LINZ 2015 – EUSAAT 2015

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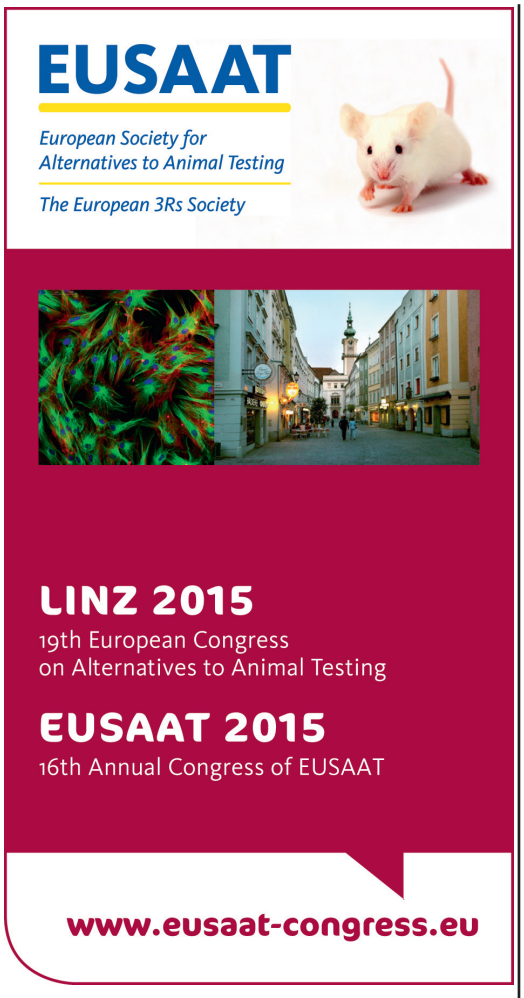

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Welcome address

Dear friends and colleagues,

On behalf of EUSAAT, the European Society for Alternatives to Animal Testing, I welcome you to the "EUSAAT 2015 - Linz 2015" congress, which is actually the "16th Annual Congress of EUSAAT" and the "19th European Congress on Alternatives to Animal Testing" on 20-23 of September 2015 in Linz.

During the past two decades the "Linz-Congress" has emerged in Europe as one of the major scientific events in the field of the 3Rs. EUSAAT 2015 is hosting presentations, discussions and exchange of new ideas for the benefit of alternative methods to animal experiments. The Scientific Committee has identified the most important subjects related to the 3Rs, which are of interest to scientists in Europe.

The EUSAAT 2015 conference is focusing in oral and poster sessions on animal-free disease models, non-animal tools for basic biomedical research, -omics-techniques and advanced 3D models including recent progress on developing a "human-on-a-chip". Thus EUSAAT 2015 is providing a forum for discussing the new EU Directive 2010/63/EU on the protection of animals used for scientific purposes, which is currently being implemented in EU member states. Colleagues from the EU Commission, from member states and from the animal welfare movement will discuss the high expectations that the public at large and the animal welfare movement have for improving the current situation on behalf of the experimental animals.

This year we are happy that two satellite meetings will be held right after the EUSAAT 2015 congress on 23-24 September 2015. Taking into account the successful first practical training course at the last EUSAAT congress in 2013 we will hold the second "EUSAAT 2015 Practical Training Course on Alternative Methods" focusing on "Assessment of *in vitro* eye irritation potential – focusing on methods accepted for the regulatory purposes". We are grateful to all speakers for contributing to the training course and in particular Helena Kandarova for organizing the training EUSAAT course and also for a financial contribution via MatTek IVLSL.

In addition we do appreciate that the German Foundation SET (Stiftung zum Ersatz von Tierversuchen, engl. "*Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing*") will hold the satellite SET Workshop "Translational Aspects of *in vitro* and *in vivo* Models for Inflammatory Diseases" on 23-24 September 2015. We want to thank the SET foundation for sponsoring the workshop and in particular for covering the travel expenses of invited experts, several of whom have agreed to give lectures at the EUSAAT 2015 congress in sessions on "disease models". We are particularly indebted to Manfred Liebsch, chair of the Scientific Advisory Board of the SET Foundation, for organising the workshop and for inviting speakers to our congress.

Taking into account the successful program for "Travel grants for students and young scientists" of WC9, the 9th World Congress on Alternatives, which was held last year in Prague, EUSAAT 2015 has this for the first time offered a Young Scientists Travel Award (YSTA) program. Therefore we will for the first time at an EUSAAT conference hold two sessions with "Young Scientists Short Lectures" and also present YSTA Lecture Awards. We want to express our thanks to the organizers of the YSTA program Helena Kandarova, Lucia Lee and Manfred Liebsch, who also organized the students and young scientists program at WC9 in Prague last year. The YSTA program has been quite attractive for young scientists and due to the generous funding by the SET Foundation, MatTek IVLSL and BASF we will be able to offer around 30 travel awards.

The EUSAAT Board is quite happy that the number of sponsors of the EUSAAT congresses has increased over the years, since without their continuous support we would not be able to maintain the high scientific standard and to keep the congress fee low. Both elements are equally important in order to attract young scientists from all over Europe and beyond. Therefore, the EUSAAT Board and Scientific Committee want to thank all of the sponsors of EUSAAT 2015 on behalf of the participants.

I finally want to thank my colleagues on the EUSAAT Board and in the Scientific Committee for their continuous support in planning EUSAAT 2015.

Horst Spielmann
President EUSAAT

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CON4EI: consortium for *in vitro* Eye Irritation testing strategy

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Measurement of ocular irritancy is a necessary step in the safety evaluation of both industrial and consumer products. Assessment of the acute eye irritation potential is therefore part of the international regulatory requirements for testing of chemicals. All *in vitro* assays have specific strengths and limitations whether this relates to ranges of irritancy, types of chemical classes or physical nature of the materials. Therefore, combinations of *in vitro* assays are needed for hazard identification and complex safety assessment. Today, these combinations of assays are used by individual companies as an integral part of their safety assessments, but often with limited scientific knowledge covering their own specific chemical properties/portfolio needs. This can contribute to a lack of consistency among *in vitro* test results within a battery approach or a conflict with available *in vivo* data. Despite efforts to compare *in vitro* methods with *in vivo* data, the problem is that most of the so far proposed testing strategies for assessment of eye irritation were not evaluated with the same adequately large set of chemicals. This is consequently resulting in a lack of information on predictive capacity of the *in vitro* assays in a testing strategy. All available information confirms that the strategy is as strong as the weakest part of it. The main objective of this project (CEFIC LRI-AIMT6-VITO CON4EI) is to develop tiered testing strategies for eye irritation assessment for all eye irritation drivers of classifications. In this project the irritancy potency of a set of 80 chemicals will be identify based on existing *in vivo* data. Following eight test methods will be included in this project: BCOP (Bovine Corneal Opacity and Permeability), ICE (Isolated Chicken Eye) and STE (Short Term Exposure), all of them can distinguish Category 1 versus No Category. EpiOcular EIT (EpiOcular Eye Irritation Test) and SkinEthic™ HCE (Human Corneal Epithelial) can distinguish between Not classified versus classified (Category 1/ Category 2). To distinguish between Category 1 and Category 2, the following methods are relevant to include: histopathology in association with the BCOP and ICE, EpiOcular ET-50 (EpiOcular time-to-toxicity test), SMI (Slug Mucosal Irritation), and BCOP-LLBO (BCOP-laser light-based opacitometer). This project will assess the reliability of these *in vitro* test methods, define applicability domains in terms of "drivers of classification", strengths and limitations of each method. In this way, we will be able to identify methods that will fit in a tiered approach to distinguish UN GHS classified Category 1 chemicals versus No Category chemicals and address the highest industrial gaps namely distinguish between Category 2 versus Category 1 chemicals. This research is funded by CEFIC-LRI & we kindly acknowledge Cosmetics Europe for their contribution in chemical selection.

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Reconstructed Human Epidermis (RhE) monitoring via the IMOLA-IVD

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The gross disconnect between preclinical toxicity screening and clinical testing causes a low success rate for new drug candidates. Substantial numbers of redundant samples are prepared in order to analyze cellular reactions over an extended time course. Furthermore, protocols that investigate organ toxicity using animal testing are inaccurate at predicting *in vivo* toxicity and present an ethical dilemma in regards to animal welfare. The advent of more accurate 3D *in vitro* cultures like organs-on-a-chip (OOCs) has improved our ability to probe the potential effects of drug candidates *in vivo*. However, standard colorimetric assays, which are labor intensive and may affect the underlying cellular activity, are still used to assay new cultures. Cellular microphysiometry systems like the IMOLA-IVD (cellasys GmbH), a microsensor array-based assaying technique, offer a solution to these issues with the ability to noninvasively monitor biological changes in real-time. To date, the IMOLA-IVD's has been used to monitor morphology, oxygen consumption and extracellular acidification rate from a diverse array of standard cellular cultures ranging from planar cultures to biopsied tissue [1]. When combined with more complex *in vitro* cultures, these "biochips" can be converted into rapid, high-content, cell-based assays that are easily automated. In the presented work, a candidate 3D *in vitro* culture, the reconstructed human epidermis (RhE) artificial skin, was integrated with a modified IMOLA-IVD biochip for online monitoring of extracellular acidification. RhE, grown on standard polycarbonate membrane culture inserts, mimic the structure and function of standard human epidermis. A few have already been used in Organization for Economic Cooperation and Development (OECD) validated in *in vitro* skin irritation and toxicity assays. Monitoring the metabolic output of an RhE in real-time can reveal time-resolved data on the toxic effect new compounds have on the epidermis. Automatic screening can also provide a new pathway that can revolutionize the process of testing new chemicals for skin irritancy. For this reason, we developed the protocol for an automated skin corrosion/irritation assay. IMOLA Biochip-D's were modified to perfuse cell culture inserts with medium while maintaining an air-liquid interface. Metabolic signals were recorded in real-time in an incubator via the IMOLA-IVD system. RhE from CellSystems GmbH (epiCS) cultured on culture inserts were perfused automatically with medium via a peristaltic pump, IMOLA fluidic modules and the DALiA control software. Future work will involve developing this method into irritancy and corrosivity assays that follow from OECD testing guideline 431 (corrosion) and 439 (irritation), by exposing the epiCS to potentially toxic agents.

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Functional neurons from cancer cells: a novel *in vitro* model exhibits appropriate functional parameters

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Alternative animal-based *in vitro* approaches may speed up the process of drug and chemical neurotoxicity evaluation for regulatory purposes, while minimizing the animal usage. However, they have their own limitations due to the scarce predictive values in humans. Our recent studies demonstrated that, when overexpressed in human neuroblastoma cancer cells, NDM29 ncRNA is able to drive a differentiation process toward a neural phenotype (Castelnuovo et al., 2010). Here, we present a cell clone engineered to further overexpress NDM29 at its highest level so approaching the last stage of differentiation toward a functional human neuron. We used a combination of population and single-cell techniques to perform a detailed biochemical, electrophysiological, and pharmacological phenotyping of neurons derived from Neuroblastoma cells. Differentiated cells express canonical neuronal markers such as Nf68, NeuN and secreted BDNF. Electrophysiological recordings reveal the expression of a pool of ion channels resembling those of neural cells as well as MEA technology shows spontaneous spiking and, in some cases, bursting behavior. Further experiments are in progress in order to validate the suitability of this model for a human relevant screening system.

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Different concepts of Committees in EU Member States, to support the competent authorities performing the project evaluation according to Directive 2010/63/EU

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According to Directive 2010/63/EU the evaluation of projects using live animals has to be performed by the respective competent authority. A comprehensive project evaluation (PE, Directive Article 38), which forms the core of the project authorisation taking into account ethical considerations in the use of animals (Directive Recital 38) has to be performed with a degree of detail appropriate for the type of project. Within the PE the authority has to verify that the project meets certain criteria, amongst others if the project is justified from a scientific or educational point of view or required by law, if the purposes of the project justify the use of animals and if the project is designed so as to be carried out in the most humane manner possible. In particular the PE shall consist in an evaluation of the objectives, the predicted scientific benefits or educational value, an assessment of the compliance with the 3Rs, an assessment and assignment of the severity of the procedures, and a harm-benefit analysis of the project (HBA), to assess whether the harm to the animals in terms of suffering, pain and distress is justified by the expected outcome taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment. There has to be an assessment of certain justifications of the applicant as well as a determination as to whether and when the project should be assessed retrospectively. According to Article 38 (3) the competent authority shall consider expertise in particular in the following areas: scientific use for which animals will be used including the 3Rs experimental design, including statistics where appropriate veterinary practice in laboratory animal science or wildlife veterinary practice where appropriate animal husbandry and care. The PE has to be transparent, it shall be performed in an impartial manner and may integrate the opinion of independent parties (Article 38 (4)). At this point EU member states have developed different systems to support the authorities: some seek for the advise of experts if appropriate and necessary, others integrate advisory committees (so called "animal ethics committees", AEC's) in the process of project evaluation. In this presentation different concepts of committees will be presented and discussed.

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Modeling of early intestinal infection events of enterotoxigenic *Escherichia coli* using an *in vitro* system with porcine jejunal tissue

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Enterotoxigenic *Escherichia coli* (ETEC) strains are involved in piglet post-weaning diarrhea, a current problem in piglet rearing. Many prophylactic measures such as vaccines or feed additives such as probiotics have been tested in infection experiments with piglets [1-3]. In the present study, we tested if effects of ETEC can also be evoked when the strain is added *in vitro* to whole mucosal tissues which might represent a more complex model system compared to cell cultures. Furthermore it was examined if this response could be modulated by prior (*in vivo*) supplementation of the piglets with probiotics.

Material and methods: Jejunal epithelial tissues from piglets before weaning from a probiotic-supplemented (*Enterococcus faecium* NCIMB 10415) and a control group were taken and mounted into conventional Ussing chambers. The ETEC strain O149:K91:K88 was added at a concentration of 108 CFU/ml at the mucosal side of the tissues and barrier and immunological functions were examined by measuring electrophysiological parameters (transepithelial electrical resistance (Rt), potential difference and short circuit current (Isc)), as well as gene and protein expression of selected target genes by quantitative PCR and Western blots. Variance analyses and t-tests were performed for statistical evaluation of the data.

Results: The Rt initially increased in the first two hours after ETEC addition, and decreased again later. The rise in Rt coincided with reduced fluorescein fluxes as a marker of paracellular permeability in the ETEC-incubated epithelia. At the end of the experiment mRNA expression of proinflammatory cytokines and of components of the inflammasome as a potential induction pathway were elevated. Expression of the tight junction protein claudin-4 was decreased in mucosal tissues treated with the pathogenic *E. coli*. Coherent effects of the probiotic prefeeding of the pigs could not be observed.

Conclusion: The *in vitro* system can be applied to study the early events of ETEC infection. Addition of ETEC affected barrier function and components of the gut immune system. Further studies are needed to elucidate the response under different incubation conditions.

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Accomplishments and next steps for the Roundtable on Science and Welfare in Laboratory Animal Use

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The Institute for Laboratory Animal Research of the U.S. National Academy of Sciences recently established the Roundtable on Science and Welfare in Laboratory Animal Use. The vision of the Roundtable is that its activities will lead to more science-informed approaches that will improve animal care and use and further the development and implementation of techniques that refine, reduce or replace the use of laboratory animals when ethically warranted, scientifically valid and regulatory compliant. To date, the Roundtable (RT) has hosted a number of workshops on such diverse topics as reproducibility issues with research using animals transportation of laboratory animals and creation, implementation and sharing of performance standards. The RT members plan the next activities taking into account opportunities for education and advancement as well as the expansion of the RT's outreach in the global community. This poster will present the mission and vision of the Roundtable, describe past and future activities and discuss how these promote and advance the Three Rs both at the local (US) and international level.

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Intelligent inserts as scaffolds for functional cell co-culture models on a biomimetic scale

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As the first step in determining the fate of a new formulation in the human body is to observe how it crosses biological barriers (BBs), predictive, *in vitro* BB models are required. Inserts are basic supports for the development of BB models. They are responsible for all the information collected to date *in vitro* and are specific to BB models which need a mechanical, non-vascularized structure around which to organize, with a directional architecture that is functional to the definition of the inside, and outside, of the body. Fascinating technologies revolutionizing the *in vitro* perspective as "organ-on-a-chip" are expected to become reliable tools starting in 2020, being disruptive technologies with "high added value" and "high entry barrier" to both their use and to the evaluation of their information yield. Today's inserts are permeable, microporous polymer membranes with average pore densities of 5-15%, pore size variation of 0.4-8 μm, a fixed thickness of 10 μm, and randomly distributed pores. In the ergonomic, SiMPLI – silicon nitride microporous permeable inserts – the current polymer membrane is replaced by an ultrafine ceramic membrane array issuing from state-of-the-art microfabrication, making possible never-before-available design control and robustness features. SiMPLI is ergonomic when it fits standard multi-well plates exactly as for current inserts, but by providing "transparent" mechanical support for *in vitro* BB modeling. Also, handling and protocols are very close to those currently used, allowing the introduction of an innovative, "low barrier entry" product consistent with existing data collection and analysis paradigms. By reducing the mechanical support's thickness, we introduced a biomimetic membrane – a microfabricated basal membrane – which, beyond its thinness, is able to:

– define the contact surface between cell layers and enable cross-talk
– ensure mechanical support to the tight, impermeable layers of cells
– allow the passage of nutrients
– not interfere with the active (biological system energy triggered) crossing mechanism of relatively large molecules, such as particulate matter generated from combustion, or nanoparticles (smart drug carriers or nanomaterials in general).

The latter would possibly be actively blocked by the BB function. This improves the model's relevance to the point of making available information that until now was available only through animal testing. SiMPLI cell culture insert is 20 times thinner than comemrcial inserts. The membranes designed to accommodate cell growth are 500 nm thick, ceramic, and have evenly distributed pores, their size and density matching current requirements. They are transparent both in the optical sense for better viewing, and in the sense of being non-invasive regarding the functioning of the BB. Being ultrathin, SiMPLI changes the game in terms of transport, and of model relevance. The simple insert suits possible vertical integration such as providing ready-to-use tissues (cell-based assay), and more interesting horizontal integration augmenting the novel silicon nitride membranes' fabrication process versatility and integration capacity by adding sensors, actuators, and even embedded data acquisition and processing (smart insert for non-invasive remote control, lab-on-a-chip approach). The "intelligent insert" design is inspired by the vision that requires the steps that will lead to the quantum leap to "human testing".

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A decision-making framework for the grouping and testing of nanomaterials

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A Decision-making framework for the grouping and testing of nanomaterials developed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" is presented. This so-called DF4nanoGrouping consists of 3 tiers to assign nanomaterials to 4 main groups, to perform sub-grouping within the main groups and to determine and refine specific information needs. The four main groups distinguish between (1) soluble nanomaterials, (2) biopersistent high aspect ratio nanomaterials, (3) passive and (4) active nanomaterials. The DF4nanoGrouping covers all relevant aspects of a nanomaterial's life cycle and biological pathways, i.e. intrinsic material and system-dependent properties, biopersistence, uptake and biodistribution, cellular and apical toxic effects. Use, release and route of exposure may be applied as "qualifiers" to determine if, e.g. nanomaterials cannot be released from a product matrix, which may justify the waiving of testing. The DF4nanoGrouping allows grouping nanomaterials by their specific mode-of-action that results in an apical toxic effect. This is eventually directed by a nanomaterial's intrinsic properties. However, the exact correlation of intrinsic material properties and apical toxic effect is not yet established. Therefore, the DF4nanoGrouping uses the "functionality" of nanomaterials for grouping rather than relying on intrinsic material properties alone. Functionalities include system-dependent material properties, *in vitro* effects and release and exposure. The DF4nanoGrouping is a hazard and risk assessment tool that contributes to the sustainable development of nanotechnological products. It ensures that no studies are performed that do not provide crucial data thereby saving animals and resources.

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Oocyte-like cell differentiation of mouse embryonic stem cells by two-step cultivation

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Introduction: In vitro oocyte-like cell differentiation from embryonic stem (ES) cells provides a novel model for understanding oocyte development and infertility. Previous work has established that Bone morphogenetic protein 4 (BMP4) and retinoic acid (RA) are essential for germ cell differentiation and meiotic entry respectively. Moreover, granulosa cell co-culturing could improve the differentiation potential of ES cells to oocyte. Here, we demonstrate that ES cells have potential to express markers characteristic for oocyte differentiation with two-step cultivation.

Materials and methods: Mouse ES cells were remain undifferentiated in the presence of mouse embryonic fibroblast (MEF) as feeder layer and in media containing leukemia inhibitory factor. For differentiation induction, ES cells were cultured as embryoid bodies (EB) in the presence of BMP4 after 48 h (+BMP4) or absence (–BMP4) for 5 days. Then, EB cells were dissociated to single cells and co-cultured with granulosa cells in presence or absence of retinoic acid (+RA or –RA) for up to 2 weeks without passaging. Adherent cells were examined by Q real time RT-PCR for the expression of Stra8, REC8, SCP1, SCP3 and Oct4 genes at 7 and 14 days.

Results: Mouse ES cells form oocyte-like structures. These cells expressed meiotic marker genes including Stra8, REC8, SCP1, and SCP3 that were significantly higher in +RA condition compared to –RA condition. In contrast, a significant decreased in the expression of Oct4 expression was detected in +RA condition compared to –RA condition.

Conclusion: In present study, the differentiation of ES cells into oocyte-like cells is supported by supplementation of these two principal factors (BMP4 and RA) besides co-culturing with granulosa cells.

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REACH, the Cosmetics Regulation and upholding the animal testing ban

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The EU Cosmetic Products Regulation established an important precedent worldwide for assessing the human health risks of cosmetic products and ingredients with the explicit exclusion of animal-derived test data. The regulation not only bans the direct testing of cosmetic products and ingredients on animals but also the marketing of such substances within the EU. The cosmetics testing and marketing bans are of great political and ethical significance. The bans were included in the regulation, based on public support from across Europe, for the fundamental principle that the harm caused to animals can never be outweighed by the potential benefit of new cosmetic products. However, ingredients used in cosmetics continue to be tested on animals within the EU because of the provisions laid out in the Registration, Evaluation, Authorisation and Restriction of Chemicals regulation (REACH). A joint statement from the European Chemicals Agency (ECHA) and the European Commission declares that ingredients exclusively used in cosmetics can be tested on animals without breaching the cosmetics testing ban [1], where there is a possibility of workforce exposure during manufacturing processes. For cosmetic ingredients that are also used in other types of products, tests on animals are permitted as a last resort, regardless of whether there is an exposure risk to the workforce. The position taken by the Commission and ECHA undermines the ethical values enshrined in the Cosmetics Regulation and contradicts the intentions expressed in preparatory documents leading up to its adoption. The requirements of REACH should not be used by ECHA and the Commission to circumvent the purpose of the Cosmetics Regulation in relation to cosmetics ingredients. It is only the Court of Justice of the European Union that can provide a legally binding interpretation of the law. In this presentation, we discuss the Cosmetics Regulation, the impact of REACH on the manufacturer and the consumer, and the importance of eliminating the impact of REACH on animal testing for cosmetic ingredients. It is vital that we uphold both the spirit and purpose of the Cosmetics Regulation by ensuring that cosmetics ingredients manufactured and marketed in Europe are never tested on animals under any circumstances, anywhere in the world.

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Systematic assessment of different computational approaches for prediction of toxic effects of new chemical structures

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Approximately 20% of failures in the late drug development are caused by occurrences of toxicities and adverse drug reactions (ADR). Animal trials are currently the major method for determining the possible toxic effects of drug candidates. However, as an alternative, several traditional chemoinformatics approaches such as Quantitative Structure Activity Relationship modeling, ligand- and structure-based approaches, have been proposed to perform well *in silico* thus enabling the reduction of cost, time and animal experiments. Molecular similarity analysis in alliance with identification of toxic fragments was reported to show promising performance in prediction of rodent oral toxicity [1,2]. Furthermore, pharmacophore models (toxicophores) were reported to indicate possible toxicity targets associated with adverse drug effects. Predicting the *in vitro* effects solely based on structural descriptors has also received great attention in recent years. Here, we describe different computational approaches and their intrinsic limitations while comparing their performance across data sets provided in the Tox21 Data Challenge 2014. In particular, we examine different methodologies including analysis of toxic fragments, pan assay interface compound substructures and toxicophore mapping. Additionally, a case study consisting two different drugs having similar toxic class effects can cause similar ADR that result from sharing similar toxicological pathways or networks has been reported [3]. Proper understanding of tissue specificity is necessary to detect relevant genes and pathways in a specific organ and to identify the key nodes underlying the organ-specific safety profile of a particular drug.

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The use of the "Neurosphere-Assay" to study specific disturbances on FGF-2 function during neurodevelopment

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Fibroblast Growth Factor 2 (FGF-2), one of the prototypic members of the FGF group is implied in numerous complex functions of embryonic and fetal development. It plays a major role in regulating pathways that drive brain region-specific development, which are related to distinct expressions of FGF-2 and FGF receptors (FGFRs) in different brain areas. Additionally, FGF-2 modulates proliferation, maturation, migration and astrocyte reactive activation, influences astrocyte gap junction coupling and neurotransmitter sensitivity, which determine astrocyte-dependent synaptic homeostatic functions. FGF-2 signalling imbalance induced by toxicants or stress can adversely affect all the above described functions and alter neurodevelopment. Therefore, our research group has adapted the so-called "Neurosphere-Assay" consisting of three dimensional (3D) structures of neural progenitor cells (NPCs) to study specific disturbances on FGF-2 function during neurodevelopment. This adapted model is able to mimic the implications of FGF-2 on the very basic processes of brain development *in vitro*: proliferation, migration and differentiation into cells expressing markers of neurons and glial cells. The model is adapted for three species, mouse, rat and human NPCs and the basic effects of FGF-2 on them are compared. In all species, FGF-2 specifically modulates migration, proliferation and differentiation of NPCs in a concentration dependent manner, which mimicks the known distribution of FGF-2 in the brain in a ventricular-pial gradient, creating definite niches of differentiation/proliferation. The effects induced by FGF-2 in the model are shown to be specific for FGF-2, as other members from the same family of growth factors like FGF-8, do not trigger the same dose-response effects. Besides, rodent NPCs present a higher sensitivity towards FGF-2 gradients than human NPCs. This adapted "Neurosphere Assay" is postulated to be an alternative model to identify compounds disturbing FGF-2 signalling and help on the inter-species neurodevelomental risk assessment.

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Characterization of the effects of epigallocatechin gallate (EGCG) exposure during neurodevelopment within the AOP concept

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This study evaluates the developmental neurotoxicity of epigallocatechin gallate (EGCG), the major antioxidant in green tea, now commercialized in high doses as a food supplement. Although many preclinical research studies propose EGCG administration during pregnancy to prevent neurodevelopmental adverse effects related to fetal alcohol syndrome, the developmental consequences of high dose EGCG intake during gestation have not been characterized yet. Using the neurosphere culture as an *in vitro* alternative method to study developmental neurotoxicity (DNT), we identified that EGCG disturbs the development of human and rat neural progenitor cells (NPCs). As the molecular initiating event, EGCG binds to the extracellular matrix protein laminin thus interfering with the laminin-β1-integrin interaction in human and rat NPCs. At a cellular level, this deficient macromolecular interaction was translated to the impacted key event (KE) of decreased NPC adhesion. This resulted in a decreased migration distance, being human NPCs more sensitive than rat NPCs (LOAEChuman NPCs = 5 μM LOAECrat NPCs = 10 μM). Structure-activity relationships studies on laminin-dependent adhesion inhibition, showed that flavonoids containing either a galloyl group or a pyrogallol group impaired hNPC adhesion, but less potently than the EGCG containing both groups. Flavonoids without these two residues, i.e. epicatechin, hesperetin and kaempherol did not inhibit hNPC adhesion. Further characterization of the neurodevelopmental effects of EGCG in human NPCs *in vitro* revealed a decreased density of migrated cells as well as an alteration of the GFAP+ processes' orientation within the migration area (LOAEC = 5 μM). Other groups reported that β1-integrin knockout mice showed altered glial structure with lack of their orientation leading as to a lower density of neurons in cortical layers. This *in vivo* effect is postulated to be the adverse outcome at the organism level of this new putative AOP. Thus, the *in vitro* method presented in this study can easily be used to recognize potential human DNT compounds with a mechanism of action disturbing laminin-β1-integrin interaction. Besides, this human cell based model allows better drawing conclusions for human DNT than using the rodent model, as human NPCs have been shown to be more sensitive to laminin-β1-integrin interaction disturbances. Therefore, the embedding of this *in vitro* test within the AOP concept will help improve regulatory assessment of human DNT.

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Influence of brake wear particle size on the biological impact using a 3D human epithelial tissue barrier model

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Brake wear particles are a significant non-exhaust traffic-related source of air-pollution, however are poorly regulated compared to other exhaust emissions sources. Humans are exposed to brake wear particles primarily via inhalation, however the side effect of brake wear particles exposure upon the human lung remains unclear with only a few studies existing. Therefore, the correlation of brake pad formulations with possible release of constituents generated by associated friction processes and their potential biological impact must be investigated. The aim of this study was to mimic the inhalation of brake wear particles by using an advanced lung cell model and an aerosol exposure, i.e. pseudo-air-liquid interface, approach. Brake wear particles released from commercially available "low-metallic" automotive brake pads were generated in a full scale automotive brake dynamometer simulating urban driving. The collected fractions were analyzed using scanning electron microscopy with energy-dispersed spectrometry and transmission electron microscopy. Exposure of brake wear particles was tested using different particle fractions (0.25-2 μm 2-4 μm and 4-8.1 μm in medium) obtained after 17 h of braking process. The particles were suspended and 100 µl of the particle suspension was pipetted onto the apical side of an *in vitro* triple cell co-culture model of the human epithelial airway barrier (consisting of A549 epithelial cells, human blood monocyte-derived macrophages and dendritic cells) cultured at the air liquid interface. Cellular morphology was observed by laser scanning confocal microscopy, whilst the ability for the particles to cause a (pro-) inflammatory response and oxidative stress were assessed. Initial results indicate that an acute exposure to the different particle fractions does not induce any significant adverse effects, although, further investigation using additional endpoints, different particle concentrations and particles from other brake pads need to be tested to support this claim.

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Applying Adverse Outcome Pathways to support Integrated Approaches to Testing and Assessment (IATA)

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The Adverse Outcome Pathway (AOP) conceptual framework provides a systematic means of characterising and organising the relationships between key events that reflect the progression from a chemically-induced perturbation, defined as a molecular initiating event (MIE), to intermediate key events (KEs) and an adverse outcome (AO) of regulatory relevance. AOPs offer the biological context to inform the development of *in vitro* methods and computational prediction models (e.g. quantitative structure-activity relationships), and to facilitate the development of Integrated Approaches to Testing and Assessment (IATA). IATA in turn can make use of the biological and mechanistic information contained within AOP to facilitate/justify the formation of chemical categories supporting read-across of (eco)toxicological data between analogues, facilitate the identification of information gaps and the strategic/cost effective generation of new data, and inform how different types of relevant information sources can be combined and weighted to support regulatory decision-making regarding the hazard and/or risk of chemicals. Within an IATA, generation of new data should be informed by the initial hypothesis formulated on the basis of the collected existing data, thus contributing to a more tailored assessment and increased efficiency in the testing of chemicals. Ultimately, the development of AOP and their regulatory application within IATA will stimulate an increased acceptance of non-animal approaches and potentially reduce the reliance on animal-based methods. This presentation will reflect on the opportunities and challenges of translating AOP knowledge into tools for regulatory decision making. Reference will be made to ongoing activities at the European Commission's Joint Research Centre, which provides independent scientific and technical support to policy makers in the European Union.

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

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Cosmetics Europe's Task Force Eye Irritation is actively involved in the development and evaluation of *in vitro* methods to assess serious eye damage/eye irritation potential of cosmetic ingredients. A thorough understanding of which of the effects assessed in the *in vivo* Draize eye test are responsible for driving UN GHS classification is critical for an adequate selection of chemicals to be used in the development and/or evaluation of *in vitro* methods and for properly assessing test method predictive capacity and limitations [1]. For this reason, Cosmetics Europe has compiled a database of historical Draize data from external lists that were created to support past validation activities. This database contains 681 independent *in vivo* studies on 634 individual chemicals representing a wide range of chemical classes. The chemicals were all screened for commercial availability. A full description of all the ocular effects observed *in vivo*, i.e. degree of severity and persistence of corneal opacity, iritis, and/or conjunctival effects, was added to each individual study in the database and the studies were categorised according to their UN GHS classification and the main effect driving the classification. An evaluation of the various *in vivo* drivers of classification compiled in the database was performed to establish which of these are most important from a regulatory point of view. These analyses demonstrate that persistence of effects on day 21 is the only driver of classification in 47% of the Cat 1 studies, whilst severe lesions occurring in the first 3 days generate a Cat 1 classification in only 27% of the studies. In both cases, corneal opacity is the main observed effect. Both corneal opacity and conjunctival effects are important in driving Cat 2 classification (70% and 84% of Cat 2 studies, respectively) but conjunctival effects appear more often alone than corneal opacity (30% vs. 16%). It follows that *in vitro* methods able to identify persistence of effects and/or address conjunctival effects are also needed to achieve full replacement of the Draize eye test. Most importantly, this database will be an invaluable tool for selecting reference chemicals with an adequate coverage of the relevant *in vivo* drivers of classification for use in the development/validation of *in vitro* methods.

