techniques that might be painful and induce suffering but do not affect the animal's survival are still deemed acceptable.

The 3Rs principles (replacement, reduction, refinement) can provide scientists with guidelines on the ethical use of animals in research and education. However, the process of incorporating the 3Rs into ecological research has been very slow: while non-invasive techniques are available, their implementation is often lagging behind.

In order to increase awareness of this issue present an overview of the most common invasive practices in ecological research will be presented. I will also provide suggestions for the available non-invasive alternatives that are less likely to negatively affect the animal's welfare, while not compromising the quality of research data.

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Human intestinal organoids as model for studying drug transport

Tamara Zietek [1,2], Eva Rath [3], Michael Weinmüller [4], Maren Ewers [5], Florian Reichart [4], Güralp Ceyhan [6], Ihsan Demir [6], Horst Kessler [4], Hannelore Daniel [2]

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Research on intestinal transport processes is of high interest for studies on nutrient absorption as well as for drug uptake studies. We previously showed that 3-dimensional intestinal organoids are a useful tool for assessing intestinal nutrient transport *in vitro* and that compounds up to 4 kDa in size are able to enter the organoid lumen. Now we could demonstrate that 3D human intestinal organoids are a valuable *in vitro* model for the screening of pharmacological compounds.

3D organoids were used as a screening platform for studying absorption of the antibiotic cefadroxil and bioavailability of novel peptide drugs and prodrugs. These novel compounds are promising candidates for cancer therapy and tumor characterization targeting specific integrin subtypes on tumor cells. We could demonstrate that some of these drugs are transported by the peptide transporter PEPT1. We tested absorption of the drugs in human intestinal organoid cultures derived from healthy human subjects. Transport studies targeting different intestinal transporters were conducted in organoid cultures derived from different parts of the human small intestine. Human organoids turn out to be a highly promising *in vitro* model for drug bioavailability screenings and a much better approach compared to animal experiments.

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Severity classification: Curse or blessing?

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Classifying the severity of procedures and phenotypes of GA lines is a core component of Directive 2010/63/EU and nowadays a prerequisite for project approval of animal experiments across Europe.

Defining severity categories is beneficial for raising awareness for the impact of procedures on the wellbeing of animals and emphasize the need for refining especially moderate and severe procedures. Accordingly, severity classification is a matter of animal welfare! Moreover, communicating the impact of research techniques on animals allows to inform the public transparently and to draw a realistic picture of animal research.

Consistency in severity classification is a key requirement for an adequate harm-benefit-analysis and a sufficient recognition of pain, suffering and distress. For severe procedures, a retrospective assessment is obligatory and in case of severe pain, suffering or distress that cannot be ameliorated or is long-lasting, project approval may be revoked by the European Commission. Furthermore, the classification of harmful versus non-harmful phenotypes determines whether the breeding of a line is subject to approval. These EU-wide requirements illustrate the importance of an appropriate and consistent assessment by scientists, animal welfare bodies and authorities.

While experience acquired during the past years has shown that there is still a considerable lack of consistency in severity classification, several efforts are ongoing to overcome this issue. Example-driven guidelines have been published by Expert Working Groups to give a better idea on the approach and support the harmonization of classification of severity.

Currently challenges remain in the harmonization process. Relating to phenotypes of genetically altered animals, severity classification is new and rules of classifying common procedures cannot simply be transferred. In example when defining the threshold of a harmful phenotype. Which stage of obesity is considered as harmful? Shall phenotypes be classified as harmful even though they are not clinically detectable?

The presentation will give an overview on severity classification and existing guidelines while focusing on genetically altered rodents. The audience will conduct interactive severity classifications on the basis of which current challenges and possible solutions will be discussed.

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Human cell-based tumor microenvironment models for improved preclinical drug development

Christian Zoschke [1], Leonie Gronbach [1], Christopher Wolff [1], Konrad Klinghammer [2], Ulrich Keilholz [3] and Monika Schäfer-Korting [1]

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Determining the optimal dose for anti-cancer treatments remains a key challenge in drug development. However, preclinical testing frequently fails to correctly predict an effective and safe dose for clinical applications (Matthijssen et al., 2014). In oncology, only 5% of investigational new anti-cancer drugs obtain marketing authorization, although they passed preclinical testing. While the molecular understanding of tumor heterogenicity improved considerably, patient-derived xenograft models often loose human stroma (Schütte et al., 2017). Thus, these models might underestimate tumor-stroma interactions as well as the drug effects on tumor stroma and normal tissues. Herein, we investigated the cancer-related changes following the co-culture of human squamous cell carcinoma cells with reconstructed human skin and oral mucosa, respectively.

Human skin cancer was reconstructed from normal human keratinocytes and fibroblasts and SCC-12 cells (Rheinwald et al., 1981). Stratum corneum lipids and tight junctions were investigated. The penetration of Nile red or indocarbocyanine-tagged nanocarriers (Radowski et al., 2007) was studied by fluorescence microscopy. Radiolabelled caffeine was used for barrier function assessment. Human oral cancer was reconstructed from normal human keratinocytes and fibroblasts and SCC-25 cells (Rheinwald et al., 1981) or patient-derived cancer cells (Klinghammer et al., 2015). Normal models were built for reference.

Models with SCC-12 cancer cells showed disturbed epidermal differentiation and increased skin surface pH values as well as altered stratum corneum lipids and tight junction protein expression (Zoschke et al., 2016). These alterations enhanced caffeine permeation as well as the penetration of Nile red and the 70 kDa nanocarrier (Alnasif et al., 2014).

Oral mucosa models with patient-derived cancer cells reliably preserved the tumor grading. The G2 tumor models comprise heterogeneous cancer, growing more invasive than the G1 or the SCC-25 tumor. Nile red penetration into the SCC-25 tumor model exceeded the penetration into the G1 cancer and into the normal mucosa model. In conclusion, patient-derived allograft cancer models may bridge the pharmacokinetic gulf between animal-based models and clinical trials.

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