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A multi-targeted *in vitro* approach to predict the potential risks to human health and ecosystems during exposure to mixtures (CER 16 05 06*) from disposal of wastes

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The assessment of human health risk requires identification and integration of information on the health hazards exposure of a chemical compound and relationships among exposure, dose and adverse outcome effects (AOE). The development of risk-assessment strategies for hazardous waste is an emerging issue at European level and the European Chemical Agency is applying the REACH Directive as recently issued (Regulation 1357/2014/EU Decision 2014/955/EU). Up-to-date, due to the lack information of toxicity, the evaluation of the biological potential of chemical mixtures may not be reliable, because little is known about AOE related to what happens when these toxic substances are mixed together, as routinely happens, for example, in discarded laboratory chemicals. Here we reported the preliminary results of a multi-targeted *in vitro* approach designed in compliance with 3R policy, to verify the potential toxicity of inorganic and organic mixtures (IM and OM, CER 16 05 06*, associated with a dangerous laboratory chemicals), from waste collection of analytical laboratories. The composition of such mixtures is subjected to change over the time, so we did not considered the toxicity of single compounds, but that of IM and OM random samples, from a chemical laboratory. Basic toxicity was evaluated, in terms of NRU and MTT viability indexes, on 2-D human keratinocyte (NCTC2544) and endothelial cells (HECV) after 4-12-24 hours exposure to 0.025-1% IM and OM. In compliance to OECD TG431 and 439, skin corrosion and irritation were evaluated on commercial 3D human epidermis reconstructed (RHE) models. Since chemicals act as stressors in cell environment, gene expressions of pro-inflammatory cytokines (TNFα and IL-18) and of Hsp70, as signal marker of cell stress response, were assessed in NCTC2544. After 24 hours exposure. Moreover, several recent evidences highlight the possible link between neurological disorders and neurotoxins in the environment. So, neurotoxicity was investigated by micro electrodes arrays-based electrophysiology (MEA) on rat neuronal networks. NRU index evidenced that OM induced cytotoxicity only on HECV, whilst IM affected both human cell lines, resulting more powerful than OM. Mitochondrial functionality (MTT index) resulted affected by IM and OM, in both cells. According to the EU classification scheme, only IM was highly corrosive (viability reduced to 90 and 3% after 3 and 60 minutes, respectively), while OM resulted irritant (viability decreased to 45%) in RHE models. In NCTC2544 cells, 24 h exposure to 0.5 and 1% OM and IM markedly increased TNFα levels, and did not affected IL-18. Hsp70 decreased only by OM. Besides both mixtures affected neuronal network spontaneous electrical activity, among which IM was the most effective in provoking a rapid block. Our multi-targeted approach can represent an high content sensitive tool to assess the safety of chemical mixtures. The improved identification of new biomarkers to estimate the hazards posed by mixtures can contribute to the development of array of alternative methods, to predict the real potential risks to human health and ecosystems.

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Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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The developing brain is highly vulnerable to the adverse effects of chemicals resulting in neurodevelopmental disorders in humans. Currently, animal experiments in the rat are the gold standard for developmental neurotoxicity testing (DNT), however, these guideline studies are insufficient in terms of animal use, time and costs and bear the issue of species extrapolation. Therefore, the necessity for alternative methods that predict the DNT of chemicals faster, cheaper and with a high predictivity for humans is internationally agreed on. In this respect we developed an *in vitro* model for DNT key event screening, which is based on primary human and rat neural progenitor cells grown as neurospheres. They are able to mimic basic processes of early fetal brain development and enable an investigation of species differences between humans and rodents in corresponding cellular models. The goal of this study was to investigate to what extent human and rat neurospheres were able to correctly predict the DNT potential of a well-characterized training set of nine chemicals by investigating effects on progenitor cell proliferation, migration and neuronal differentiation in parallel to cell viability, and to compare these chemical responses between human and rat neurospheres. We demonstrate that by correlating human and rat *in vitro* results to existing internal *in vivo* exposure data, human and rat neurospheres classified seven out of nine compounds correctly, and human and rat neurospheres differed in their sensitivity to most of the chemicals. By investigating toxicodynamic species differences between human and rat neurospheres towards the antiepileptic drug valproic acid on a molecular level, we identified a differential expression of HDAC1 and the mitochondrial membrane transition pore component ANT1 in the two species as potential molecular reasoning for the divergent sensitivities. In conclusion, our results showed that human and rat neurospheres may serve as a suitable *in vitro* method for developmental neurotoxicity screening of chemicals and enables an investigation of molecular species differences. The integration of such results into the "adverse outcome pathway" concept may contribute to a deeper understanding of species differences in developmental neurotoxicity and thus enable an improved chemical risk assessment for human health.

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Microheated thermoresponsive cell culture substrate for spatiotemporal control of cell growth

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Organotypic cell culture systems that represent the *in vivo* situation of a highly organized and dynamic cell environment are of undisputed value for the replacement of animal testing. To achieve such systems with a defined architecture, cell patterning techniques for the control of adhesion and outgrowth of different cell types *in vitro* are under continuous development. Very promising approaches are based on surface coatings of thermoresponsive polymers (TRP) that can be switched between a cell-repellent and a cell-adhesive state in a temperature-dependent manner. Patterns of TRP have been used to control cell adhesion in time and space and to create heterotypic cell sheets for tissue engineering (Hirose et al., 2000; Yamato et al., 2001). However, resolution and flexibility of such cell patterns are still limited by the lack of an appropriate, high resolution temperature control for local switching of the TRP. Here, we present a microstructured thermoresponsive cell culture substrate that offers local control of the surface temperature on the microscale with a resolution of < 1°C for precisely and dynamically regulating adhesion and outgrowth of cells. Local heating is realized by microelectrodes that are integrated beneath the chip surface. Prototype microelectrodes of 50 μm width were fabricated from ITO, thus being transparent to allow microscopy and connected to a power supply for resistive heating. In order to analyze the generated temperature distribution on the surface we established a thermometry method using thin sol-gel films containing the temperature-dependent fluorophore Rhodamine B. Using microscopy analysis of Rhodamine B fluorescence, voltage- and time-dependent temperature gradients could be measured close to the surface in aqueous conditions with high resolution in time (ms), space (μm) and temperature (0.5°C). By that we could show for example, that applying 20 V to the prototype 50 μm - electrode generated a surface temperature increase of 3°C ±0.2°C above the microelectrode and a surface gradient with a half maximal width of 85 μm. By applying the pre-defined voltage to a specific microelectrode for a certain local surface temperature, the TRP that is immobilized on a gold layer above the microelectrode can now be switched locally – within well defined dimensions. By that we were able to direct adhesion of L-929 fibroblast cells and SH-SY5Y neuronal cells at defined spots or control migration out of defined regions. This can further be used to generate dynamic, heterotypic cell patterns or study dynamic cell-cell interactions. Finally, also the outgrowth of cell extensions such as neurites of neuronal cells shall be navigated with this microchip. By guiding the axon growth between patterned neuronal cells the direction of synapse formation and thus of synaptic transmission could be controlled. The goal of this approach is to regulate the direction of neuronal connectivity to better mimic the ordered connectivity of native neuronal circuits found in the brain. This could help to further study mechanisms of synaptic transmission, synaptic plasticity and many other topics of neuroscience in detail, while reducing the amount of animal experiments for such investigations.

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Characterization of 3D reconstructed human airway models used for biomarker and inflammatory response studies

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In vitro studies for inhalation toxicology benefit from target tissues that closely recapitulate the cell types and morphology that exist in the human lung. Human reconstructed, 3-dimensional (3D) airway tissues are now available to researchers and are an important step towards providing an *in vitro* human lung model. These organotypic, air-liquid interface (ALI), tissues are comprised of heterogeneous cell types and more accurately reflect the pulmonary airway *in vivo* than do previously used monolayer cultures. They are now increasingly applied to assess aerosol exposures from diverse materials, including those from tobacco-based products such as whole cigarette smoke and E-cigarette vapors. However more characterization of the response patterns of the tissues is needed. We conducted an exploratory study to assess the impact of several acclimation conditions on the performance of the MatTek EpiAirway™ model tissue following apical challenge. Tissues were received and maintained using hydrocortisone (HC) free or HC inclusive (1 µg/mL) medium, for 24 or 48 hr prior to apical challenge with two reference chemicals known to elicit an inflammatory response. Following the designated acclimation period, groups (comprised of triplicate ALI tissues) were treated with either 15 µg/mL Poly I:C or 5 µg/mL lipopolysaccharide (LPS) for 24 hr. After measuring TEER, we collected an apical rinse, the tissue lysate, and medium from each ALI insert. Total protein, LDH, IL-6, IL-8, and IP-10 were assayed from each sample. Results indicate there was no consistent correlation between tissue protein content and biomarker expression level. A comparison between 24 hr and 48 hr acclimation lengths indicated that 48 hr acclimation could result in a greater baseline TEER value and 6-fold greater average biomarker increase (over negative control) following challenge. The 48 hr HC inclusive group typically had the greatest average cytokine response, and this was found in the apical rinse where markers were up to ~5-fold higher than in either tissue lysate or medium samples. The marker with greatest increase over control was IP-10 (160-fold in tissue 139-fold in apical rinse, and 25-fold in medium) following Poly I:C challenge in the 48 hr HC inclusive group. As more products are being evaluated using the 3D airway models, researchers should consider experimental approaches and marker sampling options that will best demonstrate relevant changes in the tissue following challenge. An optimized acclimation protocol may deliver a more robust response, and the marker sampling location may reflect relevant events that should be considered when using this useful model of the human airway.

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Development of a shipping protocol for human hemicornea constructs to facilitate their widespread use in *in vitro* drug absorption studies

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During the last decades the demand on *in vitro* methods as alternatives to animal testing has risen dramatically. Especially, after the implementation of the EU directive 2010/63/EU, which legally bound all EU member states to support the 3Rs, the research in this field became more and more prominent. Our institute started already in the beginning of the 21st century with the development of different *in vitro* models [1,2]. One of those established models was the human hemicornea construct (HC), which was prevalidated as an *in vivo* method for the testing of transcorneal drug absorption processes in 2012 [3]. In this prevalidation study the model showed excellent *in vivo*-like permeability characteristics and lower intra- and interlaboratory variances than common *ex vivo* methods. However, the project underlined that the interlaboratory transfer of methods is costly and time-consuming. For these reasons, we developed a standardized shipping protocol to achieve quality maintaining transport conditions. This protocol should allow a ready-to-use application of the prevalidated model which would significantly improve its applicability and would support the reduction of animal experiments in *in vitro* drug absorption studies. For this study the HCs were cultivated under serum-free conditions on permeable polycarbonate filters (Transwells®) using SV40 immortalized human keratocytes (HCK-Ca) and human corneal epithelial cells (HCE-T) as described before [3]. After cultivation, the HCs were exposed to varying ambient conditions. Apart from different temperatures and varying CO_2 content, alternative transport containers were tested. According to commercially available ready to use models [4], the effect of medium immobilization by agar-agar and the influence of submerse transport conditions were investigated. Subsequent to those single parameter studies, the laboratory simulations were altered to rebuild more complex interactions and, moreover, shipping to another laboratory was tested by real time transport. Cell viability after simulated transport was evaluated via MTT testing. Barrier properties of the HCs were estimated by transepithelial electrical resistance (TEER) measurements and permeability studies with sodium fluorescein. The obtained results have shown that the influence and the interplay of single parameters are complex and not easy to predict. Especially, the forces resulting from movement or changes in position have a great impact on the epithelial barrier properties, which are one key factor for quality control. Depending on the forces which have occurred during shipping, 0-33% of the transported models fulfilled the requirements for pharmacokinetic studies which were set in the prevalidation study. In contrast to the epithelial barrier properties, the viability of the HC was much more robust to differing environmental factors. Because of this higher robustness, it can be presumed that the yield of the developed shipping procedure for toxicological studies will be considerably higher than for pharmacokinetic experiments. All in all, the results allow a better understanding of the influences during shipping and can particularly be used for the improvement of common shipping procedures of the HC and other *in vitro* models.

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RTgill-W1 cell line assay for predicting fish acute toxicity: evaluation of a round-robin test

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The rainbow trout (Oncorhynchus mykiss) gill cell line, RTgill-W1, has been tested to predict fish (*in vivo*) acute toxicity, hence, an animal alternative to tests used for chemical risk assessment. In a previous study, 34 organic industrial chemicals (selected using pre-defined criteria), were tested and compared to the acute toxicity reported in the US EPA fathead minnow data base. The majority of data was within a 10-fold range versus acute fish lethality when taking into account bioavailable exposure concentrations. Moreover, the outcome of the cell line assay compared very well to results obtained in the zebrafish embryo toxicity test (FET), which has meanwhile been accepted as the OECD test guideline 236. These findings provided the impetus to initiate the RTgill-W1 cell line assay as a potential animal replacement method to the same level of international acceptance. Through support of CEFIC-LRI and UK NC3Rs, an Eawag-led round-robin test, involving six industrial and academic research laboratories from Europe and the USA was conducted. The overall goal was to test the robustness of the established methodology. In particular, the transferability of the RTgill-W1 cell line assay and the intra- and inter-laboratory reproducibility was evaluated. Seven laboratories provided data for the round-robin testing, either for the cell line assays and/or analyses of chemical concentrations. Chemical selection and quantitative analysis as well as conductance of the cell line assay followed criteria and protocols used in the initial study. Experiments with the cell line assay include a minimum of three biological replicates (cells from different passages) with three technical replicates in each test run. Results of cell viability measurements are based on verified exposure concentrations. Indeed, the RTgill-W1 cell line assay procedure was transferred successfully to each of the participating laboratories. Improvements to the original standard protocols were implemented based on the suggestions provided by the participants – leading to further simplification. Data was analysed using diverse statistical methods with a specific focus on inter- and intra-laboratory variability, providing a solid basis that will likely establish this method as an animal-free alternative to the fish acute toxicity test.

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KeratinoSens Skin Sensitisation Test: adaptation to animal-free culture conditions

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Skin sensitisation is defined as an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS). The test described here is fully accepted at a regulatory level (OECD Test Guideline 442d). It is appropriate for compliance with a range of legislation including the EU Cosmetics Regulation 1223/2009 and the CLP Regulation 1272/2008. It is expected to be incorporated into REACH (Registration, Evaluation, Authorisation and restriction of CHemicals) legislation by 2016 there is currently scope to apply the test under the provisions of Annex XI. This test method addresses the activation of keratinocytes – the second key event of the skin sensitization Adverse Outcome Pathway defined by ECVAM. The KeratinoSens™ cell line is a human keratinocyte cell line (HaCat), containing a luciferase gene that is under control of a constitutive promoter fused with an Antioxidant Response Element (ARE) from a gene that is known to be up-regulated by contact sensitisers. Therefore, the luciferase signal reflects the activation by sensitisers of endogenous Nrf2 dependent genes. The standard KeratinoSens™ test described in the guideline included the use of animal-derived components in the test system, such as bovine serum in the cell culture medium. XCellR8 has adapted the test to animal product-free conditions, within the parameters allowed under OECD Test Guideline 442d. KeratinoSens™ cells are cultured medium containing human serum and sub-cultured using animal product-free reagents. For the test, 12 concentrations of each test item are evaluated, and incubated for 48 hr. 3 independent runs (n = 3) of the test are performed. A range of acceptance criteria must be satisfied in order for the test to be considered valid. Cinnamic aldehyde is used as the positive reference. To ensure that the prediction of sensitisation potential is made at sub-cytotoxic concentrations, a cytotoxicity test is performed in parallel. Proficiency Testing as per OECD TestGuideline 442d demonstrated successful classification of all 10 reference chemicals in terms of skin sensitisation potential, using the new animal product-free conditions. The 10 reference chemicals were: isopropanol salicylic acid lactic acid glycerol cinnamyl alcohol ethylene glycol dimethacrylate 2-mercaptobenzothiazole methyldibromo glutaronitrile 4-methylaminophenol sulfate 2,4-dinitro-chlorobenzene. Data analysis provided the following parameters: The EC1.5 value is the lowest concentration of test item that causes a luciferase induction greater than 1.5-fold above the negative control (the threshold for classification as a Sensitiser). EC1.5 values for the 10 reference chemicals ranged between 2.7 μM and 62.5 μM. The IMAX value is the maximum induction achieved at any test concentration. IMAX values for the 10 reference chemicals ranged between 1.32 and 26.1. The correct classification of all 10 reference chemicals in Proficiency Testing exercise demonstrated that the KeratinoSens™ test can be successfully performed in animal product-free conditions, providing a fully human replacement for animal tests for skin sensitisation. XCellR8 now offers this test from its GLP accredited lab in the UK, as an ethical testing option for compliance with a range of legislation. This work was performed with kind support from Lush Ltd

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BlueScreen™ AF (animal-free) – the first truly animal-free *in vitro* test for human genotoxicity

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Genotoxicity can be defined as the ability of a test item to cause damage to the genetic material of cells. A genotoxic test item may be a mutagen (changing the DNA code), a clastogen (damaging or breaking the chromosomes), or an aneugen (changing the number of chromosomes). To assess ultimate carcinogenic potential, it is important to test for all major mechanisms of genotoxicity. BlueScreen™ is a non-regulatory method for the assessment of the genotoxic potential. The test captures all types of genotoxin, but does not identify which of the mechanisms is responsible for the specific genotoxic potential of the test item. The BlueScreen™ assay utilises the p53 competent human TK6 lymphoblastoid cell line. The cells host a patented Gaussia luciferase (GLuc) reporter system which exploits the normal regulation of the GADD45a gene. Exposure to a genotoxic test item increases the expression of GADD45a (as part of the DNA damage response) and hence Gaussia luciferase synthesis in the cells is also induced. The addition of a light producing luciferase substrate allows the signal to be quantified by luminescence detection, which is compared with controls to predict genotoxic potential. The test is performed both with and without liver extract (S9) so that any genotoxic potential due to metabolism can also be predicted. Simultaneous assessment of cytotoxicity ensures that the results for genotoxicity are not adversely affected by basic toxicity to the human cell cultures. In common with other *in vitro* genotoxicity tests, the standard BlueScreen™ test included the use of animal-derived components. XCellR8 and Gentronix collaborated to adapt the cell culture to animal product-free conditions, utilizing human serum and human liver extract (S9). A validation exercise assessed the ability of the adapted test to classify the genotoxic potential of a range of reference chemicals, covering various mechanisms of genotoxicity. The reference chemicals without S9 were 5-fluorouracil and 4-nitroquinoline-1-oxide (mutagens) mitomycin C, etoposide and methylmethane sulfonate (clastogens) colchicine (aneugen) phenformin, sodium idioacetate, phthalic anhydride and sodium chloride (non-genotoxic). The reference chemicals with S9 were 2-aminoanthracene and benzo[a]pyrene (mutagens) cyclophosphamide (clastogen) aflatoxin (unknown mechanism) sodium idioacetate, phthalic anhydride, 2,4-dinitrophenol, sulfisoxazole, D-mannitol and sucrose (non-genotoxic). BlueScreen cells were seeded into 96-well plates and incubated with 8 concentrations of test items for 48 hours prior to data collection. Proficiency Testing demonstrated successful classification of 17 out of 20 reference chemicals. 2 out of 3 of the "incorrectly" classified chemicals are also considered "false positives" in the Micronucleus Test (OECD Test Guideline 487). In summary, the successful classification of the majority of the panel of reference chemicals demonstrated the adaptability of the BlueScreen™ test to animal-free conditions and a comparable predictive ability with existing methods. BlueScreen™ Animal-Free (BS-AF) is the first truly animal-free *in vitro* test for human genotoxicity, capable of predicting all major classes of genotoxin. XCellR8 now offers this as a non-regulatory test from its GLP accredited lab in the UK. Further validation is ongoing. This work has been kindly supported by Lush Ltd.

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Small Airway-on-a-Chip: A Novel Human Inflammatory Disease Model

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Lung diseases are associated with significant mortality and morbidity worldwide; chronic obstructive pulmonary disease (COPD) and lower respiratory tract infections (LTIs) constitute two of the top five causes of death in man. Unfortunately, current pharmacotherapies fail to reverse disease progression and little progress has been made towards developing new therapeutics. In part, this is due to inter-species differences hampering efforts to extrapolate therapeutic targets and drugs from animals to humans, as well as inability of current *in vitro* model systems to capture complex organ-level inflammatory immune responses. Organs-on-chips are microfluidic cell culture devices that contain hollow micrometer-sized chambers seeded with human cells, which recreate specialized multicellular architectures, tissue-tissue interfaces, physicochemical microenvironments and vascular perfusion necessary to recapitulate organ-level physiology *in vitro*. Here, we describe a human lung 'small airway-on-a-chip' that is lined with human pseudostratified mucociliary bronchiolar epithelium at air-liquid interface in an 'airway lumen' mimic, and pulmonary vascular endothelial cells that experience continuous fluid blood-like flow in a microvascular channel. We used this platform to mimic flu-like inflammation, pathogenic COPD exacerbations, to circulate blood immune cells at physiological vascular shear, to study real-time complex tissue-tissue interactions, and to test for efficacy of a new experimental compound. Moreover, we engineered an advanced electromechanical setup to 'breathe' whole cigarette smoke from burning cigarettes in and out of our living airway-embedded microdevices. We were able to recapitulate smoke-induced oxidative stress and genotoxicity, and characterize ciliopathic phenotypes. Application of whole transcriptome profiling on cultured diseased cells on-chip enabled us identify a potentially novel set of genes that were differentially expressed in COPD cells. We believe these genes may serve as putative therapeutic or diagnostic targets. In conclusion, the human 'small airway-on-a-chip' microsystem offers great potential in modeling human COPD pathophysiology *in vitro* that will ultimately accelerate advances in lung biology and drug discovery and development.

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Assessment of sensitization and photosensitization of benzophenone-3 based on DPRA

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Contact allergies are considered complex diseases that reach 15-20% of the population. Among the cosmetic allergens, UV-filters have been considered emerging allergens and benzophenone-3 (BZP-3) is one of the most used UV-filter. The commonly methods to assess skin sensitization still use animals and among the few non-animal methods is the DPRA (direct peptide reactivity assay, [1,2,3]), which considers that if a substance can react with skin protein it has the potential to act as a skin allergen. Besides this, since sunscreens will be exposed to sunlight, their complete trials must address both sensitization and photosensitization potential. However there is no validated *in vitro* study to assess photosensitization potential. Thus, this study is very important, once it aims to assess the sensitization and photosensitization of BZP-3 based on DPRA. For this purpose, a BZP-3 solution was prepared in DMSO/acetonitrile and reacted with synthetic heptapeptides containing cysteine (CYS) or lysine (LYS) (reactive samples). For photosensitization study, the reactivity samples were submitted to 9 J/cm^2 of UVA radiation in a SOL- 500 solar simulator Dr Hönle (Planegg, Alemanha). After 24 h of reaction, triplicate samples of both studies were analyzed by HPLC (Shimadzu, Japan) on C18 column with detection at 220 nm (Shimadzu). Peptide reactivity was reported as percent depletion in the sample relative to its control. The positive controls were dinitrochlorobenzene (sensitization) and ketoprofen (photosensitization). Results showed that at analysis based on CYS or both CYS/LYS, BZP-3 presented minimal reactivity (CYS depletion average: 0% mean of CYS/LYS depletion: 3.9%). In the photosensitization assay, BZP-3 presented low reactivity (CYS depletion average: 14.5%), which means that BZP-3 can be considered a photosensitizer. Positive controls presented acceptable values in both studies. In conclusion, BZP-3 was considered a potential photosensitizer under experimental conditions. The heptapeptide CYS was more efficient than LYS to the photosensitization assay. These results are very important, once they evaluate initial important parameters in the complete safety assessment and enable the development of new tools to assess photosensitization based on DPRA.

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The Danish 3R-Center

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The Danish 3R-Center was established November 2013 in order to focus on alternatives to animal testing and to create better conditions for laboratory animals. The center is funded by Ministry for the Environment and Food, pharmaceutical industry and animal welfare organizations. The role of the Danish 3R center is to:

- Promote the development of alternatives to animal testing (Replacement)
- Develop methods that use as few animals as possible (Reduction)
- Improve conditions of laboratory animals, such as minimizing pain, suffering and distress experienced by the animals in all aspects of animal use from the housing and husbandry to the scientific use (Refinement)
- Collect and disseminate knowledge on all the 3R's
- Initiate and support research in laboratory animal studies and the development of alternatives
- Cooperate with similar centers abroad The Danish 3R Center stimulates, in co-operation with the Ministry for the Environment and Food of Denmark, research into the application of the 3Rs by funding relevant projects. The Danish 3R-Center currently supports seven projects with an expected impact on the 3R's
- Refinement of animal models of pain: Establishment of strategies to alleviate avoidable pain in rat models for pain and inflammation
- Pathological and immunological consequences of basic experimental animal procedures in mice
- A Refined Approach to Producing Polyclonal Antibodies in Chickens – Completely Replacing All Invasive Elements by Combining Immunizations with Routine Aerosol-based Vaccinations
- An alternative to animal experiments: Development of an *in vitro* human skin model for evaluation of topical antimicrobial compounds
- Reducing the group size in studies of dermatitis by standardization of the gut microbiota
- An *in vitro* method to predict acute lung toxicity due to pulmonary surfactant inhibition
- Establishment of an *in vitro* model for diabetic atherosclerosis

Furthermore The Danish 3R-Center is currently working on three different Projects:

- Producing educational material on the use of laboratory animals and the 3Rs. The material may be used in the education of high school students (biology or biotechnology line). This initiative will hopefully create increased awareness on animal welfare and the 3Rs in scientific use at possible future scientific researchers.
- A statistical analyzes of ten years of licenses data from the Danish Animal Experiments Inspectorate, being the single licensing and evaluation body in Denmark. For the last ten years all applications and annual statistics have been forwarded by web based platform and the data are hence stored in a database. The project aims detecting trends, identify reduction potential and evaluate development of predicted severity.
- Map the use and knowledge of 3R's in academia. This is a scientific study performed by University of Copenhagen using a questionnaire to mapping the understanding and use of the 3R's in the scientific community – being the animal users often not attending conferences, symposiums and meetings concerning the 3R's. Finally the Danish 3R-Center has developed a web site focusing on promoting international news on the 3R's. The site will draw on information from similar centers in Europe and overseas.

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The Future of the QSAR Toolbox: moving to less uncertainty in predictive toxicology

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Classification of untested chemicals is not possible without some form of prediction based on a simplified alternative biological model or a simplified chemical structure-activity relationship. The OECD QSAR Toolbox is a freely-available software that implements structure-based approaches to the prediction of toxicological data: it exploits the possibility of combining chemical and biological information to assess the toxicity of a query chemical, starting from the toxicity and structural data of analogue chemicals. The project was launched in 2003, and has grown as to include a wide range of information and tools. Recent developments include new functions to extend the possibility to analyse the data and to select the most appropriate analogues (e.g., endpoint versus endpoint correlation, filtering chemicals in data gap filling by making use of measured data). The Toolbox also includes new data bases (e.g., Toxcast results). In 2014 a new phase of improvements have started, that will lead to a system completely renovated from an IT point of view, together with usability improvements, new scientific development, and additional functionalities. Another aspect is that in order to minimize the uncertainty proper to modeling and to provide transparent mechanistic justification to the predictions, the OECD has developed the concept of Adverse Outcome Pathways (AOP). AOPs delineate the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses that describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal, and population levels of observation. The implementation of AOPs into the Toolbox will provide additional tools to predict complex toxicological endpoints.

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The role of the National Committee in Germany – severity assessment of genetically altered animals

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With the amendment to the German Animal Welfare Act in 13 July 2013 and the adoption of a new regulation dealing with the protection of animals used for scientific purposes in 1 August 2013, the Directive 2010/63/EU was implemented into German law. Article 49 of the Directive 2010/63/EU provides that all EU member states shall establish a national committee (NC) for the protection of animals used for scientific purposes. The NCs shall advise the competent authorities and animal-welfare bodies on matters dealing with the acquisition, breeding, accommodation, care and use of animals in procedures and ensure sharing of best practice. Additionally, they shall exchange information on the operation of animal-welfare bodies and project evaluation and also share best practices within the EU. In Germany, the NC was established at the Federal Institute for Risk Assessment (BfR). Within the federal structure of Germany, the NC has an advisory role with the aim of harmonizing the implementation of the new regulation across all federal states. Here, we will briefly outline the modus operandi of the German NC. One issue that was recently brought forward to the NC is the severity classification of genetically modified laboratory animals, as the breeding of these animals now needs to be approved by the authorities if the animals might experience pain, suffering or lasting harm due to their genetic modifications. In June 2013, the BfR initiated a workshop on the severity assessment of genetically altered mouse lines with experts from universities and non-university research institutes as well as members of the licensing authorities. The result of this workshop is an official statement which contains four forms for the severity assessment of new born litters, of litters on weaning, of individual animals, and for the final assessment. The statement and forms are available in English and German. As the number of transgenic fish models is constantly increasing over the last years there is a strong need for the severity classification of genetically modified fish species, especially Zebrafish. Therefore, a second workshop concerning this topic will take place in June 2015 at the BfR. The first results of this workshop will be summarized at this meeting.

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New guidance on information requirements for skin corrosion/irritation and serious eye damage/eye irritation under REACH

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One of the objectives of the REACH Regulation is that vertebrate animal testing should be undertaken only as a last resort. All existing information needs to be considered and data generated whenever possible by means of alternative methods. The recent update of ECHA's guidance addresses the latest developments in alternative test methods and assessment strategies related to skin corrosion/irritation, serious eye damage/irritation and their suitability for the purposes of fulfilling information requirements of REACH. REACH imposes in its Annexes VII to X standard information requirements (usually as test types) depending on the amount of the registered substance produced or imported annually. According to Annex VII, *in vitro* tests for skin corrosion/irritation and serious eye damage/irritation are foreseen for substances produced or imported at less than 10 tonnes per year. Currently according to Annex VIII an *in vivo* test (skin irritation and eye irritation) is a standard requirement at 10 or more tonnes per year. However, Annex VIII is under revision which is expected to strengthen the role of *in vitro* methods and remove the standard requirement for an *in vivo* study at Annex VIII level. Registrants should also consider fulfilling the information requirements if the conditions specified in column 2 of Annexes VII to X (e.g. if the substance is a strong acid/base) or Annex XI (e.g. using a weight-of-evidence) can be met. Due to the sequential nature of the REACH standard information requirements, the requirements of Annex VII also apply for substances at 10 or more tonnes per year. Thus, information from the *in vitro* tests specified in Annex VII must be considered before it is decided that the corresponding *in vivo* test specified in Annex VIII is needed. In the guidance update, the previous rather rigid integrated testing strategy is replaced by a more flexible integrated testing and assessment strategy. The integrated testing and assessment strategy for skin corrosion/irritation in the guidance is compatible with that of the OECD (2014) and allows assessment of the classification according to CLP. It comprises three steps: retrieving existing information, weight of evidence analysis and expert judgement, and generation of new information by testing if necessary. Depending on the available information, either a top-down or bottom-up approach to testing is recommended. More importantly, the registrant will be then able to use the *in vitro* data to fulfil the information requirements not only for Annex VII, but also for Annex VIII requirement for an *in vivo* test if the substance falls under the scope of the *in vitro* tests and the registrant formulates an adequate weight of evidence adaptation. In doing so, obligations under REACH to use alternatives to animal testing can be fulfilled. For serious eye damage/eye irritation, the testing and assessment strategy is analogous to that for skin corrosion/irritation. There is currently, however, no validated *in vitro* eye irritation method available to identify Eye irritants Category 2 under CLP and thus after having exhausted the possible alternatives *in vivo* testing for serious eye damage/irritation may be necessary.

The guidance is available at http://www.echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf
Supporting information can be viewed at http://echa.europa.eu/support/testing-methods-and-alternatives

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Epithelial-endothelal co-cultures to mimic the air-blood-barrier

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Exposure to chemicals, fine and ultra-fine ambient particles including engineered nanomaterials and to biologicals such as proteins, enzymes or lipids needs hazard and risk assessment independent of their application domain. In this context, inhalation is an important route of uptake due to occupational and/or non-occupational exposures. Great progress has been made in establishing new methods, assays and batteries of assays to address wanted and unwanted cytotoxic effects of compounds in the human lung. Depending on the size, shape and other physicochemical properties, molecules may translocate through the alveolar-capillary barrier into the bloodstream, and potentially reach their targets via this route and consequently damage cells and organs. Particularly the alveolar-capillary barrier has several defense strategies: surfactant, alveolar macrophages, epithelial cells, a basal lamina, and the endothelium of blood micro-vessels. Consequently, damage of the respiratory tract happens through disturbed barrier functions, and is triggered either by nonspecific irritation or by specific immune-mediated mechanisms. Based on this background it is commonly agreed that the behavior of co-culture methods that mimic the situation at the epithelial-endothelial barriers is closer to physiology. During the last ten years *in vitro* cell culture models with various cells to mimic this complex barrier have become available. They represent a promising non-animal *in vitro* alternative and could be used for studies on the mode of action or to evaluate *in vitro* absorption kinetics of compounds, their translocation into the system as well as to investigate local and/or systemic effects close to the *in vivo* situation. Here I provide an overview and present *in vitro* co-culture models of biological barriers and their reaction in response to external stimuli.

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Functional characterization of reconstructed skin containing *in vitro* generated Langerhans cells

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The sensitizing potential of xenobiotics is determined using animal tests such as the Guinea Pig maximization test, Buehler test, or the local lymph node assay. Recently, the OECD has published an adverse outcome pathway of skin sensitization, identifying the key events leading to allergic contact dermatitis. *In vitro* tests address these key events and two assays are now regulatory adopted (OECD 442C and 442D). The use of the current *in chemico* and *in vitro* models is, however, limited since they do not reflect dermal penetration, complete biotransformation and cell cross-talk in a 3D environment. In this study, we aimed to overcome these limitations by establishing reconstructed skin tissues containing Langerhans cells (LC). *In vitro* generated immature monocyte-derived (MoLC) or MUTZ-3-derived cells (MUTZ-LC) cultivated with keratinocytes on a dermal compartment with fibroblasts form a stratified epidermis after 14 days as indicated by the expression of epidermal differentiation markers. $CD1a^+$ $CD207^+$ $HLA\text{-}DR^+$ MoLC or MUTZ-LC were mainly localized in suprabasal layers of the epidermis and distributed homogeneously in accordance with native human skin. Topical application of the highly potent contact sensitizer 2,4-dinitrochlorobenzene (DNCB) resulted in increased LC mobility out of the epidermis toward the dermal compartment. DNCB exposure induced IL-6 but not IL-8 secretion in skin models with LC, whereas no change was observed in control tissues lacking immune cells. Increased gene expression levels of CD83, PD-L1, and CCR7 in the dermis indicated LC maturation. MoLC and MUTZ-LC containing skin models gave similar results confirming the applicability of non-immortalized immune cells. In summary, we have successfully integrated immature and functional Langerhans cells into reconstructed skin equivalents. Further studies are needed to determine if the models can contribute to the assessment of potency and identification of irritants or pro-haptens. These models should overcome species-related failings and offer also the opportunity to test the hazard potential of more susceptible individuals with diseased skin.

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The implementation problem: barriers to and opportunities for the use of non-animal veterinary vaccine potency tests

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As new strategies emerge for the consistent production and testing of biologics batches using non-animal methods, currently available non-animal approaches have still not been widely implemented. In some cases, available non-animal methods have not been implemented at all, despite significant public investments toward validation. Resistance to consistency measures also contributes to the difficulty in implementing non-animal batch tests for manufacturers and regulators alike. Referencing the non-animal methods validated for use by the U.S. Department of Agriculture Center for Veterinary Biologics (CVB), we describe progress and obstacles influencing the implementation of these methods since 2008. Our work in this area has shown that non-governmental organizations operating outside of the direct regulation or manufacturing of biologics are uniquely poised to identify and resolve implementation issues not directly addressed by manufacturers, regulators, or cooperative efforts between these groups. Here, we present our work towards eliminating barriers to the implementation of non-animal methods. With particular focus on leptospira vaccine batch potency testing, we discuss the challenges and opportunities faced by manufacturers and regulators in the pursuit of integrating consistency measures and non-animal batch tests into biologics production and testing guidelines.

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Nanoparticles at the human placental barrier: advancing *in vitro* placenta models using co-cultures and mimicking the 3D and/or dynamic microenvironment

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With the growing use of nanotechnology, the placenta is likely to come into contact with nanoparticles (NPs) either accidentally through exposure or intentionally in the case of nanomedical applications. Therefore studies dealing with the placental translocation and effects of NPs are of utmost importance in particular if considering the significant knowledge gap in this field of research. *In vitro* placenta models are needed to pre-screen the large variety of NPs but are often highly oversimplified making it unclear in how far they are predictive of the highly complex and dynamic *in vivo* situation. We are developing novel *in vitro* placenta models to study if the use of co-cultures and the recreation of a 3D and/or perfused microenvironment improve their predictive value. For NP translocation studies, we have established co-cultures of endothelial and trophoblast cells on Transwell inserts that will be inserted into a perfusion chamber to mimic maternal and fetal circulation. Preliminary studies with static co-cultures and paracellular markers indicate that the trophoblast layer constitutes the main barrier layer, and addition of endothelial cells did not further reduce permeability. To assess effects of NPs on placental tissue we developed 3D co-culture microspheres from placental fibroblasts and trophoblast cells using a hanging-drop approach. First studies with cytotoxic quantum dots indicate that placental microspheres exhibit a higher resistance than 2D monocultures. Identification of the relevant parameters that have a key influence on NP translocation and effects will support the design of superior advanced *in vitro* models with a high predictive power. This knowledge will not only be relevant for the design of placental barrier models but also for their integration into body-on-a-chip platforms or for other biological barriers.

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Aligning nanotoxicology with the 3Rs: What is needed to realise the short, medium and long term opportunities?

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Nanomaterials (NMs) pose numerous advantages over their bulk chemical counterparts, and there has therefore been a considerable rise in the production and type of NMs in the last decade proposed for use within a vast array of different, human-based applications. Although the potential advantages posed by these nano-sized materials are clear, concerns have been raised in recent years concerning the health implications of both human and environmental exposure. It is therefore crucial that concerted, science-led efforts are made to understand the genuine effects of exposure to NMs, to ensure that protection goals are met. Despite extensive research efforts, whole organisms continue to be the preferred test system as they capture site of administration, systemic distribution and the target tissues where the NMs accumulate. There is however much ongoing discussion within the field around how best to assess the safety of NMs going forward. It is currently not possible to extrapolate from *in vitro* or short-term *in vivo* studies to chronic effects, due to limited knowledge of particle biokinetics and accumulation in the body and the progression of short-term effects. Hence, not only may a large number of different nanoforms need to be tested but also many long-term studies could be required, demanding vast numbers of animals and resources. Such an approach is not practical furthermore there are other business, ethical and legislative drivers to re-evaluate the use of animal toxicity tests. An expert working group of regulators, academics and industry scientists was recently brought together by the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) to discuss the opportunities being offered in the short, medium and long term to advance the science underlying nanotoxicology, whilst reducing the reliance on animal models. Immediately, and in the short term these include refinement and reduction within existing animal models in the medium term, reduction through the leveraging of existing information and development of more robust *in vitro* approaches and predictive computational models and in the long term, replacement with accepted non-animal methods. The discussions also explored the key objectives that must be achieved to realise the vision of aligning the 3Rs with improved safety assessment of NMs. This presentation will provide a summary of the topics raised during these expert discussions.

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Update on 3Rs activities at the EU institutional level and beyond the borders

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Since the ban on animal testing to cosmetics in 2013, EU has set new standards for the rest of the world. The work on implementing 3Rs has progressed in many other sectors. New directions and opportunities have arisen within EU agencies to strengthen current actions. This oral presentation intends therefore to provide to the audience a global overview of the current and latest development at the EU institutional level and EU agencies (e.g. EFSA, ECHA) regarding 3Rs activities within the policy arena and beyond. Among others, this will be an opportunity to give an update on the European Parliament with the newly elected Members as well as the legislative proposals being currently discussed such as TTIP (transatlantic trade and investment partnership), the European citizenship initiative "stop vivisection", the revised structure of the new Commission and the latest news linked with endocrine disrupters since dialogue with stakeholders has started again. It is also intended to present upcoming research calls under Horizon2020 and IMI2 to maximize 3Rs community participation.

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Teaching alternative methods in Toxicology in Veterinary Science

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In AY 2014/2015, a mandatory module was introduced for students with a Bachelor's degree in the sciences at the University of Milan, Master of Veterinary Biotechnology Sciences titled "Alternative Methods in Toxicology". The module is 4 credits, consists of 30 hours including 18 hours oflectures and 12 hours of practice. The theoretical part provides the description of methods and 3Rs application in toxicological research, with particular focus on new perspectives and features the participation of experts in the field (including videoconferencing). The practical part includes demonstrations of *in vitro* replacement models, bioreactors and examples of Integrated Testing Strategy. A specific lesson is reserved for the illustration of *in silico* models provided in ad hoc facilities. Students are also hosted by the Italian Reference Center for Alternative Methods, Welfare and Care of Laboratory Animal. A dictionary with an updated nomenclature is also provided to students. At the end of the course, suggestions are collected from students through a questionnaire, on the possibleimplementation of 3Rs in toxicology in different application, specific methodologies and priorities. In the second year of this Master course, from AY 2015/2016, a module specifically focused on *in vitro* toxicology titled Toxicology and *in vitro* models will be introduced. The course is mainly practical, consists of 24 hours including laboratory activity supported by lectures (6 hours).

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In vitro effects of zinc oxide nanoparticles on human intestinal Caco-2 cells

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The field of nanotechnology is rapidly growing with the continuous development of nanomaterial-based commercial products. Zinc oxide nanoparticles (ZnO NPs) are one of the most abundantly used NPs in commercial products such as cosmetics and sunscreens, electronic materials, rubber production, pharmaceuticals, textiles, paints and dental cements. Moreover, ZnO NPs are used in the food industry as additives and in packaging due to their antimicrobial properties. Since ingestion may be an important route of human exposure to ZnO NPs, the aim of the present study was to investigate the toxicological effects of ZnO NPs on human intestinal Caco-2 cells cultured on semi-permeable inserts. These inserts allow for the separation of apical (Ap) and basolateral (Bl) compartments, corresponding to the *in vivo* lumen and the interstitial space/vascular systems of intestinal mucosa, respectively. ZnO NPs were synthesized by sonochemistry and fully characterized by HRTEM, XRD, TCA and ICP-MS. The experiment was performed after 21 days of culture when the differentiation process was completed. Caco-2 cells were treated for 48 h with different concentrations (1 μg/kg 10 μg/kg 25 μg/kg) of sono ZnO NPs and commercial ZnO NPs (Sigma-Aldrich, St. Louis, MO, USA) on both Ap and Bl sides. Both ZnO NPs were suspended in the culture medium and sonicated. Barrier impairment after exposure to ZnO NPs was assessed by measuring the trans-epithelial electrical resistance (TEER) after 1 h, 2 h, 24 h and 48 h of treatment. TEER values were recorded using an epithelial voltohmmeter. At the end of the experiment, interleukin-6 (IL-6) and interleukin-8 (IL-8) cytokine release in the culture medium was determined using a commercial ELISA kit. The results indicate that TEER was not significantly affected by Ap or Bl exposure to sono ZnO NPs or commercial ones at any tested concentrations. No significant release of the inflammatory mediator IL-6 and IL-8 was observed after 48 h of incubation. Further investigations are needed to better evaluate the effects of ZnO NPs on intestinal cells.

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Toxicological effects of copper oxide nanoparticles on Caco-2 intestinal cells

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The increased production and use of metal oxide nanoparticles (NPs) increases the probability of human exposure by inhalation, ingestion and dermal contact. The potential hazardous effects of copper oxide (CuO) NPs have not been adequately studied so far. The aim of the present study was to investigate the possible effects of CuO NPs on the intestine and barrier integrity by using Caco-2 cell line, a well-known *in vitro* model of intestinal barrier. CuO NPs were synthesized by sonochemistry and fully characterized by HRTEM, XRD, TCA and ICP-MS. Caco-2 cells were cultured for 21 days on semi-permeable inserts which separated the apical (Ap) compartment (corresponding to the *in vivo* intestinal lumen) from the basolateral (Bl) compartment (which *in vivo* faces the interstitial space and the vascular systems). At the end of the differentiation process, Caco-2 cells were treated for 24 h with different concentrations (10 μg/kg 50 μg/kg 100 μg/kg) of sono CuO NPs and commercial CuO NPs (Sigma-Aldrich, St. Louis, MO, USA) on both Ap and Bl sides. Both CuO NPs were suspended in the culture medium and sonicated. To assess barrier integrity after exposure to CuO NPs, the trans-epithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter after 1 h, 2 h and 24 h of treatment. At the end of the incubation time, the culture medium was collected for interleukin-6 (IL-6) and interleukin-8 (IL-8) cytokine determination (Human IL-6 ELISA Kit and Human IL-8 / CXCL8 ELISA Kit, Sigma-Aldrich, St. Louis, MO, USA). The results indicate that TEER was not affected by Ap and Bl exposure to commercial CuO NPs at any of the tested concentrations. On the contrary, sono CuO NPs at 50 μg/kg and 100 μg/kg were found to significantly decrease TEER. This decrease in TEER occurred after 24 h of incubation when sono CuO NPs were applied on the Ap compartment and occurred from the first hour of treatment when sono CuO NPs were applied on the Bl compartment. After 24 h of Bl treatment, both the sono and commercial CuO NPs at the highest dose tested (100 μg/kg) induced a slight IL-6 production. At 50 μg/kg and 100 μg/kg, both the sono and commercial CuO NPs markedly induced IL-8 release after Ap or Bl treatment. A notable release of IL-8 was also observed after Bl exposure to the lowest concentration of sono CuO NPs (10 μg/kg). In conclusion, the results indicate that only sono CuO NPs were able to impair Caco-2 cell barrier integrity, and the effects were observable even after a short period of exposure (1 h). Both sono and commercial CuO NPs induced release of IL-6 and IL-8, with the latter to a greater extent, suggesting that these NPs may cause intestinal inflammation.

2.

Skin sensitisation AOP and structured approaches to data integration

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cancelled

The understanding of the skin sensitisation Adverse Outcome Pathway (AOP) provides a solid scientific basis for the development of integrated approaches to testing and assessment (IATA). Within such AOP-based IATA the different information sources used (e.g. *in silico*, *in chemico*, and *in vitro* data/predictions) would target key events along the toxicity pathway. For skin sensitisation assessment, several possibilities exist on how to combine different information sources depending on the specific regulatory need and the chemical(s) under investigation. A number of approaches are being proposed on how to integrate data from various information sources within IATA for both skin sensitisation hazard and risk assessment purposes. Given the multitude of possible solutions, as an initial step to consistent evaluation and application of IATA within countries, the OECD is currently developing a Guidance Document that aims to facilitate a harmonised approach for the reporting of IATA by providing: 1) a set of principles for describing and evaluating IATA, 2) templates for reporting structured approaches to data integration and individual information sources used within IATA and 3) case studies exemplifying how approaches for skin sensitisation hazard identification and risk assessment should be documented.

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An advanced 3D human epithelial airway model to study the biokinetics of aerosolized biomedical nanoparticles after repeated exposures

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The exceptional characteristics of gold nanoparticles (AuNPs) are considered as attractive candidates for biomedical applications. In particular the inhalation route has gained interest as an innovative approach for diagnosis and treatment of respiratory tract disorders. Thus the potential use of AuNPs as carriers of inhalative drugs or as pulmonary vaccines requires a better understanding of their interaction with lung cells and possible adverse effects. In addition, there is no data available regarding the biodistribution and accumulation of AuNPs after repeated exposures. Our aim was therefore to investigate the uptake, translocation and possible effects of aerosolized fluorescently labelled and polymer surface modified AuNPs (NH2-(PVA+PEG)-AuNPs) to a sophisticated triple cell co-culture of the human epithelial airway barrier composed of epithelial cells, macrophages and dendritic cells, cultivated at the air liquid interface (ALI) upon repeated exposures (every 24 h for three days). Assessment of the particle uptake and translocation by inductively coupled plasma optical emission spectroscopy (ICP-OES) has shown that AuNPs were accumulated inside the cells over three days of repeated administration, while particle translocation beyond the epithelium significantly decreased on day 3 compared to day 1. Flow cytometry measurements revealed that particles were in all cell types, however, macrophages showed a significant higher particle uptake compared to epithelial and dendritic cells, during the exposure period. Repeated exposures of AuNPs did not show evidence of increased cytotoxicity, nor did they elicit an increase in the secretion of the pro-inflammatory mediators (IL-1β, TNF-α and IL-8). However, cells exposed repeatedly to AuNPs for three days, did showed a significant increase in cell apoptosis on day 3. In conclusion, our approach has allowed for the first time the investigation of the biokinetics and possible effects of AuNPs aerosols after repeated administration in an advanced 3D *in vitro* model of the human epithelial airway barrier. We have shown that there is an accumulation of AuNPs after repeated exposure inside the cells resulting in an apoptotic response. Further experiments are ongoing to evaluate if the effects are reversible and if the response is induced by the polymer only.

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Legal issues in the work of the German National Committee: an overview

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The scope of activity of the National Committee (NC) includes legal advice on the interpretation of German and European animal welfare law. The inquiries coming from the competent authorities and animal welfare bodies show, which issues need clarification. The presentation will give a brief overview on the current legal issues arising under the German law after the implementation of the Directive 2010/63/EU and have been addressed by the competent authorities and animal welfare bodies. One of the questions submitted to the NC concerns the legality of killing surplus of genetically altered animals bred for scientific purposes. § 1 of the German Animal Welfare Act states, that no animal shall be killed without a "reasonable cause" (*vernünftiger Grund*). This indefinite legal term is understood as a specific form of the principle of proportionality, so that the legality of killing must be examined taking into account the exact circumstances of every particular case. Since the breeding of genetically altered animals that may experience pain, suffering or lasting harm is considered as an animal experiment under the Directive, so that an authorization is needed, the problem of the animal surplus emerging from breeding procedures became more visible. As there is neither jurisprudence nor literature discussing this problem, the NC has been asked for a statement. The German NC is pursuing an approach of a close cooperation of legal and scientific experts while interpreting the animal welfare law. Only having those two competences combined it is possible to deliver solutions that are lawful and feasible at the same time. The example mentioned above will illustrate how the NC is working on the legal field.

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Predicting protein targets for chemical toxins using molecular similarity search, *in silico* docking and *in vitro* validation

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The adverse outcome pathway (AOP) framework starts with the molecular initiating event that describes the interaction of a chemical with its macromolecular targets. Although significant amounts of information about the biological activities of toxins and toxin-target interactions are already collected using experimental methods and stored in a variety of public databases, a lot of specific information remains missing. Computational methods can be used to fill up this data gap and to provide hypotheses for future experiments. We have developed an approach for predicting the protein targets of chemical toxins using a molecular similarity search of toxin-target information collected in the Toxin and Toxin-Target Database (http://www.t3db.ca). The developed method identified new targets for toluene which could be used to predict potential cellular toxicities and to validate the approach with *in vitro* laboratory studies. With this new method we obtained 124 potential targets for toluene from a molecular similarity search and the results were further analyzed using *in silico* molecular docking methods. The binding of toluene to two proteins, hemoglobin and serum albumin, was validated by the measurement of binding using microscale thermophoresis. These results demonstrate the applicability of this exploratory *in silico* toxicity tool, based on a molecular similarity search and protein-ligand docking for identification of potential targets for chemical toxins.

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Good In Vitro Method Practices (GIVIMP) as a basis for the development of harmonised standards for *in vitro* bio-barrier methods

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Good In Vitro Method Practice (GIVIMP) is a proposal from EURL ECVAM for an international Guidance document on good *in vitro* method practices for the development and implementation of *in vitro* methods for regulatory use in human safety assessment. The project was recently accepted and added to the OECD Test Guideline work programme. The purpose of the GIVIMP guidance is, taking into account all necessary good scientific, technical and quality practices, to ensure that the overall process from *in vitro* method development to *in vitro* method implementation for regulatory use becomes more efficient and effective. Some of the elements of GIVIMP are put into practice with the follow-up activities of EURL ECVAM's strategy for achieving 3Rs impact in the assessment of toxicokinetics and toxicity. It is felt that progress in the field of toxicokinetics and the availability of relevant and reliable *in vitro* methods for processes of absorption, distribution, metabolism and excretion (ADME) will benefit from the establishment of standards for human *in vitro* ADME methods. An approach will be presented aiming at harmonising and standardising *in vitro* methods for human external biological barriers (skin, lung and intestine) to increase their reliability and relevance for regulatory decisions. The presentation will describe how GIVIMP lays the fundaments for emerging standards for *in vitro* bio-barrier methods.

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Non-animal replacements for acute toxicity testing

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Human adverse reactions to chemicals currently accounts for a large volume of *in vivo* research through animal modelling. In spite of the EU Cosmetics Regulation, REACH still necessitates the use of animals to predict human acute toxicity in response to cosmetic ingredients, when those ingredients are also destined for use in other industries. Notable scientific deficiencies and low transferability to humans are widely recognised characteristics of *in vivo* approaches, such as the LD_{50} test, the Up and Down Procedure, and the Fixed-Dose Procedure. There is a clear vacancy for suitable methodology to forecast acute toxicity responses that replaces the demand for animal use. This project aims to develop a truly animal-free test for acute toxicity to cosmetic ingredients in humans. It will do so by using human-derived cells to expand on previous *in vitro* tests such as the animal cell-based 3T3 Neutral Red Uptake (NRU) assay, using a prediction model that does not rely on animal data. Generation of an *in vitro* protocol that can be validated as an accepted replacement to the currently available tests will be accomplished by incorporating the NRU assay with human dermal fibroblasts in animal product-free culture, with funding provided by Innovate UK. Using sodium lauryl sulphate (SLS) as a positive control, the project has so far established successful assessments of the robustness and reproducibility of the methods. The IC_{50} values for SLS generated for the human dermal fibroblasts in animal-free culture ranged from 37.7 μg/ml to 75.3 μg/ml and are similar to the historical mouse 3T3 cell studies (27.7 μg/ml to 64.7 μg/ml) [1]. As the next step, five known ingredients from key functional groups (surfactants, preservatives, fragrances, colours, and emulsifiers) were selected and put through initial range-finding runs followed by three valid runs. The results further confirmed the reproducibility of the SLS runs and displayed variable toxicity between ingredients, as anticipated. This helped to establish appropriate concentration ranges, as well as IC_{50} and IC_{20} values. Expanded blind trials of 20 ingredients will follow, allowing the development of a robust prediction model. Early results indicate that this human cell-based test holds the potential to replace aspects of *in vivo* animal acute toxicity testing, particularly with reference to cosmetic ingredients.

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Cellular human based systems for investigating the toxicity effects of inhalable substances

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Until now, only animal models are validated and used in OECD test guidelines for inhalation toxicity testing. In order to promote and implement the 3Rs principles, *in vitro* alternative methods are urgently needed and should be developed. However, the *in vitro* alternative methods have to be relevant and reliable: they should closely resemble and mimic the key events that are known to occur *in vivo*. Ideally, the *in vitro* assays should be human cells/tissues-based, with specific readouts/endpoints targeting known signaling pathways. Nevertheless, any relevant *in vitro* alternative methods should be developed and used [1]. Thus, a survey of the existing models relevant for *in vitro* inhalation toxicity testing will be presented, including cell lines, primary cells, 3D Air-Liquid Interface tissues, co-cultures models and explants [2]. Pros and cons of each model as appropriate tools for acute and repeated dose inhalation toxicity assessment will be discussed.

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Assess the bio-barrier function of the human respiratory system using an *in vitro* 3D model of airway epithelial model (MucilAir™)

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The human airway epithelium is an essential biobarrier which protects the human body against external insults, such as pathogens and all kinds of chemicals. In order to better preserve the normal function of this important biobarrier, it is crucial to understand how pathogens and chemicals interact with the human airway epithelial cells. Using MucilAir™ [1], a standardized human airway epithelium model, both morphologically and functionally fully differentiated, which can be maintained at a homeostatic state for more than a year, we studied:

– The effect of viral infection on respiratory biobarrier function
– The ability of various chemicals to across the respiratory biobarrier

We showed that MucilAir™ can be infected by various respiratory viruses such as influenza virus, rhino-viruses, even the type-C human Rhinoviruses, which is extremely difficult to grow on other *in vitro* cell models. Moreover, the replication is almost completely inhibited by novel antivirals agents such as Rupintrivir. These results demonstrate that MucilAir™ is a reliable and powerful tool for anti-viral and anti-bacterial drug development [2]. To assess the ability of different chemicals to across the respiratory biobarrier, we developed a standard operating procedure and its transferability and reproducibility was evaluated using 6 chemicals (propranolol, atenolol, nicotine, cadmium-chloride, cobalt-chloride and ammonium-hexachloroplatinate) in two independent laboratories. A panel of 30 compounds was further tested to evaluate the ability of the assay to rank relative permeability. A comparative permeability study between nasal and bronchial epithelium has been performed. The results showed: (i) a higher permeability of the airway epithelium for organic compared to inorganic compounds and (ii) a low transporter-mediated efflux involved in the permeability. The study indicates that this MucilAir™ -based assay represents a promising tool to evaluate respiratory absorption giving input parameters for PBTK modeling.

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Evaluation of selumetinib and trametinib MEK inhibitors antitumor efficacy in a preclinical *in vitro* human lung cancer model

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Rationale: With more than 1 million deaths worldwide every year, lung cancer remains an area of unmet need. Accessible human *in vitro* 3D tissue models are required to improve preclinical predictivity [1]. OncoCilAir™ is a new *in vitro* model of Non Small Cell Lung Cancer which combines a reconstituted human airway epithelium, human lung fibroblasts and lung adenocarcinoma cell lines. Remarkably, we found that in this 3D microenvironment tumor cells expand by forming nodules, mimicking a human lung cancer feature. In this study we used this human *in vitro* lung cancer model to evaluate the antitumor efficacy of the MEK inhibitors selumetinib and trametinib compared to the EGFR-TK inhibitor erlotinib and the taxane derived anticancer agent docetaxel [2].

Methods: OncoCilAir™ tissue cultures mutated for KRAS and expressing the green fluorescent protein were treated with the investigational drugs over a period of 35 days. As primary endpoint, changes in tumour size were assessed by fluorescence measurements. Drug activity was confirmed by western blot on the activated (phosphorylated) form of the MAPK pathway intermediate ERK and KI67 immunostainings. Side-toxicity was evaluated by monitoring changes in cilia beat frequency and transepithelial electrical resistance (TEER) and by measuring the amount of lactate dehydrogenase released during the treatment.

Results: Tumors showed a reduced growth in response to the MEK inhibitors (TGI > 90%), but halting selumetinib dosing resulted in tumour relapse. As expected, erlotinib treatment did not show any effect on KRASmut tumors, validating the specificity of the model. Importantly, histological examination and toxicity study on the normal part of the cultures revealed that the airway epithelium integrity was differently affected by chronic anticancer drug treatments, with docetaxel showing the most significant toxicity.

Conclusions: OncoCilAir™ is a unique integrated *in vitro* model which reproduces human tumour pulmonary nodules surrounded by a functional airway epithelium and fibroblasts. This long term tissue culture modelling a KRAS-mutant adenocarcinoma showed responsiveness to anticancer drugs in agreement with previously reported data, and therefore can be used as a predictive tool for anticancer drug evaluation, while reducing animal testing.

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Biophysical and metabolic properties of striae distensae evaluated *ex vivo* compared to normal skin

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BACKGROUND: When testing transcutaneous passage, company works mainly on abdominal skin, and they use skin disks that are striae distensae (SD) free. Yet it is interesting to know whether one can extrapolate the results obtain from normal skin disks to skin with SD, as it is now assumed. Moreover, 70% of women in their midlife have SD, and with the obesity rate increasing, a larger number of men and women will be concerned. The aim of this study was to determine whether the barrier property was comparable between the two skin types. Moreover, the metabolic activities, also important when studying the toxicology of a cosmetological product with a topical application, were also compared in the skin with and without SD. METHODS: Biological materials were obtained from abdominoplasty and all studies were performed *ex vivo*. Different parameters were compared between donors' skin with SD and adjacent uninvolved skin to characterize: skin barrier function by measuring transepidermal water loss (TEWL), skin surface hydration using corneometry (skin capacitance), PH skin surface, and CYP1A and esterase activity. RESULTS: No difference was observed in skin barrier function when looking at transepithelial water lost. Other biophysical properties such as PH or hydration were also similar when compared to adjacent skin. Yet the metabolic activity of the phase I enzyme was different. Esterase activity is greater in SD compared to normal skin from the same donors. Likewise, CYP1A is more inducible in SD by 3-methylcholanthrene (3MC). When looking closer at these anatomical differences, it seems that the differences come from both the dermis and the epidermis. When the epidermis is less responsive to induction for SD, the basal level of CYP1A activity is greater in the dermis of the SD. CONCLUSIONS: Based on these results, the distinct metabolic features characterizing SD lesions are to be taken into consideration when making toxicological studies. These changes however do not seem to affect the skin barrier, making skin with SD acceptable material to work on, when testing transcutaneous passage.

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Refinement of orofacial surgical experiments in the Göttingen minipig™: morphometry of the mandibula by 3D-computed tomography

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Introduction The Göttingen Minipig™ is a frequently used large animal model for orofacial research, in particular for dental implant surgery. Requests from experimental surgeons for detailed anatomical information is unavailable, because the data required does not exist. Thus, surgical interventions fail or lead to post-operative suffering. The aim of this study is to obtain detailed anatomical data of the mandibula without sacrificing pigs for this reason. Animals, materials and methods CT scans of a 64-slice scanner were collected from 18 female minipigs, consisting of 6 animals aged 12 months (group 1, n = 6) and 12 animals (group 2 n = 12) examined at the age of 17 and 21 months. These minipigs were involved in experiments, approved by the Regional Office for Health and Social affairs, Berlin. Image analysis was performed using Vitrea Advanced® (Vital images). More than 50 parameters concerning teeth, the mandibular body, frame and canal, coronoid process and mandibular condyle were defined and measured. For example, we focused on the distance between the dorsal border of the mandibular canal to the alveolar ridge at the most posterior mental foramen, a parameter immensely important testing new dental implants. Results Measurements of the CTs of the minipigs mandibles' demonstrate variations of several parameters between left and right ramus mandibulae and within the different age groups. The distance between the dorsal border of the mandibular canal to the alveolar ridge decreases between 12 and 17 months of age. Comparing group 2 and group 3 no significant difference in distance could be observed. From the age of 17 months the position of the mandibular canal in relation to the alveolar ridge remains constant. Conclusion The decrease of the distance between the mandibular canal and the alveolar ridge between 12 and 17 months of age indicates ongoing anatomical changes of this parameter until the age of 17 months. Therefore animals should be older than 17 months if included in long-term studies after orofacial operations, like implant surgery of the mandibula.

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A microfluidics integrated electronic monitoring system of a 3D *in vitro* model of the renal tubule

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The development of reliable *in vitro* models for toxicological testing and pharmacological investigation of chemical substances is urgently required to move away from animal testing and animal models. However, the predictive ability of *in vitro* based models is limited by poor reproducibility of the microenvironments and the physiological behaviour of tissue in organs. Limitations in technology are a major factor in the failure of the cell-based models. "organ-on-a-chip" technology represents enormous potential to transition from stand-alone 3D tissue models to more integrated micro-fluidic devices, in which a network of micro-channels is used to transport nutrients and other soluble substances to 3D cell culture, e.g. cell cues and drugs. In this context, the emergence of organic bioelectronics provides a unique opportunity to interface *in vitro* "organ-on-chips" with a dynamic in-line monitoring system. Organic bioelectronics has, in the past decades, made available materials and devices that interface with biology [1]. In particular, the organic electrochemical transistor (OECT) is a device that has been used extremely successfully to monitor brain activity *in vivo* [2] and also to monitor the integrity of epithelial tissue with state-of-the-art sensitivity and rapidity [3]. Here we demonstrate a highly performant 3D model of the kidney tubule integrated with microfluidics and in-line monitoring with on-board electronics. Multi-parameter monitoring of relevant parameters, including TEER, and glucose levels, are being monitor under fluidic conditions to predict renal tubule toxicology. The system has been designed in a multi-well format, for simultaneous optical and electronic acquisition mode.

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Characterization of molecular and cellular effects of thyroid hormone within an AOP frame for thyroid hormone disruption using human and murine neural progenitor cells

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Modern risk assessment requires building of adverse outcome pathways (AOPs), which describe the effects of toxicants starting from a molecular initiating event (MIE) and ending with the adverse outcome (AO) of an individual or a population by affecting causally related key events (KE). We have created putative AOPs for thyroid hormone (TH) disruption, which, by distinct initiating events, cause decreased TH levels in the developing brain leading to the AO of mental retardation. The causes of decreased brain TH levels (early KEs) are partially understood and the pathophysiological findings of decreased white matter due to reduced myelin (late KEs) are at least one cause of the observed mental retardation in humans with brain TH deficiency. However, intermediate KEs related to the cellular effects of TH deficiency, especially in a species-specific context, are so far poorly understood. This project thus aimed at closing this gap by studying TH effects on human and murine neural progenitor cell (h/mNPC) functions. Moreover, from the generated knowledge an *in vitro* test for predicting human TH disruption was supposed to be developed. NPCs are cultured as floating neurospheres and mimic the basic processes of brain development: proliferation, migration and differentiation into neurons, astrocytes and oligodendrocytes. Due to the necessity of TH for myelin production, we first evaluated the effects of the TH triiodothyronine (T3) on oligodendrocyte formation and maturation of h/mNPCs. T3 induced the formation of oligodendrocytes from mNPCs but not from hNPCs, indicating that murine, in contrast to human oligodendrocyte formation, is TH dependent. In addition, the maturation of oligodendrocytes was increased by T3 in both species as T3 induced human myelin basic protein (MBP) or murine myelin oligodendrocyte glycoprotein (MOG) expression normalized to the amount of produced oligodendrocytes. Analyses of T3 effects on mNPCs lacking TH receptors (THR) revealed that THRalpha mediates mouse T3-dependent induction of oligodendrocyte formation and maturation. Based on these data, we determined that forming the ratio (Q) of the TH-regulated process MBP expression and the process oligodendrocyte formation could provide us with the information if a compound is a TH disruptor for human NPCs. Thereby, Q of TH and test compound (Q-TH&TC) has to lie in-between Q TH alone (Q-TH) and Q of the control (Q-C). We tested this hypothesis by employing two putative endocrine disruptors, NH-3, a THR antagonist, and BDE-99, a putative TH disrupting flame retardant. As expected, NH-3 reduced Q-TH&TC to the expected margin, while unexpectedly BDE-99 did not. Although BDE-99 did reduce human MBP expression, this was due to a TH-independent reduction in oligodendrocytes in the cultures. In summary, our work added to a putative AOP framework for TH disruption by unraveling species-specific TH effects on oligodendrocyte formation and myelin production. Since human oligodendrocyte maturation is a specific, TH-dependent process, we developed an *in vitro* test for studying this endpoint for elucidation of human TH disruption in developing brain cells. More compounds are needed to calculate the predictivity of this assay.

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Induced cytotoxicity revealed species diversity of rat and human lung tissue by repeated chemical exposure

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The use of high animal numbers and the suffering of animals in experiments such as repeated dose toxicity studies (RDT) demand for alternative test approaches. This can be done by grouping of chemicals to reduce *in vivo* studies. Therefore, read-across (RAX) approach is purposed to reduce animal numbers as one or several tested source compound(s) are used to predict the toxicity of "similar" non-tested target compound(s). Similar compounds shall share structural- and physico-chemical properties as well as a similar mode of action. RAX approach can be used for filling data gaps in human risk assessment. This study was designed to evaluate the use of data from *ex vivo* experiments such as rat or human precision-cut lung slices (PCLS). Three RAX-categories were tested, namely vicinal halogenides, naphthalene derivatives and vinyl esters. Each RAX category was selected based on shared structural properties and similar toxicological effects in RDT studies extracted from the FhG RepDose database (http://www.fraunhofer-repdose.de). Repeated chemical exposure of rat and human PCLS was performed on three days for three hours daily. The cytotoxicity of chemicals was assessed by LDH and WST-1 assay. *Ex vivo* IC_{50} values were calculated by sigmoidal curve fitting. These values were correlated to the public available *in vivo* LD_{50} values. Cytotoxic effect was assessed in dose dependent manner. Vicinal halogenides and naphthalene derivatives were less toxic in human lung sections compared to rat sections, whereas the vinyl esters showed the comparable cytotoxicity in both species. Linear regression analysis of public available LD_{50} values to the obtained *ex vivo* IC_{50} values showed good linear correlation. Thus only one substance group showed similar cytotoxicity in both tested species, whereas two other groups revealed interspecies diversity based on cytotoxicity endpoint. This first evaluation of rat and human and PCLS show that *ex vivo* chemical testing in human lung sections result in a promising approach for toxicity profiling as no human *in vivo* reference data exist.

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Optimization of a new *in vitro* method for inhalation toxicology and its application for the study of zinc oxide nanoparticle toxicity

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Nanoparticle toxicity and, more specifically, inhalation toxicology is becoming a profound concern to human health and requires many experimental animals. Hence, the development of *in vitro* screening methods to reduce animal use is much-needed. Currently, most of the screening methods ignore the lung surfactant (LS) layer, the first barrier inhaled particles meet. LS serves the vital function of reducing the surface tension in the air-liquid interface at the end of the expiration. Damage to LS function, resulting in minimal surface tension increase, will induce alveolar collapse *in vivo*. The force needed to open the collapsed areas will induce shear stress to the alveolar epithelium, leading to serum proteins leakage and further inhibition of the LS function, starting a vicious cycle. Here we introduce an innovative method adapted from the constrained drop surfactometer (CDS) [1]. The modifications made allow directly exposing a surfactant drop to an aerosol of particles. The potential of the adapted CDS as a new tool supporting the 3Rs principle to minimize animal testing is evaluated by studying zinc oxide nanoparticles (ZnO NP) toxicity. The CDS consists of a drop of surfactant (Curosurf®) sitting on a pedestal connected to a stepper motor syringe. A dry-powder inhaler, Turbuhaler®, generates an aerosol of particles which deposits on the LS drop. A quartz crystal microbalance located in the chamber directly relates the change in frequency of the crystal to the deposited mass per unit area. The minimal surface tension of the drop after compression (γmin) is determined by the means of a drop shape image analysis software. The air in the chamber was continuously exchanged, simulating breathing. Curosurf® reached low values of minimal surface tension (around 5 mN/m) under these conditions, with a physiologically relevant compression rate below 50%. The volume, and thus the surface area, of the drop were cycled in accurate increments and frequency to mimic lungs movements during breathing. The minimal surface tension of LS increased in a dose-dependent manner from 5.5 ±0.6 mN/m (control) to 15.1 ±1.4 mN/m at a ZnO concentration of 400 ng/cm^2, after which the effect reached a plateau (γmin=15.5 ±0.4 mN/m from 400 to 1200 ng/cm^2) (n = 5). A 2-fold decrease in the dose-rate induced a lower increase of γmin (slighter inhibition of the lung surfactant function) (n = 5). The time required to reverse the effect depended both on the initial deposited dose and the surfactant concentration. The cycling of the drop is essential for the recovery of the LS function. Taken together, these outcomes suggest a dynamic interaction between ZnO NP and surfactant components where new, naïve and effective, components could move to the air-liquid interface of the drop and restore the surface activity of LS. Surprisingly, the exposure of Curosurf® to Zn^{2+} ions did not inhibit the surface active properties of LS. We demonstrate here the physiological relevance of the adapted CDS system. Its performance and its successful application to evaluate the toxicity of ZnO NP make this *in vitro* method a promising candidate for inhalation toxicology in line with the 3Rs principle.

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Advances in *in vitro* testing: the route to replace animal testing

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One of the biggest pressures for innovation is necessity, and this is nowhere truer than in the pharmaceutical industry which has come under increasing pressure to overhaul the drug discovery and development process. The challenge is to fix the high rate of attrition, whereby too many promising drug candidates fail at late stages in the process. Although assays developed using static cell culture techniques can provide valuable insights, the limitations of these systems in only partially replicating normal biological processes impacts on the utility and reliability of the resultant data. Advances in the area of *in vitro* testing offer one of the most promising solutions to tackle this huge attrition problem. *In vitro* models have become more sophisticated and are now at a stage where they can provide an effective alternative to some *in vivo* experiments Creating an *in vitro* environment which encourages cells to behave in the way they do in their natural situation allows for the development of more predictive models as well as providing the framework to capture more of the complexities of normal biological function and even replicate inter-individual variability. Cells experience nutrient gradients, pressure and flow, and they communicate with each other. Cells cultured in static media in well plates, whether primary cells or cell lines, cannot replicate this highly organised structure. Quasi-Vivo® perfusion systems – which provide nutrient flow and allow for co-culture and the development of 3D structures – are being used to culture cells with improved viability and function. Cells cultured in this environment have been shown to retain phenotype and exhibit long-term metabolic competency. This increases the prospect of obtaining more meaningful data from *in vitro* assays. Some preliminary results are presented that illustrate the potential for advanced *in vitro* cell culture, including the use of live cell imaging in the QV900. The Quasi-Vivo® perfusion systems provide a stepping stone towards the more speculative organ-on-a-chip or human-on-a-chip technology. A key part of the strategy to gradually replace animal testing is to find some common types of experiment that can be done better *in vitro* than *in vivo*, and that doing so will result in both scientific and ethical gains. This paper will include "case studies" that illustrate the ways in which culture models can be used to answer a range of important biological questions of direct relevance to human development, physiology, disease and healing.

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Refinement of welfare through application of a quantitative system for assessment of lifetime experience

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The EU Directive 2010/63/EU on the protection of animals used for scientific purposes requires retrospective reporting of actual severity at the end of procedures, but retrospective assessment of severity experienced by individual animals would be most beneficial if based on the collection of data as experiments progress. Furthermore, prospective ethical justification of a scientific procedure will be a balance between harm to the animals and benefit to society from the knowledge gained. Levels of harm and cumulative severity will be affected by how the work is conducted in the context of application of the 3Rs: this will include elements of contingent and direct suffering. Implementation of the refinement loop will assist in reducing the level of cumulative severity but assessment of animal welfare depends on interrelated parameters which will vary according to species, environment and procedure. Public Health England and Surrey University Veterinary School have collaborated to develop a system that uses intrinsic study data to provide a clear visualisation of the stresses involved during an animal's life history applicable to all types of studies, even those not requiring invasive techniques [1]. Thus it provides an opportunity for researchers to identify and refine key events which impact on the welfare of animals, and to explain clearly the totality of any necessary harms when justifying research to prospective funders or at ethical review. Currently there are few tools to assess the effects of lifetime events on welfare or even, in some cases, to recognise that they have an impact on the level of suffering. A matrix to assess the combined effects of environment, experimental and contingent events on welfare has been applied retrospectively to programmes of work involving macaques. Lifetime records, available for animals from their birth in the breeding colony through to experimental use in vaccine efficacy evaluation studies, were analysed as a robust validation test for the assessment matrix and refinement of the way in which information on these events is captured. The strength of the system is the ability to evaluate where improvements can be made within a study or programme of work and we have demonstrated that the system allowed areas of environment, practices, contingent events and experimental design to be identified as the particular cause of any change in welfare, enabling refinements to be focussed appropriately to maximise improvements in welfare wherever possible and assisting future study design.

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Preventing unnecessary testing by means of read-across: scope, justification and assessment

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Read-across is based on the fact that the toxicological properties of substances are ultimately based on their chemical structure. Similarity of chemical structures thus indicates possible similar effects for certain toxicological endpoints. However it is also true that apparently small differences in chemical structure can strongly influence the toxicological properties of a substance. This means that a perceived structural similarity hardly ever provides a robust basis for read-across on its own not surprising in view of the multifaceted and elusive character of structural similarity as a concept. The core of read-across should therefore always consist of an explanation why read-across is possible in a particular case. A mechanistic explanation or the analysis of data of a group of substances should convince that properties to be read across are either 1) not be influenced by the inevitable structural differences, but solely depend on common structural features, or 2) are influenced by the structural differences in a regular manner. Additional supporting evidence is often necessary to make read-across sufficiently convincing. It is more and more recognized that the new approaches in toxicology, such as *in-vitro* techniques, HTS and omics, might play a crucial role in strengthening read-across cases and might, thereby, help to make the most of already available traditional data in hazard assessment. The use of read-across introduces additional elements of uncertainty compared to actually testing the substance under consideration. Not accepting that, in many cases, there may be greater uncertainty would strongly affect the value of read-across e when it comes to the prediction of properties and the scope for reducing animal testing. It is, therefore, essential that read-across, as well as its assessment, takes account of the possible sources of uncertainty. The extent to which uncertainties are identified and addressed depends on the scope and purpose of the read-across. Obviously, prioritization for either toxicity testing or investments in further development differs in this respect from meeting obligatory information requirements. The REACH Regulation encourages the use of read-across under certain conditions as an alternative to test results for meeting the so-called standard information requirements. The outcome of the read-across should in a way be as good as the test result. In other words, it should be possible to use it in the context of REACH with the same purpose as the test result. ECHA has developed a framework (the Read-Across Assessment Framework or RAAF) to consistently assess whether a specific read-across can indeed fulfil this role in REACH. This framework separately addresses the essential aspects of read-across and results in indications of confidence on different aspects of the cases made by registrants. An outline of the RAAF will be presented.

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Reconstructed Human Epidermis Test Methods for Skin Corrosion Endpoint: How Can Changes in Prediction Models Improve Final Predictions?

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

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Human *in vitro* blood-brain barrier model generated from stem cells: reducing attrition in the central nervous system area

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Central Nervous System (CNS) disorders without effective treatment available constitute one of the key areas for drug development. Despite the ongoing development of brain targeted compounds, few drugs are today available due to the high attrition rate of this therapeutic area. With the aim to reduce attrition, Pangalos et al., highlighted two major recommendations for CNS drug discovery and development: 1. "develop *in vitro* systems physiologically relevant" and, 2. "pay careful attention to the pharmacokinetics and blood-brain barrier (BBB) penetration properties of compounds, and ensure adequate exposure of drugs to the CNS target". While many *in vitro* BBB models were developed, most were produced from animal cells. However, due to inter-species differences, cell-based drug screening and testing methods that use animal models are poorly adapted. Recently, advances in the use of human stem cells in the modeling of the BBB and the CNS has opened up opportunities to develop improved human *in vitro* physiological models able to transcribe more realistic *in vivo*-like responses to novel neuropharmaceuticals and neurotoxicants agents. With the aim to reduce the use of animal and also to avoid animal-human transposition problems, we have recently developed a human *in vitro* BBB model based on the coculture between endothelial cells derived from CD34+ stem cells isolated from cord blood and pericytes. This model: 1. display features which fit well with *in vivo* BBB data such as low permeability to small molecules (0.45 kDa), continuous expression of tight junction proteins (ZO-1, Claudin-5, Occludin, Jam-5, …) at the cell-cell contacts and expression of functional key efflux transporters (P-gp, BCRP, MRP), 2. is stable (more than 20 days in culture) and can be used in repeated dose experiments, 3. can be used to predict CNS distribution of compounds in Human, 4. achieve convincing results in many disease areas such as Alzheimer disease and Cancer brain metastases and, 5. show a very good intra- and inter-laboratory reproducibility. All these results demonstrate this human *in vitro* BBB model will improve transposition to clinical studies.

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Application of *in silico* methods for predictions of different toxicological endpoints

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To assess the toxicity of new chemicals and drugs, regulatory agencies require *in vivo* testing for many toxic endpoints, resulting in millions of animal experiments conducted each year. However, following the Replace, Reduce, Refine (3R) principle, the development and optimization of alternative methods, in particular *in silico* methods, has been put into focus in the recent years. Here, we present different *in silico* methods developed in the group which can be applied for predictions of various toxicological endpoints including median lethal doses, organ-specific toxicities including immunotoxicity and targets involved in the development of adverse drug reactions. The prediction methods comprise chemical similarity comparisons as well as so-called toxicophore models which encode structural information about binding pockets and known binders and can be used to predict possible toxicity targets. All methods have been evaluated on diverse external validation sets and displayed strong performance, indicating their universal applicability as alternative to animal toxicity testing. The methods are part of the publicly accessible ProTox toxicity prediction platform (http://tox.charite.de/tox) or will be integrated in the near future.

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Animal research: critical, challenging and creative thinking about the 3Rs

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This paper describes a new educational initiative to enable students to conduct life science research in a humane and responsible manner. Beyond the training modules needed to obtain a licence to perform regulated procedures under the revised Animal (Scientific Procedures) Act 1986 (ASPA), there is currently no requirement for 3Rs training in the UK. The RSPCA believes that all research studentships involving the use of animals or animal derived tissue, should include a clear and enforced requirement for students to undergo training in humane experimental technique, and the output of the student's progress should show clear reflection on the application of the 3Rs and ethical decision making in their work. Comprehensive training to improve skills and capabilities in ethical decision making, animal welfare and the Three Rs would reduce animal use and suffering, ensuring improved compliance with the regulations, as well as leading to increased scientific rigour. This should help to address at least some of the legitimate public concerns about the impact of science on animals, which would be a significant societal benefit. We present educational materials to deliver a two day course providing a theoretical and practical basis to facilitate the students' own ethical evaluation of the use of animals in experimentation. Students are empowered to identify their own critical research question(s), consider the best systems and methods of investigation, and openly question the validity and clinical relevance of *in vivo* work. The course is divided into four components, each comprising an introductory lecture, and a problem-based activity. Through peer-assisted learning, students are encouraged to critically evaluate current research and use this analysis to reflect on their own practice throughout the research process, from the development of a testable hypothesis through to the reporting of output. The components are: 1. Animal use in science: from a range of mora and ethical viewpoints. 2. Research Integrity and animal use: exploring factors that influence an individuals' research conduct. 3. Critical thinking about animal use: experimental design and effectively researching humane alternative. 4. Assessing animal welfare, implementing refinement and reducing suffering. This course was recently piloted as part of the Doctoral Skills Development Programme at UCL Graduate School, London. The RSPCA intends to make this set of educational resources freely available online in a format suitable for undergraduate and postgraduate training. By encouraging Universities across the world to access these resources, the RSPCA hopes to enable the next generation of life science researchers to be truly independent thinkers who challenge the status quo, and are confident when confronted with difficult ethical decisions to make appropriate choices that will minimise the suffering of other species whilst still achieving benefit to our own.

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Safety evaluation of nanosilver using reconstructed human GIT tissues

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Silver nanoparticles (AgNPs) have been used widely in food contact materials and cosmetics for their well known antimicrobial effects. Information on their toxicity has not been sufficiently evaluated yet, although there is a risk of accidental ingestion or misuse. Distinct studies suggest that silver nanoparticles may penetrate into the circulation system through the mucosa of the respiratory tract, gastrointestinal tract and/or through the skin. Oral ingestion of colloidal silver can increase the concentration of silver in the plasma and lead to accumulation of silver in the skin, which is later reflected by irreversible hyperpigmentation (argyria). Little is known about the metabolism of nanosilver and the interactions with metabolic enzymes. According to previous studies *in vitro*, silver nanoparticles may be cytotoxic for hepatocytes and may cause oxidative stress, DNA damage and apoptosis. In our study focused on safety evaluation of nanosilver the reconstructed human tissues EpiOral, EpiIntestinal, EpiIntestinal FT (MatTek) and Colon epithelium (Sterlab) were used as models mimicking the human gastrointestinal tract. Biological methods *in vitro* were applied for evaluating tissue viability, inflammation and Ag penetration in the model systems. MTT viability assay was employed for evaluating the cytotoxic effects of AgNPs in the tissues. ICP-MS and TEM were used for detecting the penetration of AgNPs into the culture medium underneath the tissues. ELISA method was employed for investigation of cytokine release suggesting inflammatory reactions. The results confirmed negative effect of nanosilver on the viability of the tissues even after exaggerated exposure. Penetration of nanosilver through the tissues, probably in the form of Ag ions, was confirmed by ICP-MS in a rate depending on the tissue type. The ELISA method did not confirm any significant (at least two-fold) increase of the inflammatory cytokines. The tested AgNPs samples did not elicit any adverse effects in the available reconstructed human GIT tissues.

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Conversion of the draft OECD guideline for the testing of chemicals: the Cytosensor Microphysiometer test method

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Because there is a high demand for alternative methods to animal experiments in the EU, efforts aimed at replacing animal testing are encouraged to find new technologies that follow the 3R approach. Traditional testing methods to determine ocular toxicity, such as the Draize Eye Test, evaluate the biological response by applying a chemical on a live rabbit´s eye. Developed as an alternative to the Draize Eye Test, the Cytosensor Microphysiometer (CM) is a cell function based *in vitro* assay that was successfully validated as a testing method for ocular irritancy [1]. As the CM is no longer available, there is a need for a new technology which is able to perform the tests. The aim of this project is to implement the draft OECD guideline using the Intelligent Mobile Lab for *In vitro* Diagnostics (IMOLA-IVD) technology [2]. Here, six fluidic modules regulate the selection of the testing and control solutions that are applied as a dilution series. One module without cells is set as a control to monitor any interactions of the testing material with the micro-sensor system, whereas two modules with cells are used as a positive and negative control. Three modules with cells are exposed to the test substance. In general, the Performance Standard (PS) of a validated test method is used to evaluate the reliability and relevance of a new test method. To transfer the PS of the CM Toxicity Test and the INVITTOX protocol #130 directly to our developed assay, we created a platform that measures the same biological effect as the CM Test Method. Following the "Principle of the Test", the assay is based on the measurement of changes in acidity of mouse L929 fibroblasts cultured on the biochip after an exposure to a dilution series. According to the "Application of the Test and Control Chemicals", Low-Buffer Medium is used as a negative control whereas Sodium lauryl sulfate is used for the positive control. As in the CM assay, seven increasing concentrations of a test chemical are supplied to the living cells. Further compliance with the INVITTOX protocol #130 was achieved by programming a stop & go mode of the pump. In order to introduce and measure the dilution series, different cycles (i.e. exposure, wash-out and measurement phases) with specific duration and flow rate are installed by programming the system with the specific parameters for the cycles. The presented system provides an automated platform that applies a dilution series of test substances and control solutions on living cells, and measures changes in the cellular metabolic rate mediated by exposure to a potentially toxic agent. This implementation demonstrates that the developed technology is capable of following the PS for the CM Toxicity Test to become a validated, alternative method for the Draize Eye Test.

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Integration of *in vitro* and *in silico* approaches to characterize endocrine activities of zearalenone and metabolites

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Within the framework of reduction, refinement and replacement of animal experiments, new approaches for identification and characterization of chemical hazards have been developed. Grouping and read across has been promoted as a promising alternative approach. It uses existing toxicological information on a group of chemicals to make predictions on the toxicity of uncharacterized ones. In the present work, the feasibility of applying *in vitro* and *in silico* techniques to group chemicals for read across was studied using the food mycotoxin zearalenone (ZEN) and metabolites as a case study. ZEN and its reduced metabolites are known to act through activation of the estrogen receptor α (ERα). The ranking of their estrogenic potencies appeared highly conserved across test systems including binding, *in vitro* and *in vivo* assays. This data suggests that activation of ERα may play a role in the molecular initiating event (MIE) and be predictive of adverse effects and provides the rationale to model receptor-binding for hazard identification. The investigation of receptor-ligand interactions through docking simulation proved to accurately rank estrogenic potencies of ZEN and reduced metabolites, showing the suitability of the model to address estrogenic potency for this group of compounds. Therefore, the model was further applied to biologically uncharacterized, commercially unavailable, oxidized ZEN metabolites (6α-, 6β-, 8α-, 8β-, 13- and 15-OH-ZEN). Except for 15-OH-ZEN, the data indicate that in general, the oxidized metabolites would be considered a lower estrogenic concern than ZEN and reduced metabolites.

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The official German "AnimalTestInfo" – a role model for more transparency on animal experiments through NTPs?

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Next to the ultimate objective of fully replacing the use of live animals for scientific and educational purposes, another important goal of Directive 2010/63/EU is to improve transparency. To achieve this goal it requires EU member states to ensure that applicants for an authorization of a project involving the use of animals submit a non-technical project summary (NTP) with their application. The NTPs are supposed to provide information on the purpose of the project, the desired prospective benefit, the number and types of animals to be used and the predicted harm the experiments will cause to them. Additionally, the implementation of the 3Rs principle must be demonstrated. According to the Directive, EU member states shall inform the public by publishing the NTPs. The EU-Commission solely made recommendations what the NTPs should contain the implementation of the Directive is each member state's own responsibility. The German Federal Institute for Risk Assessment (BfR) also published a guideline on creating NTPs which is a good basis to correctly fulfil the requirements of the Directive. The BfR has developed a database called AnimalTestInfo that contains the NTPs from Germany and released it in December 2014. We analysed NTPs published on AnimalTestInfo and judged them by transparency and fulfilment of expectations from animal welfare NGOs. We found that the database management system that informs AnimalTestInfo is a convenient tool to get an overview of ongoing animal experiments in Germany since it is user-friendly, clearly structured and includes a comprehensive search function. However, there is need of subsequent improvement of the NTPs' content. According to the BfR recommendations the entries will be deleted from the database after five years – the maximum time-frame of a project authorisation – thus, no follow-up inquiries or observations of the development of animal research in Germany are possible. Thus, in many cases, new NTPs will not allow identifying if a project is a follow-up project and thus finding out about the advancement of a project. In addition, there is a possible lapse of 15 months between authorisation and publication of the NTPs in AnimalTestInfo – that way the database will not deliver an up-to-date picture of the current situation of authorized projects involving animals in Germany. Our major criticism however is concerning the actual content of the NTPs. At present, the NTPs available in AnimalTestInfo are not a neutral description of projects but only display the subjective applicant's point of view because not the BfR but the applicants themselves are responsible for the content of the summaries. Thus, the NTPs provide only part of the required and relevant information. Many NTPs use technical language and terminology– against the European Commission's guideline recommendation and that of the BfR – and most of them do not include sufficient information about the supposed indispensability of the experiments and possible alternative approaches. Some even claim that the degree of pain and suffering is not assessable in advance. Thus, the NTPs misinform the public and do not comply with the EU Directives' aims for transparency on animal experiments.

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Depletion of neural stem cells from the sub-ventricular zone of adult mouse brain using cytarabine

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Introduction: Neural stem cells (NSCs) reside along the ventricular axis of the mammalian brain. They divide infrequently to maintain themselves and the down-stream progenitors. Due to the quiescent property of NSCs, attempts to deplete these cells using anti-mitotic agents such as Cytarabine (Ara-C) have not been successful. We hypothesized that implementing infusion gaps in Ara-C kill paradigms would recruit the quiescent NSCs and subsequently eliminate them from their niches in the subventricular zone (SVZ).

Methods: Five weeks old male C57-BL6 mice (N = 45) were randomly divided into control, sham and experimental groups. Under stereotaxic condition inserted a cannula of a brain kit 3 in right side of lateral ventricle and cannula was attached via a connecting tube to an osmotic mini-pump. We infused Ara-C 2% and vehicle in the right lateral ventricle of adult mice brain using four different paradigms: 1- one-week, 2- two-weeks, 3,4- two-weeks with an infusion gap of 6 and 12 hours on day seven. Animals from the experimental groups were sacrificed immediately or one week after the completion of Ara-C treatment then right and left side of SVZ cultured separately by neurospher assay method. In second step animal treated by two-weeks with 6 hour an infusion gap on day seven paradigms then sacrificed immediately or one week after the completion of Ara-C treatment for neural colony forming assay and immunohistochemistry method.

Result: Neurosphere formation dramatically decreased in all paradigms immediately after Ara-C infusion. Reduction in neurosphere formation was more pronounced in the 3rd and 4th paradigms. Interestingly one week after Ara-C infusion, neurosphere formation recovered towards control values implying the presence of bona fide NSCs in the harvested SVZ tissue. Unexpectedly, neural colony forming cell assay (N-CFCA) in the 3rd paradigm, as one of the most effective paradigms, did not result in formation of bona fide NSC-derived colonies (colonies > 2 mm) even from SVZs harvested one week after completion of Ara-C infusion. However, formation of big colonies with serial passaging capability, again confirmed the presence of bona fide NSCs. Immunohistochemistry analysis also confirms other data of our sudy.

Conclusion: Our study data suggest that Ara-C kill paradigms with infusion gaps deplete NSCs in the SVZ more efficiently but the niches would repopulate from the remaining NSCs or from an unidentified cell source.

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Romanian Center for Alternative Test Methods (ROCAM) – a newly established 3Rs Center to promote and support the alternative methods field in Romania

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Promotion, development, validation and implementation of alternative methods require a close collaboration between all actors involved in the process of integrating the 3Rs approach (Replacement, Reducing and Refinement) into testing strategies, which need to be further aligned to the current legislative framework. Therefore, Romanian Center for Alternative Test Methods (ROCAM) was established with the main goal to support this process and promote the 3Rs principles in Romania and regionally. ROCAM is hosted by the University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca (USAMV Cluj-Napoca) at the Institute of Life Sciences. USAMV Cluj-Napoca is a leading academic institution in Romania with a long tradition in life sciences research activities and its main goal is to promote the excellence in the field of education, research and innovation. With the aspiration towards excellence and in order to become competitive, USAMV Cluj-Napoca established various research centers in order to polarize and attract national and international funding and knowledge. To achieve its goals ROCAM aims form a collaborative network of national laboratories and institutions active in this area. ROCAM activities are structured in four Working Groups (WG): WG on Regulatory Affairs, WG on Education & Training, WG on Research and WG on Dissemination, in order to coordinate and implement its mission in agreement with the Romanian Law no. 43/2014 corresponding to the Directive 2010/63/EU on the protection of animals used for scientific purposes. The activities included in the Work programme are established by the Executive Committee together with the WG leaders. Also, ROCAM establishes a Scientific Advisory Board to follow and advise ROCAM's activity and mission. ROCAM's main goal include promoting the development of alternative methods and 3Rs approaches, their application in industry and their acceptance by regulators in Romania. ROCAM will act as a bottom-up and top-down hub in order to disseminate 3Rs approaches, will facilitate training and educational programs in the area of 3Rs and will support research activities for the development, optimization, validation and application of alternative methods (e.g. *in vitro* and *in silico* models). ROCAM will cover among others, any sector (e.g. chemicals, pharmaceuticals, cosmetics, food and feed, etc.) that can be linked with the implementation of 3Rs principles in hazard and risk assessment. Finally, ROCAM act as a national main contact point in Romania in relation with other international "3Rs Centers".

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Quantitative analysis of cell migration in the wound healing scratched assay by using active contour model and speeded up robust features

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Scratch wound healing assay has been widely adapted and modified by researchers to study the effects of a variety of experimental conditions, for instance, gene-knockdown or chemical compound treatment, on cell migration and proliferation. In this study, we propose an automatic method for segmenting the scratched areas for quantitative determination of fibroblast and other cells migration to and proliferation into the wounded monolayer. This is achieved by combining the Speeded-Up Robust Features (SURF) and Active Contour Model (ACM). Cell migrating and growth towards the center of the gap was photographed and measured using digital image processing. The scratched area was segmented from the image and then some features like the mean, variance, RGB and HSV parameters are extracted. The work has been tested on different cell monolayer at first and 3 times after scratching. Comparing the segmentation results by use of Otsu thresholding method, ACM and SURF show the superiority of ACM method over the two others. The proposed method for scratched cell segmentation which is a combination of SURF and ACM gives the best results. To assess the practical robustness of our method, we have used it for segmentation of different types of cell migration. Result of applying the proposed method on different cells shows the high performance, speed and accuracy of it. We believe that this work is applied the most powerful and newest methods of image processing to estimate the relative migration cells in wound healing *in vitro* test.

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Effects of glycine and taurine on cell viability and function of cryopreserved primary rat hepatocytes

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Hepatocytes are used as an *in vitro* model to evaluate drug metabolism. Human hepatocyte transplant has been considered as the temporary treatment of acute liver failure. Quality of the cells before cryopreservation is important for viability and function after freezing. The present study aimed to investigate the cryoprotective effects of Glycine and Taurine on primary rat hepatocytes. Freshly isolated rat hepatocytes were incubated in Krebs-Henseleit buffer under a flow of 95% O_2 and 5% CO_2 with Glycine and Taurine (50-1000 μM) at 37°C for 1 and 3 hours, respectively. The preincubated hepatocytes were cryopreserved for one week. Hepatocytes viability and function were determined post thawing and the results were compared with the control group. The viability of rat hepatocytes was significantly increased after one hour preincubation with higher doses Glycine 500-1000 μM. Preincubation with all doses of Taurine (50, 100, 250, 500 and 1000 μM) improved the viability and function upon thawing in isolated hepatocytes. In rat hepatocytes, albumin and urea production were significantly increased after preincubation with Taurine (500-1000 μM, 1 hour). The GSH content was significantly increased in Taurine (250-1000 μM, 1 hour) group in rat hepatocyte. Incubation of hepatocytes with Glycine and Taurine prior to the cryopreservation can increase the cell viability and function after thawing.

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In vitro evaluation of photoprotective efficacy of commercial sunscreens by biological parameters

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Introduction: The use of sunscreens increases significantly worldwide. According to World Health Organization (WHO), the people has risen popular outdoor activities and recreational exposure. Overexposure to sunlight is widely accepted as the underlying cause for harmful effects as skin cancer, photoaging and alterations in the immune system. In industrial production of sunscreens, the evaluation of the effectiveness is performed in stage of development of products conducted *in vivo*, using human and using test recommended by the Food and Drug Administration (FDA) or the International Standardisation Organisation (ISO). During production batch to batch, the protective efficacy evaluation is carried out by analytical determination of the concentration of UV filters added to the formulation. However, the determination of UV filters concentration do not guarantee that their protective efficacy is ensured.

Objective: Therefore, this study aimed to analyze different cell lines and biological parameters, intended to develop a quick test, precise, reproductive and inexpensive to allow differentiate the susncreens with different SPF or SPF/PPDs according to their photoprotective effectiveness.

Methods: The culture cells utilized in this work were L929 (mouse fibroblastos), HACAT (human keratinocytes) and MCR5 (human fibroblastos) and the biological assays were (i) cell viability using the resazurin dye reduction method, (ii) the formation of lipid peroxides by fluorimetric reaction of the thiobarbituric acid (TBA) to malondialdehyde (MDA) and ultimately, (iii) the production of reactive oxygen species (ROS) based on the oxidative conversion of 2.7 diclorodihidrofluoresceína-diacetate (DCFH2-DA) to dichlorofluorescein (DCF).

Results: Among the cell lines and biological parameters tested, the L929 cell line associated with cell viability and lipid peroxidation parameters were the most promising to evaluate the efficacy of photoprotective sunscreen and to discriminate the photoprotective potential the sunscreens with different SPF or SPF/PPDs against UVB radiation. The formation of ROS, expressed in HaCaT lineage, proved to be a promising biological test to discriminate the photoprotective potential the sunscreens with different SPF or SPF/PPDs exposed to UVA radiation.

Conclusion: Thus, the quality control of sunscreens in industrial production may be assessed by an *in vitro* method using cell culture.

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Enhancing effect of alternating electric fields of different frequencies on biological activity of 5FU, HuIFN-αN3, Royal Jelly and combinations of them on CaCo-2 cells

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Alternating electric fields show different biological effects on drug delivery and cancer therapy. The low-intensity intermediate frequency currents (100-300 kHz) exhibit an inhibitory effect on the growth rate of a variety of human and rodent cancer cell lines [1,2]. The efficacy of the chemotherapeutic treatment can be increased by adjuvant alternating electric fields by 1-3 fold of basic magnitude [3,4]. Experiments were performed with aim to evaluate the enhancing effect of different selected therapeutic frequencies: 0.6 kHz,1.1 kHz 5.0 kHz and 100.0 kHz of the alternating electric fields with square pulses of power 4 mW. ("Defender" device, BIOEL, Republic of Serbia) on biological activity of 5FU, (widely used chemotherapeutic in oncology) (100 mM), HuIFN-αN3 (multifunctional protein with antiviral and antitumor activity) (1000 I.U./ml) and Royal Jelly (complex bee product with antiproliferative activity) (10% solution in 0.87 g NaCl/100ml H_2O) on CaCo-2 cells. The enhancing effects of these frequencies were also measured on combinations (1:1) of these substances. Treated and nontreated cells were exposed to different frequencies for two minutes at room temperature and cultivated for 24 hours at 37°C. Colorimetric proliferation assay was used to determine the percentage of cell growth inhibition [5] and percent of apoptotic cells by the use of dye mix: ethidium bromide (EB)/Acridine orange (AO) [6]. The percentage of viable cells were determined by Trypan blue staining [7]. As the main measure of the biological effects, the highest percentage of growth inhibition was used. In addition, the percentage of apoptotic and viable cells was determined. The tests were performed in the separate glass tubes with CaCo-2 cells. The adjuvant maximal effect of the current on the biological activity of 5FU (79 ±3.5% of growth inhibition, 73 ±2.7% of apoptotic cells and 52 ±4.1% of cell viability) was at 1.1 kHz. The biological effect of HuIFN-αN3 (88 ±9.8% of growth inhibition, 61 ±1.0% of apoptotic cells and 75 ±4.4% of cell viability) was affected by 100.0 kHz. The maximal enhancement of biological effect of Royal Jelly (65 ±5.9% of growth inhibition, 56 ±2.5% of apoptotic cells and 85 ±9.9% of cell viability) was at 5.0 kHz. When the enhancements of biological effects of combinations of substances were analysed, the following data were found: 5FU + HuIFN-αN3 (1:1) biological activity (71 ±6.9% of growth inhibition, 50 ±4.3% of apoptotic cells and 65 ±3.8% of viable cells) was affected by 100.0 kHz. In case of 5FU: Royal Jelly (1:1) the biological activity (90 ±8.6% of growth inhibition, 37 ±5.1% of apoptotic cells and 79 ±9.1% of viable cells) was affected by 1.1 kHz. The enhancement of biological activity of HuIFN-αN3: Royal Jelly (1:1) (77 ±4.5% of growth inhibition, 99 ±9.8% of apoptotic cells and 69 ±7.8% of viable cells) was found at 100.0 kHz. We found that maximal enhancing frequencies differ with substance like: 5FU 1.1 kHz, HuIFN-αN3 100.0 kHz and Royal Jelly 5.0 kHz. In combinations (1:1) of these substances, the adjuvant frequencies were: 5FU: HuIFN-αN3 100.0 kHz, 5FU:Royal Jelly 1.1 kHz and HuIFN-αN3: Royal Jelly 100.0 kHz. Used substances differ for the adjuvant frequencies on the same cell line, the CaCo-2 in this case.

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The chicken chorioallantoic membrane (CAM) as model for tumour-stroma interaction in human skin cancer can widely replace the mouse in cancer research

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Introduction / Background: Grafting of mammalian cells and tissues to the chick embryo chorioallantoic membrane (CAM) is a well-established experimental system to evaluate various parameters of tumour growth. The lack of nerve cells in the CAM and the natural immunodeficiency during early development of the chick embryo makes this extra-embryonic membrane amenable to tumour xenografting. Furthermore, the introduction of *ex ovo* cultures enhances the applicability of the CAM enabling *in vivo* documentation of effects and facilitating experimental manipulation.

Materials/Methods: Fertilized eggs from a local commercial hatchery were incubated for 3d (37.8°C, 50% humidity). Eggs were cracked and incubated in plastic dishes for further 7d until xenotransplantation. Skin cancer samples (melanoma) and cell lines (melanoma and Merkel cell carcinoma) were grafted onto vascular branches of the CAM. At 3-7d post-transplantation, xenografts were fixed with paraformaldehyde followed by FFPE-tissue embedding. For subsequent analyses haematoxilin/eosin (HE)- and immune-staining of 6µm sections were done to determine the tumour cell morphology, the expression of tumour antigens and to distinguish between transplanted and primordial avian cells.

Results: Both Merkel cell and melanoma cell lines formed macroscopically visible tumour masses within 3d, whereby melanoma cells developed into intensively pigmented tumours on the CAM. Tissue xenografts grew well and induced angiogenesis within the CAM stroma. Macroscopic observation revealed attraction of numerous allantoic vessels that developed radially towards the onplants in a "spoke-wheel" pattern. HE staining revealed the presence of necrotic areas in the centre of the tumour. Moreover, loco-regional metastases with solitary evading tumour cells surrounded by newly formed blood vessels were present. Solid tumour samples were strongly connate to the CAM and showed outgrowth of the tumour invading avian vasculature. Xenotransplantation resulted in alterations of fibroblast, epithelial and endothelial cell morphology. Tumour-stroma interactions however, also affected tumour cell morphology: those cells directly in contact with the CAM surface showed changes, suggesting epithelial-to-mesenchymal transition.

Conclusion: Commonly used for neovascularization and toxicity studies, the CAM model represents an intermediate system between *in vivo* and *in vitro* systems. It also allows addressing biological features of various cancer types, such as tumour-stroma interaction, invasion and metastasis. Especially the strong brown staining of melanoma cells facilitates visualization of invading cells in the CAM stoma.

Further experiments are aimed to investigate the effect of pro- and antiangiogenic and antitumorigenic substances on growth and invasiveness of skin cancer cells using the CAM assay. There is a strong need to proof weather CAM system can replace the mouse model and help the translation of new therapies into the clinic.

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Towards the replacement of *in vivo* repeated dose systemic toxicity: achievements of the SEURAT-1 research initiative

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SEURAT-1 is a major European research consortium that was established to develop the science needed to evaluate the safety of chemicals for repeated exposure in humans without using animals. Inspired by the fundamental considerations published in the report of the U.S. National Research Council (NRC) entitled "Toxicity Testing in the 21st century: A Vision and a Strategy", SEURAT-1 aims to use the mechanistic understanding of toxicological effects for the development of innovative testing methods and, ultimately, improved safety assessment. A research strategy was formulated based on the guiding principle to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health. The proof of the initiative will be in demonstrating the applicability of the concepts on which SEURAT-1 is built. This is done on three levels: (i) theoretical prototypes for adverse outcome pathways were formulated based on knowledge already available in the scientific literature on investigating the toxicological mode-of-actions leading to adverse outcomes (addressing mainly liver toxicity) (ii) adverse outcome pathway descriptions are used as a guide for the formulation of case studies to develop integrated testing strategies for the prediction of certain toxicological effects, which also as a consequence might further elucidate the theoretical model (iii) further case studies targeting the application of knowledge gained within SEURAT-1 in the context of safety assessment [1]. The ultimate goal would be to perform safety assessment based on ab initio predictions grounded on a sufficient understanding of toxicological mechanisms. In the near-term, it is more realistic that data from innovative testing methods will support in strengthening read-across arguments.

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Using human organotypic glioblastoma tissue slices for therapy improvement

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Cancer is one of the major causes of death worldwide. Research to improve therapy is mostly done with xenograft models by injecting (human) tumor cell lines into the flanks of mice or rats. This means a great burden for the animals, especially when the endpoints of the experiments are represented by Kaplan-Meier-curves which depict the time point where half of the animals in the study died. It also involves problems like the lack of cellular heterogeneity or inter-species differences. Therefore, only few animal studies can be successfully translated into a clinical setting for humans. We have previously established a human test system consisting of organotypic 3D-tissue slice cultures of tumor tissue from surgeries which can be kept in culture for several weeks. It maintains the natural structure of the investigated tissue including extracellular matrix and all cell types which reflects the *in vivo* - situation more closely than 2D cell cultures. So far, this system is used for human tonsils, glioblastoma, squamous cell carcinoma and gastric cancer tissue from resections as well as fat from plastic surgery. In these settings, we can monitor response to known radio- or chemotherapeutic approaches, test new compounds, analyse cell proliferation or death, or track behavior of special cell types via live imaging over time. Here, we focus on glioblastoma (GBM), which is one of the most aggressive brain tumors with a mean patient survival of 15 months under standard radio-chemotherapy. We have previously established an organotypic 3D-tissue slice culture system of human GBM tissue from resection which can be kept in culture for several weeks and used for radio- and chemotherapeutic experiments. Here, we present preliminary data of Next Generation Sequencing (NGS) analysis of GBM slices which will be matched to patient's responses in a 3 year-project. The influence of X-irradiation and temozolomide as well as a natural compound, Carnosine, are investigated and changes in mRNA of GBM slices are measured. Further, we established a co-culture system of human GBM and mouse brain tissue to monitor cancer cell migration into healthy tissue as well as microglia involvement by using different live stains and time-lapse confocal live imaging. With our model system, animal testing could be reduced and species differences are eliminated. For cancer research, it can be used as an approach for personalized therapies.

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Evaluation of inflammatory and genotoxic effects of smokeless tobacco using a human organotypic model of oral epithelium

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In addition to the well-known effects of tobacco use on the causation of lung and cardiovascular disease, tobacco use is also implicated as a major cause of oral cavity disease that leads to thousands of deaths per year. Snus, a smokeless tobacco applied to the oral cavity, has been proposed as a less harmful alternative to smoking although its safety has not been adequately evaluated. The objective of this study was to evaluate the cytotoxic, genotoxic and inflammatory effects of snus using an *in vitro* model of human oral mucosa (EpiOral). EpiOral tissues were treated topically with 5 or 25 milligrams of snus for 24-48 hours and evaluated for cytotoxicity by MTT. Tissues treated with 5 mg of snus had comparable viability to vehicle treated controls while those treated with 25 mg displayed approximately a 20% decrease in viability after 24 and 48 hours of exposure. Histological analysis revealed hyperchromic staining in tissues treated with 5 mg of snus at 24 hours post-treatment whereas tissues treated with 25 mg of snus displayed a significant amount of sloughing of the apical layers. Following treatment, an inflammation-specific cytokine panel was used to analyze markers of inflammation at 24 and 48 hours post treatment. Of the cytokines analyzed, significant increases (1.5-2 fold) in IP-10, GM-CSF and RANTES were observed at both 24 and 48 hours post treatment in tissues treated with 25 mg of snus. As a measure of genotoxicity, the presence of γ-H2AX foci (specifically, phosphorlation at Serine 139) was evaluated in treated tissues. γ-H2AX is a phosphorylated derivative of the H2AX histone and is tightly bound to double strand DNA break sites, therefore serving as a biomarker of genotoxic insult. γ-H2AX foci were readily detected in the apical layer of tissues treated with 25 mg of snus at 24 and 48 hours post treatment. These results demonstrate the utility of this organotypic oral tissue model to evaluate smokeless tobacco product safety.

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In vitro induction of airway goblet cell hyperplasia by TH2 cytokines, viral exposure or cigarette smoke

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Introduction: Goblet cell hyperplasia and increased mucus production are common features of respiratory epithelial response to numerous environmental challenges (e.g. cigarette smoke, air pollution, viral infection) and are commonly observed in respiratory diseases such as chronic obstructive pulmonary disease (COPD) and asthma. Azithromycin and related macrolide antibiotics are known to possess anti-inflammatory and immunomodulatory properties, and to provide clinical improvement in COPD exacerbations. In the current work we evaluated effects of azithromycin on goblet cell hyperplasia and gene expression changes caused by simulated viral exposures, TH2 cytokines or cigarette smoke in an organotypic *in vitro* model of the human airway epithelium.

Methods: The EpiAirway *in vitro* human airway model was exposed for up to six days to Poly(I:C) to simulate viral exposure, to IL-13 to simulate TH2 imbalance, or to cigarette smoke. Induction of goblet cell hyperplasia was determined by mucin staining with Anti-MUC5AC or alcian blue (AB)/periodic acid Schiff (PAS). Gene expression changes associated with goblet cell hyperplasia were determined by qPCR utilizing a custom qPCR array containing 45 mucin related genes. The effect of azithromycin on goblet cell hyperplasia and gene expression was evaluated.

Results: Treatment with the TH2 cytokine IL-13 caused a dramatic induction of goblet cell hyperplasia after 6 days of exposure. Quantitative PCR experiments identified several genes that were associated with TH2 cytokine-induced goblet cell hyperplasia, including 15-LOX, CLCA1, Mucin 5AC, SPEDF and TFF3. Azithromycin effectively reduced goblet cell metaplasia and PAS staining. Azithromycin also significantly reduced expression of CLCA1, MUC5AC and TFF3 mRNA. Treatment of EpiAirway-FT™ with Poly(I:C) also induced goblet cell hyperplasia as determined by H&E and AB/PAS staining. Numerous genes including MUC5AC, MUC5B, STAT1 and TFF3 were upregulated more than 3-fold compared to untreated controls. Azthromycin caused a dose-dependent decrease in Poly(I:C) induced goblet cell number and size, and also diminished Poly(I:C) induced changes in mucin gene expression. Finally, cigarette smoke (1 cigarette/day for 5 days) induced modest goblet cell hyperplasia. However, smoke induced goblet cell hyperplasia was not effectively attenuated by azithromycin.

Conclusions: Taken together, the results show that TH2 cytokines, Poly(I:C), and cigarette smoke are each capable of inducing goblet cell hyperplasia and/or mucus production in organotypic *in vitro* human airway epithelial models. Azithromycin demonstrated a variable effect on attenuation goblet cell hyperplasia and gene expression changes caused by the environmental challenges. Detailed molecular mechanisms involved in mediating azithromycin effects remain to be elucidated.

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Journal policies on animal use – current landscape and future directions

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Scientists using animals work under a "social license", which warrants public trust that such work is relevant, competent, ethical and transparent. Along with laws and regulations [1,2], a number of self-regulatory initiatives from the scientific community itself are in place as a system of checks-and-balances to promote best practice. These include biomedical journals' policies on animal use, as scientists' motivation – and often pressure – to publish can motivate compliance with said policies [3,4], particularly of high-profile journals. To beneficially impact principles and practice, journal policies must be set to high standards. However, it has been suggested that journals often demand few details on how studies are carried out [5]. To map the landscape regarding editorial policies on animal use, the instructions for authors (or equivalent) of a sample of 170 journals were evaluated by a novel classification scheme, the "EXEMPLAR" scale [6]. The "EXEMPLAR" (acronym for "Excellence in Mandatory Policies on Animal Research") scale scores journal policies as regards four key parameters: A-Regulatory compliance, B-Quality of reporting, C-Animal welfare and ethics, and D-Criteria for the exclusion of papers, each awarding up to five points, in a total of 20 points. The sample comprised journals publishing animal studies on Amyotrophic Lateral Sclerosis (ALS, n = 76), Type-1 Diabetes (T1D, n = 27) and Tuberculosis (TB, n = 49), fields chosen for being areas in which animal experimentation is recurrent and widespread, and the subject of ongoing systematic reviews in this lab, thus allowing comparison between the principles explicitly stated by journals and the practice reported in studies published by those journals, currently ongoing. The median score of the whole sample (N = 170) was 4 points. The mean scores for ALS, T1D and TB were, respectively 4.45 (SD = 3.15), 4.52 (SD = 3.286) and 4.20 (SD = 3.54), with no significant differences between them. Most journals (92%) had a non-nil score for Category A, with 5% of journals being awarded five points in this category. For Category B, while 72% had a nil score, 18% were awarded the top score for this category, virtually in cases for referring authors to the ARRIVE guidelines. Respectively 86% and 91% had no explicit policies regarding categories C and D. Overall, only 18% of journals scored eight points or higher. Scores were not found to vary with journals' impact factor, country of origin or antiquity. Open-access journals scored higher ($p < 0.001$) than subscription-based journals, which may result of their greater exposure and consequent higher public scrutiny. The greater focus of editorial policies on regulatory compliance and neglect for other key issues suggests a transfer of journals' responsibilities – regarding the ethical and competent use of animals – to scientists, institutions and regulators. And while referees can play a relevant role in the review process by flagging ethically questionable studies, it is less likely they will do so (or that journals will consider them) if there are no strong policies in this regard, reason why journals must improve their efforts in order to become important guarantors of ethically and scientifically sound research.

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Community guidelines on animal studies – ALS research as a case-study

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Aside existing legislation and supervision by competent authorities, animal research is also regulated within the scientific community itself. Such self-regulatory mechanisms include community guidelines on best practice, commonly seen in the neurosciences. These guidelines have arisen partly as a response to several examples of translational failure of promising preclinical results. These often appear to have been false-positives in the first place – apparently as a result of poor experimental design and statistics – which, along with publication bias, misinform clinicians about the therapeutic value of candidate drugs. This raises ethical issues, since animals, time and resources are wasted in unreliable research, but particularly because patients are being enrolled in pointless clinical trials. To assess whether community guidelines influence research, we systematically reviewed all retrievable research papers (N = 382) on the SOD1 mouse model of amyotrophic lateral sclerosis (ALS) published in 2005, 2009, 2011 and 2013, to include studies carried out before and after publication of ALS guidelines in 2007 [1] and 2010 [2]. These guidelines list a number of methodological considerations for proof-of-concept and preclinical ALS studies (eg regarding randomization, blinding and sample size calculation), suggest relevant parameters for evaluating disease progression, and a "standard" surrogate endpoint for survival studies (euthanizing animals unable to right themselves after being laid recumbent for 15-30 s) Information retrieved included (self-reported) regulatory compliance status, humane endpoints and other refinements, study severity (based on disease stage at time of endpoint), as well as information regarding animals (eg sex and genotype), experimental design (eg sample size, blinding of observers, random assignment to groups), and housing and husbandry. Preclinical studies included a higher proportion of high-severity studies ($p < 0.001$). This might be explained by guidelines' suggestion that proof-of-concept studies may not warrant survival assessment. An increase in reported regulatory compliance – particularly project approval – was observed over the time period reviewed ($p < 0.001$), but not found to influence severity or reported refinement, with no changes across time. Overall, these results reflect previous findings on Tuberculosis [3] and Huntington's [4] research. However, there is one important difference: while death was used as an endpoint in 66% and 26% of studies on "lethal models" of TB and HD, respectively, this happened in less than 3% of ALS studies. Nonetheless, animals were only euthanized when reaching very late stages – either the "standard endpoint" or complete paralysis – meaning that the actual animal welfare impact was probably small [5]. The broad adherence to the "standard endpoint" opens the possibility that less severe endpoints – some already proposed in the literature – can also be readily and broadly adopted by the ALS community, provided there is ample consensus on their validity. Future guidelines should have these and other animal welfare considerations, rather than solely focusing on improving and standardizing basic scientific parameters. Until then, their potential for promoting a culture of care and increase awareness to the 3Rs remains largely untapped.

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CERST-NRW: a German state government of North Rhine Westphalia-funded initiative for the development of alternative methods to animal testing

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There is a current paradigm shift in toxicity testing from apical endpoint evaluation to human-relevant mechanism-based hazard assessment, which is based on the observation that rodents do not necessarily reflect human physiology and covers the need for higher throughput testing of a large number of chemicals. This paradigm shift requires methods predicting adverse effects of compounds on human physiology with high confidence. One endpoint required from regulatory authorities with regards to safety testing is reproductive/developmental toxicity (R/DT). Such studies require a large amount of animals and thus methods are needed that have the ability to reduce/replace animal tests in this area. Therefore, the state government of North Rhine Westphalia, Germany, has initiated CERST-NRW ("Center für Ersatzmethoden zum Tierversuch – Nordrhein Westfalen") for establishing human-based *in vitro* alternative methods for R/DT. CERST-NRW will thus, in a 5-year time frame, establish a human induced pluripotent stem cell (hiPSC)-based embryonic stem cell test (hEST). Building on the neurodevelopmental expertise of the IUF, CERST-NRW also aims at establishing hiPSCs as the basis for neuronal network formation, resulting in the human neuronal network formation assay (hNNFA). CERST-NRW intends to obtain close collaboration with industry with the goal to "develop close to the market". Thus, the research area will not be limited to R/DT, but can be adapted due to industrial needs.

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Generation of the molecular basis for studying thyroid hormone disruption in neural progenitor cells of humans and rats

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Despite the long knowledge on the importance of thyroid hormones (TH) for proper brain development, there is still a profound gap in the understanding of molecular and cellular TH effects on developing brain cells. Moreover, species-specific cellular TH actions are an understudied field of research. Therefore, the goals of this project are (1) to generate molecular signatures of TH signaling components in primary human (h) and rat (r) neural progenitor cells (NPC) growing as neurospheres, (2) to identify the actions of the TH T3 (triiodothyronine) and T4 (thyroxine) on basic processes of brain development (proliferation, migration, differentiation) in a species-specific manner, and (3) to identify the signaling pathways guiding TH-dependent neurodevelopmental processes. Understanding basic physiology of TH signaling is a prerequisite for the identification of TH disrupting chemicals. For generation of molecular signatures we compared gene expression levels of TH signaling components (TRα1/2, TRβ RXRα/β/γ, NCOR1/2, MCT8, OATP1c1, LAT1, LAT2, DIO2, DIO3) of h/rNPC on a copy number basis by qRT-PCR. The receptors and co-repressors TRα1, RXRα, NCOR1 as well as the TH transporter OATP1c1 are lower expressed in rat than human NPC. Compared to human neurospheres, the TH transporter MCT8 is lower expressed in proliferating rat spheres, but present with higher copy numbers in the 5 days differentiated NPC. The transporters LAT1 and LAT2 and the TH metabolizing enzyme DIO2 are higher expressed in the rat than in human NPC. Quantitatively distinct responses of the TH-regulated genes HR and DIO3 indicate that these species differences might be of functional relevance. For studying TH effects on neurodevelopmental processes, h/rNPC development was monitored in presence of different T3 and T4 concentrations. T3 reduced proliferation of hNPC and induced oligodendrocyte maturation in h/rNPC neurogenesis was only induced in hNPC by T4. Neural migration was not affected in either species. Elucidation of signaling pathways guiding these TH-dependent neurodevelopmental processes is the scope of current experiments. In summary, we found that molecular signatures of TH signaling components differ between species possibly leading to distinct regulation of the TH-dependent genes HR and DIO3. Once TH-related pathways relevant for human neurodevelopmental processes are identified, the ground for chemical testing for TH disrupting compounds is prepared.

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The HuALN model for testing immune reactivity *in vitro*

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As modern biopharmaceuticals show a very high degree of species-specificity, animal models are inadequate to assess drug safety and drug efficacy. Drawbacks in the pharmaceutical arena during the last years have raised significant concerns about the predictability of animal models for immune reactivity and immunotoxicity testing of biological therapeutics such as antibodies, glycoproteins, cytokines or vaccines in humans. The Human Artificial Lymph Node Model (HuALN) is a micro physiological system (MPS) mimicking immunity in a continuously perfused 3D culture system and suitable for long-term treatment (e.g. 28 d) and repeated dosing. The MPS serves as a human micro organoid lymph node model for induction or modulation of cellular and humoral immune responses. The implementation of stromal cells improves organoid formation. The HuALN model is designed for testing immunomodulation (e.g. MoA of checkpoint modulators), to assess unwanted immunogenicity reactions (e.g. ADA formation, sensitization) or efficacy of vaccines, adjuvants and formulation. T cell responses and shifts in the TH1/TH2 pathway are continuously monitored by cytokine secretion profiles. The induction of primary humoral responses is demonstrated by B cell activation, plasma cell formation and antibody secretion profiles for IgM and IgG. Cells can be harvested from 3D matrix at the end of the MPS culture time and used for flowcytometric analysis and functional tests, e.g. ELISPOT assays. We will introduce into the HuALN model and present recent data in the relevant applications of vaccine testing, immunomodulation and immunogenicity assessment.

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CERST-NRW – a platform for developing human *in vitro* methods for (neuro)developmental toxicitiy testing

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According to the recent European Union (EU) legislation for regulation, evaluation authorization and restriction of chemicals (REACH), approximately 30,000 chemicals need to be tested for safety over the next years. Of those, approximately 80% will be used for examining reproductive and developmental toxicity testing. With the current *in vivo* guideline studies this is very resource-intensive when it comes to the number of animals used and the time and costs required. In addition, current state-of-the-art strongly suggests that in many cases animals respond differently to compounds than humans. Therefore, alternative testing strategies based on human cells are needed that refine, reduce and replace animal testing by creating affordable, sensitive, and mechanism-based methods predictive for humans that are suitable for high- and medium-throughput screenings. CERST-NRW aims to establish human cell-based *in vitro* tests for (neuro) developmental toxicity testing: the human embryonic stem cell test (hEST) and the human neuronal network formation assay (hNNFA). The tests will be based on human induced pluripotent stem cells (hiPSCs), a human cell source without any ethical concerns. Our preliminary work demonstrates that hiPSC are pluripotent in culture because the majority of the cells express TRA-1-60 and/or SSCA4. By using the Hanging-drop method they form embryoid bodies (EBs). Plating of EB on gelatine causes differentiation into beating cardiomyocytes, which is a prerequisite for the hEST. In the next steps we will explore a suitable method for quantifying cardiomyocyte differentiation in a way compatible with medium and high throughput screening techniques as a readout for the hEST. For the hNNFA, hiPSC were differentiated into free-floating neurospheres with a neural induction protocol. When plated onto PDL-laminin-coated multielectrode arrays (MEAs), hiPSC-derived neural progenitor cells (NPC) form neuronal networks. First MEA recordings revealed action potentials of three weeks differentiated cells. These networks will be thoroughly characterized and control cultures standardized as a prerequisite for toxicity testing in the future. Readouts of both assays will feed into the theoretical frame of the "Adverse Outcome Pathway" (AOP) concept, which currently is receiving significant attention from regulatory agencies worldwide.

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Isolated human intervertebral disc cells as a useful platform for *in vitro* toxicology tests

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Introduction

The diagnostics of discogenic low back pain may employ also radioopaque contrast agents, such as iodixanol. It is known that a local application of such agents may cause cytotoxicity to intervertebral disc cells following the intradiscal injections, which may provoke or accelerate the intervertebral disc degeneration. However, the potential effects of these agents in humans still remain unclear, as many studies so far have been accomplished on animal cells. The study aim was to evaluate the effect of iodixanol on human intervertebral disc cells *in vitro* and to compare its cytotoxicity to saline solution control.

Materials and methods

From human lumbar intervertebral disc fragments obtained during discectomis, annulus fibrosus and nucleus pulposus cells were isolated. They were cultured in the microtitre plates and exposed to various concentrations of iodixanol. The saline solution was used as a control. Three different dilutions (undiluted, 1:2 and 1:4) of iodixanol were tested. The cells were treated for 6, 24 and 48 hours and then examined for viability. The cells were treated for 6, 24 and 48 hours. They were then examined with the crystal violet cell staining. The percentage of live cells after treatment was determined.

Results

A time and dose depended response to iodixanol exposure was observed in human intervertebral disc cells. The nulceus pulposus cells were more susceptible than annulus fibrosus cells to the toxic effects of the tested agent. Iodixanol was cytotoxic in all three tested concentrations (undiluted, 1:2 and 1:4) with the cell survival of 0%, 8% and 14%, respectively. The cell death was caused mainly by necrosis rather than apoptosis.

Conclusions

Human intervertebral disc cells *in vitro* are useful for various toxicology tests. In combination with iodixanol, commonly used for discography, the cytotoxic effects were observed in a dose- and time-dependent manner. According to our study, concentrated iodixanol should be avoided due to its high toxicity to the intervertebral disc cells. The 1:4 dilution was least toxic and may be thus recommended for the intradiscal diagnostics. However, high dilutions are questionable due to loss of resolution yield. It is assumed that the genesis of disc degeneration might be contributed also by the toxic effects of the contrast agents used, culminating to progressive tissue damage after the diagnostic measures.

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Human induced pluripotent stem cells as tools for studying the etiology of Non Alcoholic Fatty Liver Disease

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The prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) is on the rise as a consequence of our high fat diets and sedentary life style. NAFLD encompasses a range of disease states starting with simple steatosis and developing into steatohepatitis (NASH), which can in turn lead to liver cirrhosis and hepatocellular carcinoma (HCC). An individual's susceptibility to steatosis and NASH can be influenced by both genetic and environmental factors that vary widely. This and the fact that a complex interplay of pathways are involved in the pathogenesis of NAFLD have made it difficult to predict individual susceptibility or to determine the genes involved in the disease. In a previous consortium- "LIVSYSiPS, we adopted a systems biology approach employing patient liver biopsy-derived transcriptomics, serum for biomarker profiling, metabolomics, proteomics and finally mathematical models to gain insights into the etiology of NAFLD. The transcriptome data revealed several genes differentially expressed between distinct grades of steatosis, e.g. KRT18, a serum biomarker for steatohepatitis. ELISA measurements revealed significant differences in the levels of Resistin, hIGFBP3, hARCP30 (Adiponectin) and Leptin in high and low grade steatosis compared to healthy control subjects. Pathway analysis identified differentially regulated relevant pathways related to liver and fat metabolism. These include fatty acid synthesis and metabolism, cholesterol metabolism and transport, insulin receptor signalling, adipocytokine signalling, glycolysis, citrate cycle, urea cycle, arginine metabolism. With this vast knowledge at hand we now generate *in vitro* models of the disease using NAFLD patient-derived induced pluripotent stem cells differentiated into hepatocyte like cells.These cellular models are challenged with excess oleic acid. Induction of the steatotic phenotype is monitored by Oil Red O staining to confirm the production of excess lipid droplets, then followed by detailed cellular, molecular and biochemical analyses to assess downstream effects on the steatosis phenotype.We anticipate that the data generated might help in identifying drug targets that could be used for treatment of steatosis, thus avoiding development to NASH and ultimately HCC.

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Pain for profit. Can monetary reasons justify animal research ethically?

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Since the transition of the EU directive 2010/63 into national laws across the member states a great number of researchers deal with the questions, what has changed and what has to be changed in practical terms. There were many hopes connected with the new directive. For instance, that the increased status of animals in our European societies should be mirrored and the requirements to use animals should become stricter. From an ethicist's point of view this can only be seen in some regards, or in other words, big changes are not on the horizon. Quite the opposite, working on a methodology for the required harm-benefit analysis (HBA) that takes ethical considerations into account, it becomes more and more clear that the new directive was a Pyrrhic victory from an ethical point of view. The example to illustrate this is the question as to whether pecuniary interests can justify animal harm in animal research. At first glance this is not possible since pecuniary interests aren't legitimate purposes. However, it will be argued that the directive provides no basis to turn down research proposals that aim at increase in profit as long as the applicant fulfills one of the legitimate purposes. In this talk we will deal with the argument in three steps: First, we will identify differences in justifying reasons within the HBA and indicate why pecuniary interests do not justify harms done to animals alone. Second, we will argue that due to the nature of animal research it can never be the case that solely pecuniary interest are at stake research interests and gaining knowledge is always part of animal experimentation. Third, we will put forward that because of the entanglement of monetary and research interests, no proposal can be turned down on basis of the directive by referring to involved monetary interests. With this contribution we aim at demonstrating that the hopes connected to the term "ethical considerations" in the directive will be frustrated and the idea that the term "ethics" settles problems is misguided as done from a different viewpoint in the recent past already [1].

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In vitro characterization of monoamine oxidases, steroid-5α-reductases, sulfotransferases and glutathione S-transferases in excised human skin and reconstructed skin

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The use of reconstructed human skin (RHS) in preclinical development of topical dermatics and transdermal therapeutic systems requires detailed knowledge on the biotransformation capacity of the constructs. In the present study, we investigated monoamine oxidases (MAO), steroid-5α-reductases (SRD5A) as well as sulfotransferases (SULT) and glutathione S-transferases (GST) in excised human skin, RHS (Phenion FT, Epiderm-FT) and in normal keratinocytes, HaCaT cells and fibroblasts. Quantitative analysis of gene expression revealed that MAO A, SRD5A type 1 and 3 were expressed in all matrices, whereas MAO B and SRD5A type 2 levels were higher in dermis than epidermis and hardly detectable in undifferentiated keratinocytes. MAO A and B protein was detected in high levels in human skin whereas in RHS mainly MAO A was expressed. Both proteins were absent in monolayer cells. MAO activity was detected in excised human skin but not in reconstructed tissues. In human skin and reconstructed tissues strong constitutive gene expression of SULT2B1b, SULT1E1, GSTP1 and GSTT1 was found. Gene expression of SULT1A1 was moderate, but protein expression and SULT activity was not detected. GST activity was comparable in RHS and human skin. Reconstructed tissues and human skin but not undifferentiated monolayer cultures share a similar expression profile of the tested phase I and phase II enzymes and GST activity. Taking into account the benefit of human-derived RHS avoiding species-related differences, the reconstructed tissues are adequate test matrices for preclinical testing and toxicology studies regarding biotransformation related processes.

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Biotransformation of 2,4-toluenediamine by human skin and reconstructed tissues

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Reconstructed human skin (RHS) gains increasing interest in preclinical drug development. But knowledge is rather poor on its biotransformation capacity, which is highly relevant for genotoxicity, skin sensitization or the efficacy of topical dermatics. In the present study, we investigated the biotransformation activity of two RHS models (Phenion FT and EpiDerm-FT) to the aromatic amine 2,4-toluenediamine (2,4-TDA) as a model compound and compared their metabolic capacity with normal human skin *ex vivo*. Besides whole skin tissues, cell metabolism activity to 2,4-TDA was also examined in primary epidermal keratinocytes and fibroblasts as well as Langerhans cells and dendritic cells. We found the mono N-acetylated derivative N-(3-amino-4-methyl-phenyl) acetamide was the only metabolite detectable in substantial amounts in all test matrices. Formation of the metabolite ranked as: RHS > excised human skin and keratinocytes > fibroblasts ~ Langerhans cells ~ dendritic cells, respectively. Phase I metabolites were not detected. Thus, metabolism of 2,4-TDA in human skin is dominated by phase II reactions whereas the contribution by phase I biotransformation seems to be minimal. In conclusion, RHS is an adequate test matrix for the investigation of N-acetylation of xenobiotics in human skin and thus may serve as test system in non-clinical drug development as well as the investigation of biotransformation-related toxicological endpoints.

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Follow-up on 2013 EUSAAT's Congress and its impact for Sri Lankan scientific community in 3Rs

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This oral presentation intends to provide a feedback to the 3Rs community on the scientific progresses and initiatives that took place since last September 2013 at the 18th EUSAAT Congress. Indeed, 2013 EUSAAT's Congress was the first milestone to present Sri Lankan 3Rs perspectives to the European audience. This event created a momentum and has triggered cooperation between EU and Asian academic researchers to acquire new knowledge and skills on 3Rs. In this context, this presentation will walk the audience through 3Rs trainings that Sri Lankan researchers underwent at a workshop organised by SLALAS (Sri Lanka Laboratory Animal Science Association) as well as the introduction of several alternative models in toxicity testing. It ranged from mock demonstrations on *in vitro* Epiderm skin irritation test, IDMOC model and introduction to the Zebrafish embryo model test for wastewater toxicity testing (ISO 15088, 2007) and at the OECD level for chemical toxicity testing (TG 236). This initiative on alternatives developed an interest for young researchers to move away from *in vivo* studies when performing toxicity studies. Zebrafish is a native species in Sri Lanka and is bred for ornamental purposes only but their use as a research model so far was not considered. Therefore suitability of zebrafish embryo model for water quality testing developed an enthusiasm since then. It led to the submission of a request for a comprehensive learning about this model at the University of Antwerp, Belgium with the intention of establishing a laboratory back in Sri Lanka when funding becomes available. In addition, a module titled "Alternatives" is being included in the course leading to PG Certificate and Diploma on Laboratory Animal Science at the University of Colombo, Sri Lanka.

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Replacement - Coming closer to a dream

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Alternatives to animal tests have emerged over the last decades mainly as cell cultures aiming to replace on by one an animal experiment, producing similar (correlating) results. To some extent, *in silico* modeling, mainly as structure activity relationships, has added to this repertoire. In fact, about 50 methods have been internationally validated and are used to some extent replacing, reducing or refining (3Rs) animal use. However, these were typically tests for topical and acute effects of substances, while the progress to substitute for long-term and systemic effect testing was quite limited. They use cells similar to the traditional animal experiments as black boxes, where the outcome of a substance exposure is recorded. More recently only, we have started to discuss the shortcomings of both the animal tests and our cell culture work in more detail. This might actually serve as a door opener for new approaches making evident the limitations of animal tests in an objective not only animal welfare driven way. At the same time, awareness of the shortcomings of our *in vitro* work helps to improve them and increases the credibility and reliability of research results. At the same time, a mechanistic, molecular research has evolved over the last decades, which is effectively relying to a large extent on methodologies which substitute or complement traditional animal tests. The biotechnology and informatics revolution and their commercialization over the last decades has made such technologies broadly available, standardized and useful. Novel approaches toward a more organo-typic cell culture open new avenues to replicate human physiology in the test tube. Regulatory toxicology, which can serve as an example for current developments, has only slowly begun to embrace these new approaches. Major validation efforts, however, have delivered the evidence that new approaches not necessarily lower safety standards and can be integrated into regulatory safety assessments, especially in integrated testing strategies. Political pressures especially in the EU, such as the REACH legislation and the 7th amendment to the cosmetic legislation, further prompt the need of new approaches. In the US, especially the NAS vision report for a toxicology in the 21st century and its most recent adaptation by EPA for their toxicity testing strategy have initiated a debate how to create a novel approach based on human cell cultures, lower species, high-throughput testing and modeling. However, major parts of toxicology have not yet found *in vitro* solutions. The lecture summarizes the lessons learned from the development, validation and acceptance of alternative methods for the creation of a new approach for research and especially regulatory toxicology. Beside the technical development of new approaches such as organo-typic cultures ("human-on-a-chip") or systems toxicology, a case is made that we need both conceptual steering and an objective assessment of current practices by evidence-based toxicology. The concept of mechanistic validation is proposed as a way forward to quality-assure new cell-based tests.

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Analysis of insulin mimetic phytochemicals with a complementary chick embryo model

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Type 2 diabetes mellitus (T2DM) is a very complex and multifactorial metabolic disease characterized by insulin resistance and β cell failure leading to elevated blood glucose levels. Hyperglycemia is suggested to be the main cause of diabetic complications, which not only decrease life quality and expectancy, but are also becoming a problem regarding the financial burden for health care systems. Therefore, and to counteract the continually increasing prevalence of diabetes, understanding the pathogenesis, the main risk factors, and the underlying molecular mechanisms may establish a basis for prevention and therapy. We have recently developed a quantitative total internal reflection fluorescence microscopy (TIRFM) based assay to identify and characterize insulin-mimetic phytochemicals in a live-cell context [1]. For this purpose, TIRFM was applied to quantify glucose transporter 4 (GLUT4) translocation in highly insulin-sensitive CHO-K1 cells expressing a GLUT4-myc-GFP fusion protein. Using this approach, we demonstrated GLUT4 translocation modulatory properties of selected substances and identified novel potential insulin mimetics. An increase in the TIRF signal was found to correlate with an elevated glucose uptake. Variations in the expression level of the human insulin receptor (hInsR) showed that the insulin mimetics identified stimulate GLUT4 translocation by a mechanism that is independent of the presence of the hInsR. The chick embryo model has been reported to be a superior *in-vivo* tool for predicting the effects of drugs in various areas of application [2,3,4]. Here we used this alternative animal model to study the effect of selected phytochemicals on the blood glucose level. Therefore, candidate substances were applied to the air bladder of fertilized white SPF (special pathogen free) chicken eggs on day 10 of incubation for various time intervals. After the incubation period the egg is opened at its blunt end and the egg membranes are abscised. Finally, blood vessels are opened and blood is collected followed by analysis of the blood glucose level by high pressure liquid chromatography (HPLC). A time dependent decrease of blood glucose levels could be determined by applying human insulin as a control substance. Preliminary results of tested phytochemicals showed similar promising effects. In summary, the chick embryo model is an optimal complementary tool for a functional *in-vivo* analysis of candidate phytochemicals, which have been identified by preceding TIRFM measurements.

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Emulating the human vasculature in a multi-organ-chip platform

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Background and novelty: Our Multi-Organ-Chip (MOC) platform contributes to the ongoing development of *in vitro* substance testing systems with the ultimate aim to replace animal models. The two-organ variant (2OC) is able to incorporate two tissue engineered micro organ equivalents. These organoids are separated, yet, interconnected through a microfluidic system. The integrated on-chip micropump provides pulsatile circulation at a microliter scale. The circuit contains only 600 μL, thus, enables autocrine and paracrine crosstalk between the organoids through the enriched medium. The organoid cultures are, however, not sufficiently vascularised to overcome limitations in size and complexity. The object of this work is to recreate a continuous endothelial barrier between a subjacent tissue and the medium or a potential blood surrogate. We will present an overview of our ongoing approaches towards the emulation of human vasculature. Experimental approach: To recreate the human vasculature three major aspects had to be addressed. (1) Providing a near-physiological, pulsatile flow that recreates an *in vivo*-like shear environment. Micro particle image velocimetry (μPIV) was used to assess and optimise the dynamic flow. In contrast to classical µPIV red blood cells (RBCs) were introduced into a pumping chip instead of polymeric beads. (2) Creating an endothelial barrier in the chip's microfluidic system. Human dermal microvascular endothelial cells (HDMECs) were seeded into the perfused channels of the 2OC to line the luminal surface. (3) Establishing capillary-like vessels in the cultivation cavities as a vascular bed for organoid integration. We used fibrin scaffolds to enable the self-organisation of human umbilical vein endothelial cells (HUVECs) to microcapillaries. The process was initiated by the addition of adipose-derived stromal cells (ASCs). Results and discussion: The velocimetry-optimised flow enabled physiological cell behaviour of the HDMECs within the microfluidic channels indicated by migration, proliferation and orientation of the cells with the direction of flow. Moreover, the cells exhibited a pronounced expression of actin and typical EC-markers like CD31, VE-Cadherin and vWF under dynamic in contrast to static cultivation conditions. The HDMECs could be cultivated for up to 40 days. Data of the colonisation and the vitality of the HDMEC layer will be presented. The co-cultivation of HUVECs with ASCs inside a fibrin scaffold led to the formation of capillary-like structures. We will address issues to dynamic versus static cultivation environments, the stability of the fibrin hydrogel, along with the influence of the medium constituents on the cell behaviour. All results demonstrate that basic structures and features of blood vessels can be emulated inside the MOC platform and are the basis for future organoid integration. We assume this to be crucial for physiological-like interactions, regulation and homeostasis within an organoid (co-)culture. Moreover, it is a prerequisite for using blood in the chip and to address immunological queries.

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Phenotypic responses of human asthmatic airway epithelial cultures to rhinovirus and poly(I:C)

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Rationale: Human rhinovirus (HRV) is a pathogen for the common cold and is implicated as an inducer of asthma exacerbations. Human airway epithelial (HAE) cells are the principal target of HRV infection. Much of our knowledge of HAE responses to HRV are derived from *in vitro* HAE studies using either HRV or viral mimetics (e.g. double-stranded RNA, poly(I:C), or "PIC"). Two technical approaches that are used to study *in vitro* responses to HRV include 1) un-differentiated HAE monolayers in submerged (Sub) cultures, and 2) differentiated HAE cultured in air-liquid interface (ALI) systems. To gain an increased understanding of mechanisms linking HRV infection of HAE to asthma, we: 1) directly compared the response of Sub and ALI cultures to HRV 2) directly compared the effects of HRV and Poly(I:C) in HAE cultures 3) evaluated inherent differences in HAEs derived from asthmatic and non-asthmatic individuals as well as responses to HRV challenge.

Methods: HAE cells were isolated from lungs donated with informed consent. Sub and ALI cultures prepared from 6 asthmatic and 6 non-asthmatic donors were exposed to HRV or PIC. Cellular gene expression (including HRV genome) and cytokine/chemokine secretion into the culture medium were determined at 1.5, 8, 24 and 48 hr post-exposure and evaluated by an 88 inflammation relevant transcript Fluidigm custom qPCR array, and a 42-Plex Millipore Luminex-based protein assay, respectively. Genome-wide changes in gene expression in ALI cultures upon live viral infection at 24 hr post exposure were further analyzed using RNA-seq technology.

Results: HRV and PIC induced significant responses in gene expression and cytokine/chemokine secretion for numerous mediators/factors. Responses were highly dependent upon the culture condition (Sub or ALI) and exposure agent (HRV or PIC). PIC elicited a stronger response in Sub cultures, but was less effective in eliciting effects from ALI cultures. HRV elicited a stronger response in ALI cultures, but was less effective in eliciting effects from Sub cultures.

Conclusions: Our studies demonstrated that, compared to other models using Sub and/or viral mimetics (PIC), the ALI culture system challenged with live HRV better recapitulates the immune response to viral infection observed *in vivo*. Further, we have identified genes that show evidence of differential expression in asthmatic vs. non-asthmatic samples at baseline and post HRV challenge. This *in vitro* model system may be useful for facilitating our understanding of different viral exacerbation mechanisms in the future.

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A relevant *in vitro* model for safety testing of chemicals and efficacy testing of pharmaceuticals

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At present, animal-based tests are the major tests systems to assess tolerability and safety of chemical substances for regulatory purposes, and also for pivotal efficacy testing during pharmaceutical development. Animal (mostly mice) models are also used in increasing amounts as disease models in biomedical research. A vast number of examples show that animal tests are not ideal test systems to predict toxicity [1,2] nor pathophysiological processes in humans [3,4]. The poor predictivity is seen as a contributing factor e.g. to the low success rate of pharmaceutical development. At present less than 10% of pharmaceuticals in development phase enter market [5]. The major reasons for failure are the insufficient efficacy in patients and adverse drug reactions observed not pre-clinically but observed in clinical trials. At present there are only a few relevant and validated tests available. Thus, tests and testing strategies that are better mimicking human biology are urgently needed. The best predictability is expected to be achieved with organotypic human cell based models that mimic the microenvironment of the human tissue [6]. To ensure that the model is human relevant following key aspects should be taken into account: The used cells should be characterized and quality controlled. The media used should be defined. Before launching the model should be characterized on structural, genetic and functional levels. Further, the performance has to be shown using known chemicals with preferably human data (pharmaceuticals). Finally the test should be validated to confirm repeatability, reproducibility and relevance to ensure the data gained is adequate and the method will be accepted in use by the end users. In the presentation novel human cell based tissue models of vasculo/angiogenesis, cardiovascular and glucose resistant adipose test, are introduced highlighting the key factors that were considered when these standardised and human relevant *in vitro* tests to be used in pharmaceutical and chemical testing and in biomedical research were developed.

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Pain management and post-operative monitoring and care of laboratory rodents in Germany – lessons learnt and recommendations to improve practice

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We carried out the first large-scale assessment of research applications which involved recovery surgical procedures with rats and mice in Germany to evaluate recent practices in anaesthesia, analgesia, post-operative monitoring, humane endpoints and killing methods. Over 500 applications were reviewed. In this paper, we will present and discuss the results concerning perioperative pain management and postoperative monitoring, and we will give recommendations for best practice approaches. Our study suggested that perioperative analgesia could still be improved. Intraoperative analgesics were not administered to approximately 25% of mice and 28% of rats when Isoflurane was used as an anaesthetic. In the cases where intraoperative analgesics was provided, the time of administration, an important welfare factor, varied greatly, with 13% of the mice and 18% of the rats only receiving the pain relief at the end of surgery. In order to provide pre-emptive analgesia and minimize side effects from avoidably high doses of anaesthetics timely pain relief is crucial. Postoperative analgesic regimens were applied in 67% of mice compared to 71% of rats. In about 30% of cases, pain relief was not given or given at the discretion of the researcher (about 10%), whereas the presence of postoperative pain is to be expected after surgical interventions. Humane endpoints were not specified in 57% of the research applications. The frequency of monitoring the animals' wellbeing after surgery was indicated in only 33% of applications, and in the majority of these cases the frequency was only once daily. When clinical score sheets were used (in only 13% of applications), only a small portion (12%) included information about monitoring intervals. Critical times when extra monitoring and care should be given were rarely specified. The quality of the score sheets varied. Important information concerning the animals, such as general potential negative effects of the procedure, were often not included, and few gave clear instructions on how to intervene and treat, and when to apply humane endpoints. The study showed huge potential for further improvement of analgesic treatment, and the necessity to apply pain assessment methods and to use health score sheets became apparent.

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Use of (Q)SARs under REACH – perspective of a regulator

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In this presentation an overview of the use of (Q)SARs under REACH will be given from the perspective of a regulator from an EU Member State authority. Compared to Read-Across approaches, (Q)SARs are much less frequently used in REACH registration dossiers and a change in the near future appears unlikely. Potential reasons for this will be discussed and important elements needed for acceptance as well as areas of future work will be highlighted.

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Building a framework of communication between industry and regulators to identify *in vitro* methods suitable for tobacco regulatory science

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The Family Smoking Prevention and Tobacco Control Act (TCA) of 2009 gave the U.S. Food and Drug Administration's Center for Tobacco Products (CTP) regulatory authority over the manufacture, marketing and distribution of tobacco products in the United States. Estimates of the potentially large number of animals that could be used in this regulation are concerning to industry and animal protection organizations alike. While other industries have been successful in working collaboratively with regulators to identify non-animal testing approaches, past behaviors of the tobacco industry precludes such close association. It is evident, however, that the most efficient path to identify and standardize *in vitro* methods must draw on industry's expertise in this area. When dealing with an industry limited in collaboration, presentation and publication opportunities, how can industry's experience with *in vitro* methods be utilized Taking past lessons of success learned from collaboration with regulators, a multi-level workshop concept was conceived to (1) foster communication among tobacco industry, regulators and other stakeholders (2) draw on the experience of the industry in identification of non-animal methods for tobacco products and (3) provide a framework to optimize and standardize these methods to make them suitable for regulatory consideration. Recommendations from the first of these informational and technical workshops will be presented.

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Training as a means to hasten the implementation of 3Rs methods for cosmetics: a case study of China

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The disparity between countries in the acceptance of data from non-animal test methods poses not only significant ethical concern among consumers, but also economic and public relations consequences for global companies. Although international pressure has been pronounced, certain countries, such as China, have been reticent to adopt non-animal methods. The reasons given for the lack of adoption are varied but include: (1) the fact that China's cosmetic industry, including safety testing and risk assessment, is relatively young, and (2) that the infrastructure to perform non-animal tests in China needs to be established before acceptance can take place. It is viewed by some officials that if adoption of 3Rs methods happens too quickly it will create an inequality between domestic Chinese cosmetic companies and multi-national companies which have the means to not only perform non-animal methods outside of China, but also the expertise to use them in a risk assessment approach. Therefore the establishment of validated methods in qualified laboratories within China is an essential factor in the adoption of non-animal test methods. Additionally, as is the case in many other countries, the availability of validated *in vitro* methods does not necessarily mean that regulators will accept data from them. Therefore, to bring about a genuine shift in policy and attitude, the scientific community at large must support the concepts that underpin the use of non-animal approaches. To address these challenges a multi-faceted approach supported by industry and NGO's is being taken which focuses on: establishing in-country testing resources, educating key decision makers on the scientific merits of non-animal tests and encouraging leadership from the Chinese scientific community.

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Reboot3R – design thinking Workshops for scientists to constructively find ways to apply 3Rs within their own career

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Many efforts have been made during the last years to implement the 3Rs in university education. One big challenge for PhD students and Postdocs performing animal experiments is to address this sensitive topic and ethical concerns in a satisfying manner and to gain deep knowledge in alternatives for animal testing. Reasons for this may be the ignorance about the 3Rs as well as the restricted time besides the pressure to finish their thesis or to publish newest findings. Nevertheless, most of the scientists working with animals are willing to use and work on Reduction, Refinement and Replacement. In order to provide knowledge and join scientific forces, we organize interdisciplinary workshops focusing on the 3Rs using Design Thinking methods. Thus, young scientists get an unbiased and playful approach to the 3Rs and find creative space to exchange experiences and work on ways to further implement these principles. The workshop includes the Design Thinking process to find key questions, perform research with a focus on gaining empathy for the users, and design a serious, realistic solution. Design Thinking is a creative process to stimulate innovation. It combines creative with analytical methods from engineering, design, arts, social sciences, and business, to exploit the intrinsic innovative potential of multi-disciplinary teams. The given challenges for the teams can be about the practical work, e.g. implementation of a specific *in vitro* assay in the lab routine, or about general purposes, e.g. integration of the 3R topic on international scientific conferences. Instead of trying to quickly find a first solution the process allows the team to extensively explore the complexity of the challenge, getting to know different perspectives and developing a common language. This means, e.g. interviewing other stakeholders, university professors, animal keepers, veterinarians, and sharing individual interview experiences within the team. The following step includes the definition of the underlying problem. This could be e.g. the difficulty of access to information about alternative methods, or the lack of time. All gathered information, different perspectives and experiences are incorporated into the solution process creating a space between user needs and feasibility. The decision for one solution is made by the team after prototyping and intensive testing of selected ideas. The whole workshop is supported by short briefings about basics concerning history, laws and bureaucratic procedures, financial support possibilities, and examples of alternatives to animal testing. This new workshop approach guides young scientists through a difficult and emotionally complex topic towards a tangible result. Moreover, it aims at training scientists in team work and creative confidence – facilitating a change within the scientific community. The first workshop Reboot 3R of two or three days will be performed at the Berlin-Brandenburg School for Regenerative Therapies in September 2015.

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What happens when the particle has landed? – *In vitro* models for studying post-deposition events of aerosolized particles

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Pulmonary drug delivery needs not only to consider the properties of generated aerosols, but also to study the fate of the particle after deposition at the air-blood barrier in the different anatomical regions of the lung. Different cell culture models were investigated, representing either the airway tissue of the central lung or the alveolar epithelium of the peripheral lung. As a model for the airways, air-liquid cultured Calu-3 were used to study particle deposition by means of correlative microscopy and to study differences compared to submerged cultured cells. In this upper area of the lungs, tracheo-broncial mucus provides another, non-cellular barrier that protects the cells from direct particle interactions, emphasizing the relevance of air-liquid culture conditions. The barrier function of the alveolar epithelium was addressed by two different coculture models. The first model is based on primary human alveolar epithelial AT-1 cells (hAEpC's) and primary alveolar macrophages. The second model was set-up as a combination of lenti-virus immortalized epithelial (hAELVi) cells and differentiated THP-macrophages. Transepithelial electrical resistance (TEER) was investigated under air-liquid and submerged conditions. In contrast to the primary model, the immortalized coculture is more robust and expresses higher TEER values. Additionally, both models were investigated with focus on cytokine release after stimulation by lipopolysaccharides. The secretion of IL-6, IL-8, IL-10, IL-1β and TNF-α were determined in order to mimic the inflamed lung. The primary model is more sensitive towards inflammatory stimuli, compared to the cell line model. In summary, the choice of the most appropriate *in vitro* model to study particle-cell interactions in the context of pulmonary drug delivery depends on both the aerodynamic particle properties as well as the active pharmaceutical ingredient.

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Modelling the air-blood-barrier in state of disease – challenges and perspectives

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Before entering clinical studies, the safety and efficacy of new drug products has to be demonstrated first in preclinical studies. These may include animal experiments as well as *in vitro* methods. Aiming to ultimately predict the human situation, both approaches suffer from certain limitations. Apart from ethical advantages, however, *in vitro* methods allow to address certain biological and non-biological factors more directly. While *in vitro* models of healthy tissues and organs are primarily suited to address safety questions, demonstrating the efficacy of new treatments requires models that show certain features ("symptoms") relevant to the diseases for which a treatment is sought. In the context of pulmonary diseases, modelling the air-blood-barrier state of inflammation and/or infection is most needed. In order to demonstrate the efficacy of anti-inflammatory drugs, a co-culture of human alveolar epithelial cells and macrophages was stimulated first with pro-inflammatory stimuli and subsequently treated with anti-inflammatory drugs, monitoring the effect by measuring the release of inflammatory markers [1]. Similar approaches could be used to demonstrate the potential of innovative nanocarriers to improve the delivery of novel antiinfectives [2,3]. Improved delivery of nuclease-encoding mRNA for genome editing to correct some otherwise lethal SP-B deficiency using non-viral nanocarriers could be demonstrated in a mouse model [4]. Modelling a similar pathological situation using human cells *in vitro* might allow reducing the use of animals for such purposes in the future.

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Stem cell-derived human dorsal root ganglia-like cells to identify peripheral neurotoxicants

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Safety sciences and the identification chemical hazard have been seen as one of the most immediate practical applications of human pluripotent stem cell technology. Protocols for the generation of many desirable human cell types have been developed, but optimization of neuronal models for toxicological use has been astonishingly slow, and the wide, clinically-important field of peripheral neurotoxicity is still largely unexplored. Here, a 2-step protocol to generate large lots of identical peripheral human neuronal precursors was characterized and adapted to the measurement of peripheral neurotoxicity. High content imaging allowed an unbiased assessment of cell morphology and viability. The computational quantification of neurite growth as functional parameter highly sensitive to disturbances by toxicants was used as endpoint reflecting specific neurotoxicity. The differentiation of cells towards dorsal root ganglia-like neurons was tracked in relation to a large background data set based on gene expression microarrays. On this basis, a peripheral neurotoxicity (PeriTox) test was developed as first toxicological assay that harnesses the potential of human pluripotent stem cells to generate cell types/tissues that are not otherwise available for prediction of human systemic organ toxicity. Testing of more than 30 chemicals showed that human neurotoxicants, as well as neurite growth enhancers, were correctly identified. Various classes of chemotherapeutics causing human peripheral neuropathies were identified, while they were missed when tested on human central neurons. The PeriTox-test established here shows the potential of human stem cells for clinically-relevant safety testing of drugs in use and of new emerging candidates.

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Systems toxicology-based assessment of aerosols using human organotypic tissue cultures of nasal and bronchial epithelium

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In 2007, the National Research Council published a new strategy plan for toxicology assessment to update and advanced our knowledge on the toxicity and the mode of action of environmental agents. This 21st Century Toxicology paradigm recommends the use of computational toxicology and systems biology in combination with human relevant *in vitro* models that could allow multiple doses testing, reduce the use of animals in research and generate better data on the potential risks to humans. Exposure to cigarette smoke (CS) is known to be a major risk of developing serious diseases like chronic obstructive pulmonary disease. The development of new tobacco product that could reduce such health impact is ongoing at Philip Morris International. Because careful safety assessment strategy is required for these new products, we first investigated if human airway *in vitro* models exposed to CS at the air-liquid interface could mimic the *in vivo* biological response. Various endpoints (e.g. cytotoxicity, CYP1A1/1B1 enzyme activity, inflammatory markers release, mucus ciliary beating frequency, morphological and transcriptomic changes) were collected at different time after exposure to identify and compare the dose- and time-dependent effect of each exposure conditions. By using systems toxicology-based risk assessment approaches combining computable biological network models and gene expression changes, the molecular perturbations triggered after the different exposure conditions were analyzed and quantified. By taking this example of 21st Century Toxicology Assessment, we will discuss the advantages, the limitations and the remaining challenges of such strategy, especially concerning repeated exposure design required to assess respiratory disease *in vitro*.

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

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The development of non-animal skin sensitisation test methods and strategies is quickly progressing. Either individually or in combination, the predictive capacity is usually described in comparison to local lymph node assay (LLNA) results. In this process the important learning from other endpoints, such as skin or eye irritation, to account for variability reference test results – here the LLNA – has not yet been fully acknowledged. In order to provide assessors as well as method and strategy developers with appropriate estimates, we investigated the variability of EC3 values from repeated substance testing using the publicly available NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods) LLNA database. Repeat experiments for more than 60 substances were analysed – once taking the vehicle into account and once combining data over all vehicles. In general, variability was higher when different vehicles were used. In terms of skin sensitisation potential, i.e. discriminating sensitiser from non-sensitisers, the false positive rate ranged from 14-20%, while the false negative rate was 4-5%. In terms of skin sensitisation potency, the rate to assign a substance to the next higher or next lower potency class was approx.10-15%. In addition, general estimates for EC3 variability are provided that can be used for modelling purposes. This analysis stresses the importance of considering the LLNA variability in the assessment of skin sensitisation test methods and strategies and provide estimates thereof.

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Transatlantic collaboration of Cosmetics Europe with NICEATM/ILS to assess and develop strategy for skin sensitisation safety assessment without animal testing

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Cosmetics Europe's Skin Tolerance Task Force has taken up the challenge to develop an approach that allows skin sensitisation risk assessment of cosmetic ingredients without the need for animal data. For this purpose, it has set-up a four-phase programme. After initial assessment of 16 test methods in the first phase, the second phase focuses on the assessment of existing testing strategies and on the development of new testing strategy. Therefore, a database of approximately 100 substances with test data of six prioritised *in vitro* and *in chemico* test methods as well as Local Lymph Node Assay (LLNA) and human reference data has been compiled. Both re-assessment and development require appropriate computational skills. In addition, good knowledge of the skin sensitisation mechanisms *in vivo* as well as of the available *in vivo* reference test methods is essential to tackle these tasks. This combined expertise was found at NICEATM/ILS, who had developed an open source version of an existing Bayesian Network Integrated Testing Strategy as well as a hazard assessment strategy, so that a collaboration between Cosmetics Europe and NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods)/ILS has been established. Coordinated by Cosmetics Europe, ILS has curated the LLNA and human reference data, while the non-animal test method data were primarily curated by the test developers. The approved data were included in a database, not only to provide a central repository, but also to ensure appropriate functionality for the subsequent uses. Six testing strategies were selected based upon their ability to integrate the *in vitro* data currently curated in the database. NICEATM is preparing to reproduce the diverse predictive algorithms that range from approaches for hazard assessment based on majority of test results to artificial neural networks and Bayesian networks for potency prediction. In the third phase, data on challenging cosmetic ingredients will become available to explore the methods' applicability domains and fine-tune strategies. Through this programme and the collaboration, Cosmetics Europe envisages to evaluate the available testing strategies for skin sensitisation safety assessment and develop new approaches tailored for cosmetic ingredients where necessary.

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Generation of 3D neurospheres from human induced pluripotent stem cells as an alternative *in vitro* method to study neurodevelopmental defects of Cockayne Syndrome patients

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Studying mechanisms causing human brain disorders remains to be challenging due to the difficulty in obtaining neural tissue from patients. Despite the availability of an enormous variety of transgenic animals, these do not necessarily reflect human disease. Thus, the novel technique of human induced pluripotent stem cells (hiPSCs) represents a promising alternative method for patient-specific disease modeling as was already demonstrated e.g. for Alzheimer's and Parkinson's disease. Cockayne Syndrome B (CSB) is a rare autosomal recessive disease caused by mutations in the CSB gene, leading to severe neurodevelopmental defects. How CSB mutations impair brain development is so far enigmatic. This instant is owed to a lack of models to investigate CSB because compared to humans CSB-deficient animal models reveal a dissimilar neurological phenotype. Therefore, the goal of this study was to create a hiPSC-derived *in vitro* method that allows investigations on the neurodevelopmental pathophysiological mechanisms underlying the severe mental retardation of CSB patients. In analogy to primary neurospheres, which represent a well-studied *in vitro* method for studying basic processes of brain development (neural progenitor cell (NPC) proliferation, migration, neuronal and glial differentiation), we created 3D neurospheres from hiPSCs. Therefore, we compared two different protocols: one neural induction protocol using noggin (Noggin protocol) and one cultivating cells in neural induction medium containing B27 and N2 medium supplements (NIM protocol). FACS analyses revealed that both methods resulted in the differentiation of hiPSCs to nestin+/Sox2+ NPC, forming neurospheres. To test their performance, we compared both hiPSC-derived neurospheres to primary neurospheres generated directly from fetal brains (Lonza, Verviers). The comparative studies revealed that with regard to NPC proliferation and neuronal differentiation hiPSC-derived neurospheres created with the NIM protocol are more similar to primary human neurospheres than hiPSC-derived neurospheres generated with the Noggin protocol. Using the NIM protocol, hiPSCs derived from two different CSB patients with two different mutations (p.0 and pArg683x) and two healthy controls were differentiated into neurospheres. CSB-deficient hiPSC-derived neuropsheres display impaired proliferation as measured by the increase of sphere diameter over a time period of 14 days. Moreover, they show a decreased migration capacity. Preliminary data suggests that also differentiation seems to be impaired in CSB-deficient hiPSC-derived neurospheres compared to healthy controls. In summary, we set up a hiPSC-based neurosphere method, which allows investigations of mechanisms causing human neurodevelopmental disease. This *in vitro* model is used for studying the pathophysiological mechanisms underlying the neurodevelopmental phenotypes of CSB patients.

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Mechanistic studies on signaling pathways contributing to human neural progenitor cell migration

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Developmental neurotoxicity (DNT) of chemicals has recently been identified as a serious issue for society. However, there is a huge lack of data concerning chemicals effecting the development stages of the nervous system, in particular in humans. For gaining insight into the effects that compounds exert on the developing nervous system our group has been establishing the neurosphere assay, a three-dimensional (3D) *in vitro* test system based on human neural progenitor cells (NPC). The assay detects disturbances of basic processes of brain development such as NPC migration. Furthermore, it enables the investigation of signaling pathways driving or altering this process. The goal of this study was the investigation of pathways contributing to NPC migration by employing specific pathway inhibitors. Human NPCs were treated with seven pathway inhibitors (blocking Rho-associated protein kinases p160ROCK, c-Jun N-terminal kinases, p38 MAP kinase, src thyrosine kinase as well as Wnt/β catenin and Phosphoinositide 3-kinase pathways) for 24 and 72 hours. Migration distances were determined microscopically by using the software ImageJ. To distinguish the results from possible influences on cell viability, the AlamarBlue® assay was performed. The JNK inhibitor SP600125, the p38 MAP kinase inhibitor SB202190, the src thyrosine kinase inhibitor PP2 and the Rho-associated protein kinase p160ROCK inhibitor Y-27632 inhibited NPC migration significantly after 24 hours. Three of them reduced migration with statistical significance and did not affect cell viability. The Wnt/β catenin inhibitor (IWP-2) and the MAPK2 pathway inhibitor (PD98059) neither displayed any influence on migration nor showed a significant influence on cell viability. In contrast, the Phosphoinositide 3-kinase pathway inhibitor (LY294002) solely decreased viability without affecting migration. These data contribute to the over-all concept of mechanism-based hazard assessment of compounds and, in combination with pathway-based information of unknown substances, will facilitate chemical risk assessment by using *in vitro* methods. However, for such data to be of value for qualitative and quantitative risk assessment they need to be embedded into the "Adverse-Outcome-Pathway" concept.

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Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells

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Vascularization in Tissue Engineering is essential to provide sufficient nutrient and growth factor supply and after implantation into target sites. Co-cultures of adipose-derived stem cells (ASC) with outgrowth endothelial cells (OEC) in fibrin gels were shown to provide an effective approach to induce vasculogenesis *in vitro*. The aim of this study was to evaluate molecular mechanisms involved in endothelial cell (EC)-ASC-induced vascular structure formation. EC were embedded in fibrin gels either containing non-coated or ASC-coated microcarrier beads as well as ASC alone. Moreover, EC-seeded constructs incubated with ASC-conditioned medium were used in addition to constructs with ASC seeded on top. Vascular network formation was monitored by green fluorescent protein (GFP) expressing cells or immunostaining for CD31. Quantitative PCR analysis of cells derived from co-cultures in fibrin was performed to evaluate changes in the expression of EC marker genes during the first week of culture. Finally, angiogenesis-related protein levels were measured by performing proteome profiler arrays. The results demonstrate that proximity of both cell types is required for network formation and ASC stabilize EC networks by developing pericyte characteristics. We further show that ASC induce vessel growth by secreting pro-angiogenic and regulatory proteins. This study reveals angiogenic protein profiles involved in EC/ASC interactions in fibrin matrices show the suitability for testing of pro- and antiangiogenic substances *in vitro*.

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3D biocompatibility test systems mimicking the *in vivo* situation: evaluation of a hydroxyapatite-nanoparticular paste

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Introduction: Nano-particular biomaterials have gained increased attention because of their potential application in medical fields including tissue engineering (TE), dentistry and pharmacy. For the evaluation of novel biomaterials, *in vitro* testsystems are often used to evaluate the cellular response. However, the capability of most cell culture testsystems to represent the *in vivo* situation is limited. Ostim® is a nano-hydroxyapatite aqueous paste approved for clinical use as bone defect filling matrix. In a previous study in a bone defect model, Ostim® showed to support bone formation with no inflammatory reaction of the surrounding tissue [1]. Interestingly, the addition of the paste on cultured cells in a typical cytotoxicity test set-up resulted in a toxic effect *in vitro*. Therefore, we aimed to establish an *in vitro* test system that is able to mimic the *in vivo* effects of nanoparticles.

Materials and Methods: Ostim® was compared to nano-hydroxyapatite powder (HAP Sigma-Aldrich). As a first step, 3-dimentional (3D) fibrin embedded cells (C2C12 cells as well as adipose tissue-derived stromal cells) were used to evaluate biomaterial cytotoxicity. As a second, new cytotoxicity test-system, we placed the biomaterials between two layers of viable, healthy and intact human amniotic membrane. Both systems were compared to standard 2-dimensional (2D) cytotoxicity evaluations. Results: A dose-dependent cytotoxic effect of nano-hydroxyapatite particle formulations on the cells was detected in standard 2D culture as well as in 3D fibrin constructs. In contrast, placing the biomaterials between two amniotic tissue layers showed no toxic effect on the membrane. Therefore, the 3D test system using human amniotic membrane may be well suited to predict the biomaterials impact on human tissue.

Discussion and Conclusion: To conclude, it was possible to establish a new test system based on amniotic membrane, which as a human tissue may hence be better suited to estimate the behaviour of the biomaterial *in vivo*. The presented test system presents a promising candidate for biomaterial evaluation.

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Development of micro-engineered 3-dimensional biomimetic hydrogel culture system by using Gelatin Methacrylate

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The cellular microenvironment plays a critical role in controlling different aspects of the cell behavior such as morphology, differentiation and proliferation. Hydrogels are the most widely used culture systems for developing 3D microenvironment [1]. However, some of the current hydrogels suffer from limitations like poor cell responsiveness, limited biodegradability, difficulty in tuning the stiffness of hydrogel and poor stability. Here, we describe a microengineered 3D cell culture system using a UV photopolymerisable gelatin derived hydrogel called Gelatin Methacrylate (Gel-MA) [2,3]. In these scoping experiments we have characterized growth and differentiation of, 2 types of human lung fibroblasts (MRC5 and primary fibroblast) encapsulated within GelMA. Our data show fibroblasts attained their native elongated morphology within microfabricated hydrogel system with different percentages of GelMA 24 hours after encapsulation and maintained high (> 80%) cell viability throughout experiments. Regarding cell proliferation, the confocal imaging of cytoskeletal staining showed that encapsulated cells became confluent around Day 10 and Alamar blue cell proliferation also showed significant increase in cell numbers and proliferation rate over 10 days, proving that encapsulation within GelMA has no restriction on cell spreading or cell proliferation. Together, both results confirm that GelMA hydrogel conserves the natural extracellular matrix (ECM) components which are necessary for biodegradability properties of hydrogel. As ECM deposition is one of main characteristics of fibroblast, detection of significant fibronectin deposition by encapsulated fibroblasts within hydrogel system again highlight the biocompatibility of cultural environmental condition within the hydrogel system. In terms of degradation, despite some degradation the microfabricated GelMA was stable for up to 10 days making long term cultures feasible. Therefore, we conclude that our biomimetic hydrogel system could be useful for developing cell responsive microtissues and cell to cell interaction within hydrogel in many research areas such as drug development, biomedical engineering and disease models to replace the animal experimental models.

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A microfluidic four-organ-chip – a potential tool for ADME profiling

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Systemic absorption and metabolism of drugs in the small intestine, metabolism by the liver as well as excretion by the kidney are key determinants of efficacy and safety for therapeutic candidates. However, these systemic responses of applied substances and their metabolites lack in most *in vitro* assays. Here, we present a new microphysiological system maintaining the homeostasis and functionality of four organ equivalents over 28 days in co-culture to overcome this problem. The Four-Organ-Chip consists of two independent microphysiological fluid flow circuits arranged on two levels, separated by a PET membrane in a bioreactor the size of a microscopic slide. Each circuit is operated by a separate peristaltic on-chip micro-pump interconnecting the four human organ equivalents through pulsatile media flow within the microfluidic channels. On the the first, surrogate blood circuit, a primary human small intestinal model was inserted. The intestinal tissue was cultured in an integrated cell culture insert and provided a barrier function from the apical side of the intestine to the first circuit, allowing absorption. The on-chip micro-pump enabled the distribution from the basolateral side of the intestinal model to 3D-based liver-spheroids, where potential substances could be metabolised. Finally, the microfluidic channel passed the bottom of the PET membrane, seeded with renal proximal tubule cells. This kidney model separated the first circuit from the second, excretory circuit. In this study, we combined this ADME approach with a skin biopsy to analyse toxicity, but this organ culture could also be replaced by any other organ equivalent, like neuronal tissue, lung tissue or others, depending on the target organ for toxicity. This four-organ-chip system assures near to physiological fluid-to-tissue ratios. The combination of the four organs was cultured for up to 28 days and results showed a steady consumption of glucose and low LDH profiles during the complete culture period, providing evidence for a stable coexistence between the four tissues. The constitutive phase I and II enzymes were expressed in liver tissues and stayed constant on protein and mRNA level over the time cultured. The intestinal tissues expressed glucose transporters and its barrier function was proven by expression of tight junction proteins and stable, near to physiologic TEER values. Renal proximal tubule cells showed polarisation, a steady expression of tight junctions and metabolic activity. To our knowledge, this is the first approach to establish a system for *in vitro* microfluidic ADME profiling and repeated dose systemic toxicity testing of drug candidates over 28 days.

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Glutamate and hypoxia as a stress model for the isolated perfused vertebrate retina

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Purpose: Neuroprotection has been a strong field of investigation in ophthalmological research in the past decades and affects diseases such as glaucoma, retinal vascular occlusion, retinal detachment, and diabetic retinopathy. One object of this study is to introduce a standardized stress model for future preclinical therapeutic testing. Methods: Bovine retinas were prepared and perfused with an oxygen saturated standard solution, and the Electroretinography (ERG) was recorded. ERG measures the electrical responses of various cell types in the retina, including the photoreceptors (a-wave response) and inner retinal cells (bipolar and amacrine cells, b-wave-response). After recording stable b-waves, hypoxia (pure N2) or glutamate stress (250 μM glutamate) was exerted for 45 min. To investigate the effects on photoreceptor function alone, 1 mM aspartate was added to obtain a-waves. ERG-recovery was monitored for 75 min. Results: For hypoxia, a decrease in a-wave amplitude of 87.0% was noted ($p < 0.01$) after an exposition time of 45 min (decrease of 36.5% after the end of the washout $p = 0.03$). Additionally, an initial decrease in b-wave amplitudes of 87.23% was recorded, that reached statistical significance ($p < 0.01$, decrease of 25.5% at the end of the washout, $p = 0.03$). For 250 μM glutamate, an initial 7.8% reduction of a-wave amplitudes ($p > 0.05$) followed by a reduction of 1.9% ($p > 0.05$). A reduction of 83.7% of b-wave amplitudes ($p < 0.01$) was noted after a washout of 75 min the reduction was 2.3% ($p = 0.62$). Conclusion In this study, a standardized stress model is presented that may be useful to identify possible neuroprotective effects in the future and replace expensive preclinical animal experiments. The isolated superfused retina is a sensitive and highly standardized tool for basic and preclinical research and can be easily expanded. It is a cost effective replacement for animal experiments and generates a pseudo *in vivo* situation. With the ERG, that provides information about the functionality of the cells a great read out tool is implemented. Because of structural and functional analogies between the human and the bovine situation and the high standardization, the method itself can be considered superior to the animal model in selected situation.

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Cytochrome C assay as a tool for determining ranking for nanomaterials surface reactivity

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Bio-physical interactions may hold the key to understanding the potential effects of nanomaterials on human health. One property which is integral to regulating bio-physical interactions of nanomaterials is the surface reactivity. It is known that the nanomaterial surface area to volume ratio is greatly increased when compared to bulk materials, the increased surface area means there is a larger surface available for reactions to occur leading to generation of reactive oxygen species generation (ROS). To quantify the surface reactivity of a nanomaterial an assay using Cytochrome c was applied, reduced Cytochrome c will be oxidised when there is a ROS present. The amount of ROS present will dictate the amount of oxidised Cytochrome c, this will give an indication to the surface reactivity of a given nanomaterial. Reduced Cytochrome c was treated with different concentrations of Mn_2O_3, TiO_2, $BaSO_4$, and CeO_2 for 24 hours at 4°C in a 96 well plate. Tecan multi plate reader was used to scan the absorbance between 400 to 600 nm and peaks at 520 nm and 549 nm were observed, which correspond to the peaks of reduced Cytochrome c. The concentration used for the assay was 5 mg/mL, ranking based on the results shows that Mn_2O_3 has the greatest oxidation potential oxidising 9.95% of the Cytochrome c. Followed by CeO_2 (8.8%), $BaSO_4$ (6.08), and finally TiO_2 (2.48). This study, developed in co-operation with Paris Diderot University [1], has produced a method giving a ranking system for surface reactivity. A number of other methods are also currently being tested to support data produced by Cytochrome c, preliminary analysis has suggested that the ranking of surface reactivity by Cytochrome c is reflected in the results attained by FRAS/FRAP methods [2] and photocatalytic assays. A combination of method that exhibit similar findings can be used to strengthen the case for bio-physical property ranking, which may be a tool to be used in grouping and read-across for risk assessment. In conclusion, surface-reactivity is one of the relevant bio-physical interactions contributing to the potential adverse health effects of nanomaterials. Thus surface reactivity, along with other relevant bio-physical interactions of nanomaterials could be used to group nanomaterials that will make risk assessments more efficient in the future [3-5].

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An animal-free batch testing method for alum-adjuvanted veterinary rabies vaccines

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Since many years efforts are ongoing at the Paul-Ehrlich-Institute to develop modern batch testing methods for vaccines and immunologicals according to the 3R concept. Here, we aim to establish an animal-free test method for the antigen quantification of alum-adjuvanted rabies vaccines. The challenge with alum-adjuvanted vaccines is to detach the adsorbed immunogenic components from the adjuvant and make them available to immunochemical quantification. To this end an electrochemical method was developed by which the antigens are detached from the aluminium salt matrix and simultaneously immobilized on a nitrocellulose membrane. In a second step the amount of desorbed antigen is determined using a quantitative immunoblot. We can demonstrate that the method is highly specific. The antigens are near quantitatively desorbed and detected. Additional antigens present in the vaccine do not influence the results. The method is reproducible and suitable for all veterinary rabies vaccines currently available on the German market. Together with additional tests assessing the quality of the adjuvant matrix this versatile and robust antigen quantification method holds promise to form an important component for a new animal-free test strategy for alum adjuvanted vaccines based on the consistency approach.

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In vitro investigation on uptake, cytotoxicity and genotoxicity of two similar sized nanoparticles used in food and packaging on the hepatic cell model hepaRG

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The application of manufactured nanomaterials (MNMs) in consumer products is expected to increase considerably in the near future, and the evaluation of their safety is thus a major concern in Europe and worldwide. Although, the toxicity of these MNMs still requires more investigation. Due to numerous different MNMs, the variability of their physic-chemical properties together with inherent limitations of animal experimentation, the toxic effects cannot be investigated *in vivo* for each MNM. Therefore *in vitro* data are required to study potential adverse effects. In this context, in the SolNanotox project, we choose to study two rutile TiO_2 nanoparticles of similar size: NM 103 (hydrophobic) and NM 104 (hydrophilic). As liver is known to be one potential organ for accumulation, uptake and several toxic and genotoxic effects were checked and compared for these two MNMs, in a range of 9 to 256 μg/ml, on the human liver cell model HepaRG, sharing close characteristics to primary human hepatocytes. Uptake and localization were investigated by Transmission Electronic Microscopy (TEM) and cytotoxicity with NRU assay. Inflammation was investigated by IL8 release detected with ELISA. Other toxicity endpoints (apoptosis, DNA breaks, inflammation …) were investigated by immunofluorescent labeling through High Content Analysis (HCA). Genotoxicity was investigated by alkaline comet assay which detects simple and double DNA strand-break as well as alkali-labile sites. This comet assay was perform with or without addition of formamido pyrimidine glycosylase (FPG), which is a protein used to assess oxidative DNA base damage because it detects 8-OH guanine and other oxidatively damaged purines. Our first results showed a large uptake of both NM 103 and NM 104 by membrane invagination. In the cytoplasm, free nanoparticles as well as trapped in vesicles were observed. No MNM was found in the nucleus. NM 103 and NM 104 induced apoptosis on HepaRG cells for the two highest concentrations without showing any modification of cell viability or cell number. No significant phosphorylation of H2Ax was detected by HCA compared to the control, as well as no significant DNA damage by the comet assay. Increase of IL-8 release was observed with both MNMs but only significant for cells treated at 28 μg/ml with NM 103. However, the MNMs may interfere with most assays and further checking is underway. Our results showed that NM 103 and NM 104 are well absorbed by HepaRG cells irrespective of their hydrophobicity and induced a slight apoptotic effect but no genotoxicity. Still, the interference of MNMs with the set up methods as well as other endpoints by HCA (oxidative stress, NFkB translocation, …) needs to be more deeply investigated.

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Characterization of stably transfected HEK-293 cells expressing uptake transporters using fluorecent substances

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Membrane transporters are major variables for disposition, efficacy and safety of many drugs. Organic anion transporting polypeptides (OATPs, gene family: SLCO) and Na^+-taurocholate co-transporting polypeptide (NTCP) belong to the uptake transporters and mediate the uptake of a broad range of substrates including several widely prescribed drugs into cells. Therefore, we developed a cell platform using stably transfected cells expressing pharmacologic relevant uptake transporters. Traditionally, the function of cells has been characterized using radiolabeled substrates, which causes potential health risks and requires a specific equipment and an isotope laboratory facility. To overcome these issues, we wanted to analyse the uptake function with fluorescent substrates, which can be performed in normal labs. Four fluorescent substances (Fluorescein methotrexate (FMTX) Fluorescein Rhodamine 123 and Cholyl-Lysyl-Fluorescein (CLF)) were analyzed with stably transfected HEK-293 cells expressing OATP1A2, 1B1, 1B3, 2B1 and NTCP. HEK-OATP1B3 showed an uptake of FMTX (Km = 1.5 ±0.4 μmol/l, V_{max} = 22.8 ±1.7 pmol/mg × min), which could be inhibited by rifampicin (IC_{50} = 0.37 μmol/l) efficiently. FMTX was also a substrate for HEK-OATP1B1 cells with a Km value of 4.7 ±1.3 μmol/l and a V_{max} value of 59.1 ±4.7 pmol/mg × min. This uptake was inhibited by rifampicin with an IC_{50} value of 0.69 μmol/l. HEK-OATP1B1 showed an affinity to fluorescein (Km = 18.2 ±6.7 μmol/l, V_{max} = 147.9 ±23.8 pmol/mg × min), which could be inhibited by rifampicin (IC_{50} = 1.05 μmol/l). HEK-OATP1A2 showed an uptake of Rhodamine 123 (Km = 3.8 ±1.3 μmol/l, V_{max} = 283.2 ±41.8 pmol/mg × min). This uptake was inhibited by rifampicin (IC_{50} = 35.4 μmol/l). HEK-NTCP showed an uptake of CLF (Km = 1.7 μmol/l, V_{max} = 2.9 pmol/mg × sec), which could be inhibited by Cholate (IC_{50} = 7.45 μmol/l) efficiently. However, OATP2B1 stably transfected HEK-293 cells showed no specific transporter-mediated uptake of all four fluorescent substrates. Thus, the transport function of stably transfected HEK-293 cells expressing OATPs and NTCP can be characterized with fluorescent substances. Having demonstrated that transporter activities in stably transfected HEK cells can be analyzed with fluorescent compounds we are currently adopting these methods for the use with primary hepatocyte cultures.

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Characterization of Transporter Activities in Different Cell Types by Using Fluorescent Substrates

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Membrane transporters are major variables for disposition, efficacy and safety of many drugs. Organic anion transporting polypeptides (OATPs, gene family: SLCO) and Na^+-taurocholate co-transporting polypeptide (NTCP, gene family SLC10A1) belong to the uptake transporters and mediate the uptake of a broad range of substrates including several widely prescribed drugs into cells. They are expressed in hepatocytes as the major drug metabolizing cells. We have established a cell platform using stably transfected cells expressing pharmacologic relevant uptake transporters to analyze drug affinities. Here, the transporter activities are analyzed with fluorescent substances instead of traditional characterization using radiolabeled substrates. This new approach avoids potential health risks and can be performed in normal laboratories. Identified fluorescent substrates are then used in characterization of transporter functions in primary hepatocytes from different species.

Transporter activities of OATP1A2, 1B1, 1B3 and NTCP were analyzed with four fluorescent compounds (Fluorescein methotrexate (FMTX); Fluorescein; Rhodamine 123 and Cholyl-Lysyl-Fluorescein (CLF)).

FMTX was specifically transported by OATP1B3 and OATP1B1; both transporters could be inhibited by rifampicin in their activities. HEK-OATP1B1 showed an affinity to fluorescein as well, which could also be inhibited by rifampicin. Rifampicin also inhibited the transport of Rhodamine 123, which is a substrate for HEK-OATP1A2. CLF was characterized as a substrate for HEK-NTCP, whose uptake functions were efficiently inhibited by cholate.

Among the specific transport in stably transfected cells, FMTX was evaluated as a suitable transporter substrate for primary hepatocytes as well. Fresh isolated human and Cynomolgus hepatocytes in culture showed a saturable time-dependent uptake of FMTX with rifampicin revealing its inhibition potential also in primary hepatcytes. In conclusion, the transporter functionalities in stably transfected HEK293 cells and primary hepatocytes can be analyzed with fluorescent compounds. These platforms can be used for identification of specific transporters involved in drug uptake.

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NanoE-Tox – in-depth database on ecotoxicity of nanomaterials

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The development of the consumer society leads to constant need of materials that are lighter, stronger and more durable than the existing ones. During the past few decades, various types of engineered nanomaterials (ENMs) have been created to fulfil these criteria and as of June 15th 2015 there were more than 1,800 ENM-containing products listed in Consumer Products Inventory (http://www.nanotechproject.org/cpi/products/). However, as the production and use of ENMs increases, more ENMs are inevitably released into the environment during the life cycle of the products. Alarmingly, as the properties of materials change at nano-scale, they might also become more harmful to organisms but the exact parameters determining the safety or toxicity of ENMs are yet to be discovered. Risk assessment of all the ENMs in the market would require enormous amount of time and test organisms of diverse range. Therefore, there is a need to refine, reduce or replace (3R's) traditional animal testing and develop alternative risk evaluation methods, e.g. computational QSARs/QNARs models (predicting toxicity based on ENP's physico-chemical properties). While development of *in silico* methods relies on good-quality experimental data on ENM toxicity, most of the available data in the literature even for the same test species are highly variable. In order to map and analyse the state-of-the art of the existing information suitable for QNARs, we created a database NanoE-Tox. The database is based on existing literature on ecotoxicity of eight ENMs: carbon nanotubes (CNTs), fullerenes, Ag, TiO_2, ZnO, CeO_2, CuO, and iron oxides (FexOx Fe_2O_3, Fe_3O_4) which were selected based on their production volumes, application in consumer products and technological potential. In addition, information on detailed physico-chemical properties of ENMs, toxicity test conditions as well as reported toxicity mechanisms and uptake of ENMs was compiled. Altogether, NanoE-Tox database consolidates data from 224 articles and lists 1,518 toxicity values (EC_{50}/LC_{50}/NOEC) for 116 different test species. Most of the data originates from studies with crustaceans (26%), bacteria (17%), fish (13%), and algae (11%). Based on the median toxicity values of the most sensitive organism (data derived from three or more articles) the toxicity order was as follows: Ag > ZnO > CuO > CeO_2 > CNTs > TiO_2 > FexOx. Interestingly, systematic collection of data revealed several gaps in the current knowledge about ENMs ecotoxicity, e.g. the physico-chemical properties of investigated NPs are often described insufficiently, and many ecotoxicity tests with standard test organisms are conducted with slightly modified versions of OECD/ISO protocols making risk assessment of ENMs difficult. To conclude, NanoE-Tox allows the analysis and comparison of toxicity of ENMs across different test species and provides valuable input for computational analysis and ENMs environmental hazard estimation. This work was supported by Estonian Research Council's "Environmental Conservation and Environmental Technology R&D Program" project "TERIKVANT" and by Estonian Ministry of Education and Research (target-financed theme IUT23-5, PUT748, and ETF9001).

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Assessment of the wound healing effect of selected flavonoids on *in vitro* irritation model

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Silymarin, an extract of *Silybum marianum* seeds has been reported to have anti-inflammatory properties [1]. Accordingly, the aim of this study was to evaluate the anti-inflammatory and wound healing potential of silybin, the main flavonolignan component of silymarin. *In vitro* model systems currently used to predict the topical irritation potential of compounds can be divided into four categories corresponding to the increasing levels of complexity, which are single cell assay, epidermal equivalents, skin equivalents and excised skin [2]. The reconstructed human epidermis (RHE) grown on inert porous filters provides the possibility for the passage of cytokines released by keratinocytes into the medium underneath to enable subsequent detection [3]. In our experimental setup, the epidermal barrier disruption has been represented by reconstructed human epidermis treated with topical application of solution of surfactant. The inhibitory effect of silybin on pro-inflammatory cytokines (interleukin 1α, interleukin-6, interleukin-8) production as compared to the traditional nonsteroidal anti-inflammatory drug indomethacin after 24 hours of topical treatment was examined. Simultaneously, the measurements were performed with flavonoid quercetin, with proven anti-inflammatory and wound healing efficiency [4]. These results were correlated with histological analysis and immunohistochemical staining with anti-cytokeratin 14 and anti-loricrin antibody. In conclusion, according to our findings, the investigated flavonolignan silybin exhibit anti-inflammatory properties, that are comparable to indomethacin. Nevertheless, silybin displays slightly different level of anti-inflammatory effect in comparison with quercetin.

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Accessibility of published QSAR models: ecotoxic endpoints

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In QSAR (Quantitative Structure-Activity Relationship), previously created experimental data is used to develop predictive models, thereby reducing the need for experimental testing which is costly and may also raise ethical issues when sacrifice of laboratory animals is required. Every year, according to Web of Science, more than a thousand papers on QSAR models are published in scientific journals. Can we make the published QSARs into easily usable tools? Development of information technology has made it possible to distribute QSAR models in executable form organized into repositories. However, reestablishing QSARs from already published scientific literature depends on how accurate and complete is the data that is compiled and made available by the authors. In this presentation, we are giving an overview of the availability and reusability of published QSAR models considering a list of ecotoxic effects on a variety of endpoints (included in JRC QMRF v1.2 classification). Web of Science was searched for TOPIC: (qs*r AND "endpoint specific term(s)"). Each paper and their supplementary material were checked for endpoint and descriptor values, precise algorithm of the model (complete with weights, scores, support vectors, etc. depending on the method) and statistical parameters. The search over all listed endpoints resulted in 791 hits, including 344 publications containing QSAR models of which approximately one third could potentially be rebuilt from the data provided. The largest number of models was found for fish toxicity, followed by Daphnia and algae toxicities. The most prevalent form of modelling methods was (multi)linear regression, followed by partial least squares, neural networks, linear discriminant analysis, support vector machines and k-nearest neighbours. We found that publications addressing ecotoxic effects were more likely to contain all the data when published after the year 2000, had less variables, smaller data set and were expressible as (multi)linear relationships. A notable obstacle was inaccessibility of earlier publications and loss of supplementary material due to certain time limits. To recognize the efforts of researchers in curating experimental data and developing QSARs, we propose a solution for storing and distributing the models in the publicly accessible QSAR DataBank (QsarDB): http://qsardb.org/

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An *in vitro* reconstructed normal human corneal tissue model for corneal drug delivery studies of ophthalmic formulations

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Permeation of topically applied ocular drugs occurs predominantly through the cornea and therefore absorption studies using corneal tissues play a critical role in ocular drug formulation. Currently, most ocular absorption studies use *in vivo* or *ex vivo* animal tissues that have many disadvantages, including poor standardization, species extrapolation, high cost, and ethical concerns. In this study, we present a new, reconstructed ocular tissue model that consists of normal human corneal epithelial cells that have been cultured using serum free medium to form a highly differentiated organotypic corneal epithelial tissue. The cultures form a multilayered structure containing tight junctions and develop barrier properties comparable to *in vivo* human cornea. Real time qPCR analysis confirmed that the reconstructed corneal tissues express ALDH-A1 and TXNRD1 (corneal epithelium specific enzymes that confer resistance to UV light damage), MUC4 (corneal glycoprotein mucin), and ABCC1 and ABCB1 (efflux transporters with important role in corneal drug distribution). The corneal tissue model permeability was evaluated using compounds with a wide range of properties, including the hydrophilic dye, sodium fluorescein (NaFl), hydrophobic dye, rhodamine B (RdB), fluorescein isothiocyanate-labeled dextran (FD-4, MW = 4000), and ophthalmic related antibiotics, ofloxacin (OFL) and voriconazole (VCZ). The permeabilities of RdB and FD-4 were 13.5 fold higher and 2.1 fold lower, respectively, compared to NaFl. The permeation enhancers, Benzalkonium Chloride (0.002-0.02%) and EDTA (0.05-0.5%) significantly increased permeation of OFL and VCZ by 10.1 and 9.3 fold, respectively. The reconstructed *in vitro* corneal tissue morphology, barrier function, and permeability resemble those of the *in vivo* human cornea, and hence this model is anticipated to be a useful tool to evaluate corneal drug permeation and its mechanisms.

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Optimization of an eye irritation assay for hazard identification and labelling of materials to address the EU Cosmetic Directive and REACH legislation

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Implementation of the 7th Amendment to the EU Cosmetics Directive and EU REACH legislation has heightened the need for predictive *in vitro* ocular test methods. To address this need, we have developed an eye irritation test (EIT) which utilizes a three dimensional organotypic tissue model based on normal human cells. The test utilizes two separate protocols, one specifically designed for liquid chemicals and a second, related protocol for solids. The protocol is able to discriminate between ocular irritant / corrosive materials (CGHS Categories 1 and 2) and those that require no labeling (GHS No Category). Over 100 substances were tested during the development of the assay and the overall results met the acceptance criteria set by the Validation Management Group (VMG) for eye irritation. Although the original eye irritation protocol was successfully pre-validated in an international, multicenter study sponsored by COLIPA (the predecessor to Cosmetics Europe), data from two larger studies (the ECVAM-COLIPA validation study [1] and an independent in-house validation at BASF SE [2]), resulted in sensitivity for solid materials below the acceptance criteria set by the VMG and indicated the need for improvement of the assay sensitivity for solids. Here we report results of the assay optimization. By changing the exposure for solid materials from 2 to 6 hours, the EIT achieved 100.0% sensitivity, 68.4% specificity, and 84.6% overall accuracy, thereby meeting all the acceptance criteria set by the VMG. An independent validation study was performed with this protocol and extended shipping times in Japan, resulting in the concordant predictions of almost all chemicals tested previously by Kaluzhny et al in 2013 [3,4]. This modified procedure together with the original liquid protocol has been approved as the new OECD TG 491.

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Development, optimization and standardization of an *in vitro* skin irritation test for medical devices using the reconstructed human tissue model EpiDerm

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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 extracts are dilute solutions with low irritation potential, therefore the validated RhE-methods needed to be modified 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. Two additional exposure times were tested along with other medical device materials. After the successful transfer and standardization of the protocol, eleven laboratories were trained from EU, USA and Korea in the use of the protocol in the preparation for the validation. All laboratories produced data with almost 100% agreement among predictions for the selected references. RIVM and MatTek IVLSL performed additional tests with heat-pressed PVC sheets spiked with Genapol X-080 (Y-4 polymer), Vicryl suture, and polymers spiked with heptanoic acid and sodium dodecyl sulfate. All materials were extracted for 24 or 72 hours in both saline and sesame oil at 37°C. Significant irritation responses were detected for Y-4 under all conditions. These results were consistent with those reported by other research groups involved in the upcoming validation study. Vicryl suture was negative and spiked polymers were either positive or negative dependent on the extraction solvent. We conclude that a modified RhE skin irritation test has the potential to address the skin irritation potential of the medical devices, however, standardization and focus on the technical issues is essential for accurate prediction.

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Health safety of food contact paper evaluated by *in vitro* toxicological methods

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Paper and paperboard are widely used as food packaging materials intended for direct contact with foodstuffs and have to comply with a basic set of criteria concerning safety. A number of chemicals, such as slimicides, bleaching agents, and inks are used during the paper production process. Consequently, the total amounts of extractables might be high and the number of compounds considerable, including many unknowns. In the recent years a number of short-term bioassays focused on cytotoxicity and genotoxicity have been proposed as an approach for safety assessment of the chemically complex food contact paper materials and a draft methodology for biological testing has been published as the outcome of the joint European Commission/Industry project known as Biosafepaper. In our screening study we have tested extracts of 141 commercially available paper and paperboard samples in the 3T3 Balb/c NRU cytotoxicity test. Extracts of 75 samples (53.2%) induced cytotoxic effects in the cell culture. Selected samples with high cytotoxicity were further tested using reconstructed human tissues EpiIntestinal, EpiIntestinal FT (MatTek) and Colon epithelium (Sterlab) as models mimicking the human gastrointestinal tract. MTT viability assay was employed for evaluating the toxic effects of paper extracts in the tissues. HPLC-TOF-MS was used for analysis of the extracted compounds and for detection of their possible penetration into the culture medium underneath the tissues. ELISA method was employed for investigation of cytokine release suggesting inflammatory reactions. The results confirmed high sensitivity of the cytotoxicity test enabling to detect minor differences in the toxicity potential of paper samples. However, the study on reconstructed human models confirmed that even highly cytotoxic extracts did not affect the viability of complex human tissues despite exaggerated exposure.

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iPS-derived human 3D cardiac microtissues to assess cardiac toxicity

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3-dimensional (3D) spheroid cultures of iPS-derived cardiomyocytes were recently shown to build homogenous microtissue structures with well-developed myofibrils, which extend throughout the whole spheroid. The microtissues showed spontaneous contractions for more than 4 weeks and were responsive to pharmacological interventions. Here, we report the electrical characteristics of these 3D cardiac microtissues in comparison to conventional 2D cultured iPS-derived cardiomyocytes. iCell Cardiomyocytes were purchased from Cellular Dynamics Inc (USA) and either tested (i) in 2D culture in a 96well plate (25,000 cells/well cultured for 10 days) or (ii) in 3D culture (2500 iCell cardiomyocytes/microtissue in co-culture with myofibroblasts), supplied in 96well GravityTRAP™ plates from InSphero AG. In both cases the cells were transferred to serum free media and transiently exposed to the voltage sensitive dye (Di-4-ANEPPS 3 μM). The Di-4-ANEPPS fluorescence was recorded at 10KHz from regions 2D and 3D cultures for periods up to 30 s in the 96 well plates on the CellOPTIQ electrophysiology platform (Clyde Biosciences Ltd). The average time between spontaneous action potential (AP) firing in the two culture formats was similar (1.5 ±0.47 s vs. 1.12 ±0.14 s 2D vs. 3D), as was the rate of depolarisation of the AP (5.9 ±1.3 ms vs. 5.1 ±0.6 ms 2D vs. 3D). But the action potential duration (APD) in 3D cardiac microtissues was significantly shorter than in 2D culture. At 90% repolarisation APD was 428 ±50 ms vs. 222 ±25 ms (2D vs. 3D $p < 0.01$). Similarly, APD at 30% repolarisation was shorter (188 ±36 ms vs. 128 ±16 ms 2D vs. 3D $p < 0.01$). In conclusion, 3D cardiac microtissues of human iPS-derived cardiomyocytes exhibit a significantly shorter APD than comparable 2D cultures, the difference may arise from increased electronic interactions within microtissues.

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Omics analysis of human liver microtissues grown over 4 weeks: implications for studies of liver functions

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3-dimensional (3D) spheroid cultures of primary human hepatocytes were previously shown to prolong viability and liver-specific functionality in comparison to 2D cultures. However, little is known about how donor-to-donor differences of primary human hepatocytes are reflected in 3D liver microtissues. Here, we provide a thorough in-depth characterization of 3D human liver microtissues derived from 3 different human hepatocyte donors in co-culture with primary liver Kupffer cells over 5 weeks. The 3D liver microtissues were analysed for viability, morphology, basal cytochrome activity, cytochrome inducibility, transcriptomic and proteomic profile over the whole culture period. The 3D liver microtissues exhibited stable ATP-content and albumin secretion over 5 weeks in culture. Histological sections indicated presence of a healthy tissue with high glycogen incorporation. Bile canaliculi were established and exemplified by BSEP expression. Distinct donor variability was seen for CYP 1A2, 3A4, 2B6, 2C8, 2C9, 2D6 and 2C19 activity. For example, CYP2B6 and CYP2D6 showed little activity in 1 of the 3 donors. However, the basal cytochrome activity remained stable for most of the CYPs over the whole culture period. Inducibility of CYPs was tested with Omeprazole, Phenobarbital and Rifampicin induction of 3D liver microtissues, which showed >2 fold induction of the corresponding CYPs even after 4 weeks of culture. Transcriptomic analysis by whole genome arrays revealed distinct similarities to native human liver gene expression, despite high inter-donor differences in liver specific gene expression. Proteomic profiling of more than 3500 proteins over the whole culture period using HRM-MS™ Technology showed that only around 350 proteins were up- or downregulated more than 2-fold from day 7 to day 35 in culture, suggesting that the 3D human liver microtissues are very stable over time. In summary, this characterization exemplifies that 3D human liver microtissues are a robust organotypic liver model and is therefore suitable for various applications in drug metabolism and toxicity testing.

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Pro-drug activation in a dynamic microfluidic systems interconnecting liver and tumor microtissues

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Rational development of more physiological in-vitro models includes the design of robust and flexible 3D-microtissue-based multi-tissue devices, which allow for studying tissue-tissue interactions. The newly developed microfluidic device consists of interconnected multiple micro-chambers, which are loaded with preformed spherical microtissues. Gravity-driven flow is generated from on-chip reservoirs through automated chip-tilting without any need for additional tubing and external pumps. This tilting concept allows for operating up to 48 devices in parallel to test various drug concentrations with a sufficient number of replicates. Rat and human liver and colorectal tumor microtissues were interconnected on the chip and cultured over 8 days in the presence of the pro-drug cyclophosphamide. Cyclophosphamide has a significant impact on tumor growth but only after bio-activation by the liver. This bio-activation-mediated effect was only observed in perfused and interconnected co-cultures of the different microtissue types on chip, whereas the discontinuous transfer of supernatant via pipetting from liver microtissues that have been treated with cyclophosphamide in culture wells did not significantly affect tumor growth. The results indicate the utility and multi-tissue functionality of this platform as well as the importance of continuous medium circulation to assess liver-mediated pro-drug activation.

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Microfluidic *in-vitro* platform for parallel human multi-tissue interaction studies

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Conventional two-dimensional cell-based assays have limitations in mimicking *in vivo*-like environments [1] and are, therefore, more and more replaced by three-dimensional (3D) cell cultures. A next step is the integration of different 3D cell cultures into microfluidic devices and to establish so-called microphysiological multi-tissue networks for pharmacokinetic studies [2]. Recently, we developed a 96-well-format-based microfluidic platform for robust and facile long-term culturing of multiple 3D microtissue spheroids (MTs) under perfusion conditions [3]. Here, we present the results of a human two-tissue network by combining primary human liver MTs and human iPS-derived cardiac MTs upon substance exposure. Both MT types, liver and cardiac, were reliably formed from the respective cell types by using InSphero's GravityPLUS hanging drop technology. MTs were then transferred into the microfluidic device with simple pipetting steps and hosted in specially designed microchambers. 6 MTs can be arranged in a flexible manner and are interconnected through one microchannel. Each microfluidic device holds 10 of those 6-MT-arrangements so that up to 10 different conditions can be tested per device. Gravity-driven flow was induced through automated chip-tilting in a standard incubator thus avoiding any tubing and external pumping technology. This simple setup allows not only for straightforward operation of the device by using existing tools, such as multichannel pipettes, but also for conducting multiple experiments in parallel. Currently up to 120 conditions with various drug concentrations, replicates, and controls can be applied simultaneously. 5 human liver and 1 cardiac MTs were interconnected and cultured under continuous perfusion in the presence of the pro-drug Terfenadine. Terfenadine is bio-inactivated by the liver and transformed to fexofenadine, an antihistamine pharmaceutical drug used in the treatment of allergy symptoms. Terfenadine itself is cardiotoxic at higher doses, whereas no cardiotoxicity is associated with fexofenadine. First of all and interestingly, human cardiac MTs showed an elevated level of beating frequency under perfusion in the device in comparison to the static condition in the well plate. We observed the cardiotoxic effect, the cessation of spontaneous contraction, of Terfenadine above 5 μM in static as well as perfusion conditions upon culturing cardiac spheres alone. The inactivation-effect of Terfenadine by liver metabolism could only be observed under perfusion conditions: Spontaneous activity of the cardiac MTs was observed up to concentrations of 20 μM of Terfenadine in the presence of liver. In contrast, discrete transfer of supernatants from liver MTs treated with Terfenadine under static conditions did not lead to any reduction of toxic effects on the cardiac microtissues. These results illustrate the importance of continuous interaction between different tissue models and the usability of the present platform concept for multi-tissue configurations in the field of compound testing.

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Replacing rodent models for investigating the influence of the microbiota upon innate immune responses and resistance to pathogens

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The intestinal microbiota has been implicated in a range of conditions in which inflammation plays a role, including inflammatory bowel disease and obesity. Presence of a microbiota is also important for normal development of the immune system. Current mouse models do not allow for high-resolution *in vivo* imaging of the interactions between the immune system and the microbiota. The translucent nature of the zebrafish larvae will be taken advantage of to investigate how the microbiota influences the innate immune response against bacterial infection. To determine this, immunity in the presence and absence of a microbiota will need to be characterised. Therefore, we will establish a protocol to derive germfree zebrafish larvae. This will allow characterisation of the immune system of germfree and conventional larvae by histology and qPCR, and their ability to resist a systemic *Streptococcus iniae* infection. Further studies will investigate whether probiotic treatment alters resistance to *S. iniae* challenge. Live imaging will allow the investigation of the interactions between the immune cells and *S. iniae*. Currently work is focused upon initial generation of germfree larvae by sterilisation post-spawning, based on a published protocol [1]. Briefly, using the natural breeding method, embryos were collected and washed with antimicrobials (kanamycin, amphotericin B, ampicillin, with or without penicillin-streptomycin), bleach, and either polyvinyl pyrrolidone-iodine complex (PVP-I) or glutaraldehyde. Survival was monitored daily and samples of media and homogenised larvae were cultured at 3dpf and 5dpf using LB agar, sabouraud dextrose broth and thioglycollate broth. 16s rRNA PCR with universal bacterial primers (1320f:1431r and 27f:1492r) was also performed using 5dpf larvae. Bleach and penicillin-streptomycin did not affect larval survival, but did reduce the amount of culturable microbes. PVP-I caused high larval mortality and inconsistent microbial growth inhibition, whereas glutaraldehyde was not detrimental to larval viability and showed distinct antimicrobial activity. Not all microbes are culturable, so such methods may yield false negative results. Although PCR data indicated bacterial presence in all samples, 16s rRNA PCR with universal primers is highly sensitive to any bacterial DNA and is therefore prone to false positives. Precautions such as using PCR reagents manufactured without bacterial DNA are necessary. qPCR will also be performed to robustly confirm germfree status. As the zebrafish immune system and gut structure is similar to mammals, establishing this model to investigate the influence of the microbiota on the immune system will allow for further work that may be translated to other species, fish or mammalian. We report our attempts to overcome some of the challenges of researching the microbiota in this zebrafish model. This work is funded by NC3Rs.

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First successful therapy with a new retinal ischemia organ culture model

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Purpose: Ischemia plays an important role in several ophthalmologic diseases. Retinal ganglion cell (RGC) death induced by ischemia can lead to irreversible loss of vision as found in central retinal artery occlusion (CRAO). To investigate neuroprotective agents and therapies against these diseases we developed an *ex-vivo* model, with an easy-to-use ischemia chamber for 6-well plates with inserts for organotypic cultures. To further evaluate the model, we tested hypothermia as a first therapy in this ischemia model.

Methods: Retinal ischemia was mimicked *in vitro* using a custom-made ischemic chamber. In this chamber, retinal explants were kept under oxygen free conditions at 37°C for different durations (45, 60, 75, 90 and 120 minutes). Briefly, the chamber was streamed with N2 for 5 minutes, then the chamber was immediately sealed and the retinas were incubated for the rest of the designated time. After the incubation the 6-well plate was adjusted to normal air conditions and incubated for 24, 48 or 72 h in an incubator under standard conditions. To analyze the amount of RGCs immunohistology was performed as well as qRT-PCR for ganglion cell marker. Apoptotic cells were visualized via TUNEL-staining and overall cell amount via DAPI-staining. Furthermore, Western-Blot analyses with GFAP- and Thy-1-antibodies were conducted. Moreover, retinal thickness via OCT measurements was determined. After the proof-of-principle of our model, experiments with retinas treated with hypothermia after ischemia, as a possible therapy was analyzed accordingly.

Results: A time- and ischemia duration-dependent decrease in the amount of RGCs after 24, 48 or 72 h was observed. Moreover, the amount of TUNEL-positive RGCs was also ischemia duration- and time-dependent. The thickness of the retina significantly decreased ischemia duration- and time-dependent as observed with OCT-measurement. Furthermore, microglial and astrocytic activation, an increase in apoptotic markers accompanied by a decrease of neuronal marker could be detected. Hypothermia counteracted the loss in retinal thickness and expression of neuronal markers of retinal ischemia. In addition, hypothermia rescued the spontaneous retinal ganglion cell activity and less glial activation was observed.

Conclusions: We successfully established a reliable, reproducible, easy-to-use organotypic culture model for retinal ischemia, following the guidelines of refinement to minimize potential distress or pain for the animals. Especially basic pretesting of new agents can be conducted *ex-vivo*, to avoid animals suffering from ischemia and surgeries. We could prove neuroprotective effects of hypothermia on the survival of retinal ganglion cells after ischemia in this *ex-vivo* model.

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Directive 2010/63/EU: five years after its adoption

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Directive 63/2010 was adopted on 22 September 2010 and came into force 20 days after its publication in the EU Journal (20 October 2010), hence on the 10th of November 2014. What happened after this date in the Member States is rather obscure but the Guidance Document of the Commission's expert working group (EWG) stated in its Guidance Document that: "In many Member States, at the time of writing (summer 2014), National Committees are still in the very early stages of development, and even those which existed previously are undergoing some restructuring to meet the requirements of the new directive". In the EU historically certain Member States have always been more involved in the welfare of laboratory animals than others. My home country, the Netherlands, was one of those. Nonetheless, it formally implemented Directive 63/2010 in its legislation only in December last year. Although this seems like a steeply declining level of interest in experimental animal welfare, the contrary is true. The Netherlands had adopted its law on animal testing as early as 1977 and the revised Dutch version of Directive 63/2010 is not essentially different from its 1977 version. However, the so-called Dutch "polder model" for reaching political consent often takes a long time as everybody knows better than anybody else! Other Member States may have different but also plausible reasons for their delay in acting. In January 2015 the Netherlands National Committee on the Protection of Animals Used for Scientific Purposes (NCad) made a kick-start. The presentation will explain the structure and network of national animal welfare committees and groups and its mandates. It will also address the pivotal role of the NCad, its independence, current assignments and its outreach to NCs in other Member States, in particular on technical approaches and best practices in the use of animals and the 3Rs. As an example the use of the so-called "synthesis of evidence" practice will be addressed. Hopefully together with other front runners the Netherlands NCad is willing and prepared to assist the European Commission in helping Member States that seem to lag behind, to get things rolling.

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The Asian Congress on Alternatives and Animal Use in the Life Sciences 2016

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The Asian Congress on Alternatives and Animal Use in the Life Sciences (Asian Congress) 2016 is being organized by the Japanese Society for Alternatives to Animal Experiments (JSAAE) with the cooperation of the Alternatives Congress Trust (ACT), and the Organizing Committee is currently making preparations to begin registering participants from China, India, Japan, and Korea. The Asian Congress is scheduled to be held in November, 2016, at venues in the cities of Karatsu, Saga, and Fukuoka, Japan. The opening session will be held in Karatsu, followed by four days of scientific sessions and plenary lectures in Saga, and a closing session in Fukuoka, that will include the 29th JSAAE annual meeting and poster sessions focusing on Reduction, Refinement and Replacement (the Three Rs) of animal experiments. The Asian Congress will be the first conference of its kind for researchers from Asia, and will afford an opportunity for promoting alternative methods to researchers in these places, where the concept of the Three Rs is just now achieving penetration. The Asian Congress is intended to achieve multiple missions, which will include disseminating information not just on the latest advances in including pure sciences but on practical applications of the Three Rs worldwide.

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Failure and success: *in vitro* ocular irritation test methods for agrochemical formulations the Bovine Cornea Opacity and Permeability Test and the EpiOcular™ Eye Irritation Test

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The Bovine Corneal Opacity and Permeability (BCOP OECD TG 437) test is commonly used for the identification of severe ocular irritants (GHS Category 1) and ocular non-irritants (No GHS Category), but it is not recommended for the identification of ocular irritants (GHS Category 2). The value of the EpiOcular™ Eye Irritation Test (EIT) for the prediction of ocular non-irritants (No GHS Category) has been demonstrated, and the OECD Test Guideline is expected to be adopted in 2015. The purpose of this study was to evaluate whether the BCOP test, in conjunction with corneal histopathology (as suggested for the evaluation of the depth of the injury) and/or the EpiOcular-EIT, could be used to predict the eye irritation potential of agrochemical formulations according to the UN GHS, US EPA and Brazil ANVISA classification schemes. We have assessed opacity, permeability and histopathology in the BCOP assay, and relative tissue viability in the EpiOcular-EIT for 97 agrochemical formulations with available *in vivo* eye irritation data. By using the OECD TG 437 protocol for liquids, the BCOP test did not result in sufficient correct predictions of severe ocular irritants for any of the three classification schemes. The lack of sensitivity could be improved somewhat by the inclusion of corneal histopathology, but the relative viability in the EpiOcular-EIT clearly outperformed the BCOP test for all three classification schemes. The predictive capacity of the EpiOcular-EIT for ocular non-irritants (UN GHS No Category) in the 97 agrochemical formulations tested (91% sensitivity, 72% specificity and 82% accuracy for UN GHS classification) was comparable to that obtained in the formal validation exercise underlying the OECD TG. We therefore conclude that the EpiOcular-EIT is currently the best *in vitro* method for the prediction of the eye irritation potential of liquid agrochemical formulations.

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Towards the assessment of skin sensitization potency: quantitative estimations by the Direct Peptide Reactivity Assay

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Evaluation of the skin sensitization potential of a chemical is one of the principal endpoints in both hazard and risk assessments. Three non-animal test methods addressing key events in the sensitization process have passed formal validation and OECD (draft) test guidelines are available. A simple prediction model using the results of the three assays to estimate the skin sensitization potential has been proposed [1]. One of these methods is the *in chemico* Direct Peptide Reactivity Assay (DPRA) assessing the ability of a chemical to bind to proteins to form a complete antigen [2-4]. The test is used to obtain a yes/no answer on whether the substance has a protein-binding potential. For a risk assessment such as done in the evaluation of cosmetic ingredients, however, an estimation of a chemical's potency is also needed. In this study we examined whether a quantitative readout of the DPRA can be used for potency assessment of skin sensitizers. The standard protocol of the DPRA was amended by testing three concentrations (i.e. 1, 10, and 100 mM) instead of only one. 51 Reference substances with available potency information from the local lymph node assay were tested. Potency classes were assigned using the interpolated concentration of a test substance that is needed to cause a peptide depletion of 6.38% (the EC6.38% value). Cut-offs for the prediction of the Globally Harmonized System sensitization classes (non-sensitizer, GHS Category 1B and GHS Category 1A) were defined and yielded an overall accuracy above 80%. In summary, using quantitative information derived from the DPRA EC6.38% value may support the assessment of the skin sensitizing potency.

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A novel human organotypic tumor invasion model of cell line MDA-MB-231 in Precision-Cut Lung Slices (PCLS) to reduce animal experiments in preclinical oncology

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Cancer such as breast carcinoma is a major public health problem worldwide. The actual cause of death is the formation of metastasis which often occurs in the lung. Among the cell types present in the tumor microenvironment, macrophages have been proven to be the dominant leukocyte population with high macrophage density correlating to poor patient prognosis. An increasing amount of publications points to evidence that a macrophage sub-population actively supports and promotes the initiation, growth and development of tumor tissue. Indeed, Bingle et al. (2002) have shown in their meta-analysis that over 80% of studies show a correlation between macrophage density and poor patient prognosis [1]. Thus, these cells are of high interest as targets for cancer therapeutics, making them a valuable research topic. In order to better understand cancer and potential drugs, multiple xenograft mouse models are currently being used with the disadvantages of less predictive, expensive or technically complicated procedures. GEM mice are an alternative to xenograft models through alteration of genes for tumorigenesis. However, both types of models have been reported by Singh et al. (2012) to possess scant prediction with over 90% of phase 3 studies in oncology failing [2]. In accordance with the 3R principle, we hereby present an innovative and translational *ex vivo* organotypic tumor invasion model using living human Precision-Cut lung slices (PCLS) and cancer cells to focus on the local immunological respose during early metastasis formation. An AdGFP-transduced human breast cancer cell line MDA-MB-231 was added to human PCLS over a period of one week. Viability assays such as LIVE/DEAD® staining and LDH measurements were performed to assure intact human tissue throughout the experimental procedure. Tissue immune staining methods against CD68 and Ki67 were used to visualize locations of macrophages and proliferating cells, respectively. Immune response and neoangiogenesis were determined by cytokines IL-10 and IL-1beta and tumor markers VEGF and GM-CSF. An *in vitro* neoangiogenesis assay was performed to observe whether the tissue is able to attract endothelial cells through a BME-coated membrane. Surrounding parenchyma remained viable during infection and invasion with cancer cells with no significant increase in LDH release or a diminished Calcein-staining. A 2.5-fold increase of Ki67- and GFP-positive cells within the first 24 hours showed cancer cell expansion and proliferation. Colocalization of CD68-positive macrophages and MDA-MB-231 was found during the entire invasion period, thereby indicating a direct tissue response towards the invading cells. The VEGF/GM-CSF release correlated with the MDA-MB-231 growth curves, further reflecting the tumor microenvironment with a 5.8-fold increase of GM-CSF within 24 hours and a 1.7-fold increase of VEGF within 48 hours, respectively. HUVEC cell invasion showed a 5-fold increase in the PCLS that had been treated with MDA-MB-231 in relation to untreated controls. Here we mimic cancer cell proliferation and immune responses in the native microenvironment of human lung tissue which will be used for testing of anti-tumor drugs in the near future. In terms of the 3R concept, this alternative model does not require any animal experiments and takes advantage of human tissue.

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Preliminary validation studies of a 3D *in vitro* inhalation model, using cytokine and gene expression responses to metal-oxide particles

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Human 3D airway models are fully differentiated and functional models of the respiratory epithelium and therefore may be positioned in safety evaluation of (nano)particles. Cultured at an air-liquid interface, they allow relevant exposure via air. It is anticipated that these models predict a more realistic bioavailability of inhaled compounds compared to mono-layered 2D-cell cultures. We performed air exposures of metallo-oxide particles using MucilAir™ human 3D bronchial model and compared these to Beas-2B and A549 2D-cell cultures. We investigated the effects of donor, exposure-unit, exposure-session and insert, on the MucilAir™ response using a statistical experimental design. In addition we investigated the biological response of both healthy and asthmatic donors upon exposure to cupper-oxide particles. MucilAir™ and A549 or Beas-2B cell cultures were exposed at ALI conditions *in vitro*cell exposure modules (3 inserts each) to metal-oxide particles (0, 50, 224, 1000 mg/m^3 simultaneously) for 1 hour. It was found that MucilAir™ cells were less affected by the air stream (0 mg/m3) compared to both cell lines. Upon metallo-oxide exposure MucilAir™ cells induced only a mild oxidative stress (HO-1) response. In contrast the Beas-2B cell line showed an inflammatory (IL-8) and cytotoxic response (LDH). Both cell lines demonstrated a genotoxic response (Comet assay). Differences were confirmed by gene expression analyses. Using cytokine and gene expression responses of MucilAir™ to metal-oxide particle exposures we show that the influence of the parameter "concentration" is the largest, followed by "donor", "unit" and "session", closed by "insert". Finally MucilAir™ of healthy and asthmatic origin were exposed to cupper-oxide particles. MucilAir™ cells of asthmatic origin were more affected by the exposure, showing a greater cytotoxicity response (LDH), but also showed a greater variation in this response. Summarizing, our results show that 3D MucilAir™ is more resistant to air stream and particles compared to 2D-cell cultures, most likely due to its *in vivo* relevant and protective morphology (cilia, mucus layer). Therefore, human 3D airway models might predict a more realistic response compared to 2D-cell cultures and can be used to assess the effects of particles, as long as donor- and session- variability are taken into account in the experimental design and subsequent statistical analyzes.

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A porcine retina organ culture model to study neuronal degeneration

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Purpose: Our aim is the replacement of animal models to test unidentified therapy agents against retinal degeneration. Therefore, porcine eyes (leftover material from butchered pigs) were used for retina organ culture. In order to test therapy agents for retinal diseases, like glaucoma, a reversible, substrate induced ex-vivo retina damage model is needed. Possible fast analysis and various options for repetition and modifications are also demanded factors and advantages of this model. N-Methyl-D-Aspartate (NMDA), a known inducer for degeneration of retinal and glia cells, was used to this porcine organ culture.

Methods: Organotypic cultures of porcine retina were cultivated and treated with different concentrations of NMDA (0 (Co), 50, 100 and 200 μM) on day 2 and 3. At day 7, retinas were cryoconserved for histological and qRT-PCR analysis. The amount of retinal ganglion cells (RGCs) was determined via Brn-3a-staining. Additionally, apoptotic RGCs were counted (cleaved caspase 3). Additionally, the mRNA ratio of Bax/Bcl2 was analyzed. Furthermore, macroglia (GFAP) were detected with histology. The mRNA level of both glia cell types, macroglia (GFAP) and microglia (CD11b), was evaluated with qRT-PCR. The groups were compared with the one-way ANOVA followed by Tukey post-hoc test.

Results: After 7 days, the number of Brn-3a$^+$ RGC was not affected by the NMDA treatment (50 μM: 21.5 ±2.4 cell/mm, $p > 0.9$, 100 μM: 17.1 ±1.0 cells/mm, $p = 0.4$, 200 μM: 18.3 ±0.6 cells/mm, $p = 0.7$). Yet, the level of cleaved caspase 3$^+$ RGCs was increased in the 50 μM group (37.4 ±2.9%, $p = 0.001$) as well as in the 100 μM (43.0 ±2.7%, $p < 0.001$) and 200 μM group (41.0 ±3.2%, $p < 0.001$) in comparison to the control group (20.1 ±1.6%). The Bax/Bcl-2 mRNA ratio was not enhanced ($p > 0.05$). Similar results were observed for the mRNA level of the microglia cells ($p > 0.05$). However, the immunhistology data demonstrated that the microglia amount (39.95 ±4.3 Iba1$^+$ cells/mm, $p = 0.02$) and their activity (5.1 ±1.5 CD16/32$^+$ cells/mm, $p = 0.03$) increased in the 100 μM group compared with the Co group (Iba1: 16.2 ±4.2 cell/mm CD16/32: 0.4 ±0.4 cells/mm). The GFAP mRNA level as well as the GFAP$^+$ signal area remained unaltered in all three NMDA treated groups ($p > 0.05$).

Conclusions: A significantly higher apoptosis rate was measured in RGCs with this model. Therefore, we established an organ culture model of moderate cell loss with reversible damage. For a stronger RGC degeneration, the cultivation or the NMDA exposure time needs to be increased. Fast analysis and various options for repetition and modifications are possible with this model. The next step of investigation will be the test of therapy agents. Now, we reduced the importance of animals for the testing phase of therapy agents in a retinal damage model.

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AniMatch – an innovative approach to reduce lab animal usage addressing article 18 of the EU directive 2010/63/EU

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Besides the general enhancement of the protection level for animals used in scientific experiments, the European directive 2010/63/EU 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). Between 2010 and 2013 the number of animals that have been used for scientific purposes in Germany has increased. The itemization of categories according to the animal welfare act (TierSchG) revealed scarcely used potential to reduce animals that are not used in experiments but for the collection of tissue and organs. In 2013 819.094 were killed for scientific purposes (§4 Abs. 3 TierSchG), 386.657 animals were used for organ and tissue collection under anaesthesia (§6 Abs. 1 Nr. 4 TierSchG) and further 64.603 animals were used for educational purposes (§10 TierSchG). The development and deployment of a web-based platform that enables scientists to connect and share organs and tissue of killed animals would directly address the request of the EU directive as well as exploit the existing potential to reduce animals and save the biological resources that are gained. Therefore, we have developed AniMatch (http://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 killed animals. To publish an offer the providing party has to quote the species, type and if necessary the genetic background as well as the number, age, sex, the organ or tissue that is used for own purposes and the timeframe for the killing. The seeking party can search a list including filters for the species and a geographical radius and request while quoting the number of animals and organs or tissue in need. With completion of the request the contact information is exchanged between both parties who are now able to arrange the details of the transfer. Optimizations of our service have been performed after intensive discussions with animal welfare officers in Berlin. Subsequently, we have implemented two safety barriers in the registration process in order to avoid abuse. The measures include approval of affiliation and account activation by the designated animal welfare officer. Furthermore, we integrated a complex matching system that focuses on the verification of the different microbiological units (hygienic management system) that have to be considered during the sharing process. In addition, besides the general solution we have developed an in-house solution. The platform is now ready to be tested for its feasibility in a pilot trial that will start in September 2015 and will be provided to interested institutions in Germany as well as Europe. Besides the moral exculpation of scientists AniMatch provides a cost efficient way to use existing infrastructure and to conserve resources in accordance with reducing lab animal usage. To our knowledge this is the first approach to address the challenge for multiple use of killed animals in science.

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Molecular modelling of osteoarthritis – evaluating pathways *in vitro*

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In 2020, osteoarthritis (OA) will be the fourth leading cause of world´s most common disabilities as a result of an increasing life expectancy and an aging population. According to the world health organization, today, 9.6% of men and 18% of women aged 60 or older suffer from OA worldwide. Therefore, a deeper knowledge about the pathogenesis of OA which is characterized by a complex interplay of inflammatory processes and cartilage degradation is needed in order to develop new therapeutic strategies. Today, a priori small animal models (e.g. rat and rabbit) and – later on – large animal models (e.g. dog, sheep, and horse) are widely used in order to examine the complex mechanism of OA pathogenesis and to test new therapeutic approaches. However, the applicability to the human is questionable. To effectively reduce the animal numbers, valid and attractive *in vitro* model systems are required to replace the animal experiments at least in the initial step of OA research. To our knowledge the already existing 3D cell-based models for OA research are either limited to reflect the complex pathogenesis or inconvenient to handle. Therefore, we generated an *in vitro* OA model that consists exclusively of chondrocytes and their metabolic products and is based on the scaffold-free 3D cartilage transplant (SFCT) technology developed at the fzmb GmbH. SFCTs were generated using equine chondrocytes and treated with IL-1β and TNF-α for 3 weeks to mimic the *in vivo* inflammatory environment of OA or were left untreated to serve as a control. Subsequently half of the constructs were fixed. A group of stimulated constructs were further cultivated without stimulation for up to 3 weeks in order to study the regenerative potential of the SFCT. Beside biochemical, histological and immunohistological investigations, quantitative PCR was performed to investigate the inflammatory and cartilage specific marker profile (IL1B, TNF, IL6, CXCL8, COX2, MMP1, MMP3, MMP9, BMP2, SOX9, TGFB1) which was normalized to the endogenous control genes (GAPDH, HPRT, SDHA) and to the unstimulated samples. After stimulation with IL-1β and TNF-α, we observed a significant increase of inflammatory marker gene expression (IL1B, TNF, IL6, CXCL8, COX2) and matrix degrading enzyme expression (MMP1, MMP3) in SFCTs as compared to the untreated controls. The observed effect was reversible after 3 weeks of regeneration. First results obtained from our 3D *in vitro* OA model are promising as they strikingly reflect comparable observations to *in vivo* findings of early stage OA pathogenesis in humans. Now, we are in the position to conduct microarray studies in order to match our data to patient data (in situ transfer) and to further confirm the applicability of our *in vitro* model to identify pathogenic pathways of OA. Finally, we aim to transfer our 3D *in vitro* approach to simulate other rheumatic disorders such as rheumatoid arthritis following the meaningful path of implementation of 3R in the scientific community.

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A mismatch between micro-motives and macro-behaviour: the inefficiency of efforts toward replacement at the individual level

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Viewed at the microscopic level of each individual lab, the principles of the 3 R's may appear to work reasonably well. Yet, turning to the macroscopic level of the entire field, the principles are essentially ignored. This is largely due to our research culture, which works on the basis of free inquiry, with every principal investigator as an autonomous entity. Researchers organize their studies at the microscopic level: each lab steers its own course, usually staying with a particular paradigm and animal model, aiming to get the smallest sample size that provides statistically reliable results for any experiment, and continuing to refine the techniques and knowledge extraction. At this level, then, we see Reduce and Refine at work. However, the investigations in one lab may overlap with those done in another lab sometimes the fear of being scooped will lead to rushing the completion of a study. As a result, the present research culture leads to considerable levels of redundancy and a vast literature with too many premature or trivial research papers. In addition to the problem of redundancy, the current research culture makes it practically impossible for an individual researcher to really consider the Replace principle. Shifting to another animal model (e.g., from nonhuman primates to rats) would imply a great individual cost, in time and effort, at the expense of the publication flow. Young scientists usually cannot afford to take this kind of risk established scientists may lack the energy, motivation, or flexibility to reconsider their way of working. However, support from the general public for animal research hinges on the quality of its output. Good research with important payoff will be welcomed poor research with suboptimal payoff will lead to disinterest and fading support. Here, it should be noted that the general public naturally views scientific research at the macroscopic level. At this level, people ask tough questions: not lab-by-lab, but for the entire field, about the returns for the vast amounts of resources that are invested in animal research. People outside science also wonder about the efficiency of the three R's, or why, for instance, nonhuman primates are being used for certain types of research when rodents, at first sight, seem a viable alternative. Indeed, several recent calls from within the neuroscience community have suggested that the levels of public support are dangerously low already (e.g., [1,2]). The calls for support, however, were limited to a demand for more effort toward public outreach. This is essentially a conservative attitude, aiming to change the communication about science, without actually addressing the 3 R's. Instead of a conservative attitude toward animal ethics, I argue that we can adopt a proactive strategy to promote the three R's at the macroscopic level. The task is to make collective decisions about which research could offer a justifiable contribution: not at the level of the Principal Investigator, but at the macroscopic level of organizations: research communities, universities, funding agencies.

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Strategies of refinement concerning the keeping and the breeding of C57BL/6

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The development of new refinement strategies for laboratory-mice is a challenging task in fulfilling the 3R-princip of laboratory animal science. Using positive reinforcement training and intensive handling, we seek to figure out if social interaction between the keeper and the mice influences their well-being. Additionally, we study the effect of different environmental designs – from an impoverished single housing to an overfilled group housing system. We choose to work with C57BL/6 mice because it is the most used strain and, therefore, our findings will be representative for the highest percentage of laboratory-mice. To get an objective prediction about the mice's well-being, we are engaged to establish the "Mainz Mouse Well-Being Score" (MMWS). As well-being is a complex interaction of elements, the MMWS includes data about the state of health, the physical development and the evaluation of stress indicators. Furthermore, it contains information about the performance of luxury behaviour, for instance, nest building and burrowing behaviour. Moreover, the mice are tested in several behavioural assessments which provide details about the intensity of their social interaction, their anxiety and explorative behaviour as well as their ability of spatial learning and memory. Primarily, all components are individually evaluated and afterwards combined in the MMWS. The evaluation of different blood parameters, such as cortisol, glucose and haemoglobin, allows us to obtain further information about the well-being of the mice during different stages of the study. To further address the influence of the different housing and handling conditions on the neuronal network, we finally perform histological analysis of the brains. In a pilot study, we try to discover the possibilities of establishing a relationship of trust between the keeper and the mice. We can see a large divergence of willingness, curiosity and courage – even among the mice of the same cage. In order to find an adequate positive reinforcer, we evaluate the mice's preference for different treats. In addition, we examine which nesting material and which burrowing behaviour setup provides us with the most significant results in our scoring sheet. Our preliminary data shows that only some of the provided nesting materials enable us to score properly. The execution of the burrowing behaviour test is quite simple and gives important results concerning the well-being. We are confident that the achievements of our study will help to improve the welfare of laboratory mice and, therefore, also improve the quality of research.

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Quantitative data on toxicant classification derived from transcriptomics studies of stem cell development

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In the field of neurodevelopmental toxicity there is an urgent need for appropriate *in vitro* tests. We established a human embryonic stem cell based test system. However, the major challenge of such a system is that gene expression patterns change already in untreated control conditions. Therefore we hypothesize that not only concentration and duration of treatment matter but also the time window. We used microarray analysis to get (i) a closer insight into the underlying processes of neurodevelopment and (ii) to investigate drug effects for different treatment scenarios. Moreover we undertook two studies examining the discrimination power of transcriptome analysis to classify toxicant classes such as histone deacetylase inhibitors and mercurials. Or to blindly categorize 6 different types of toxicants only by knowldege of altered transcriptome responses. The data from these studies were used to identify consensus genes for toxicant classes and to develop new visualization forms to use transcriptome data sets for information of toxicologists. In the context of this, a full human transcription factor network was generated to visualize the changes of subnetworks affected by toxicants, and 2-dimensional organ landscapes were generated to visualize normal cell differentiation and the toxicant-induced deviation from the healthy track. In summary, our data provide bridge between bioinfomatically generated gene lists and hazard information usable by toxicologists in the area of developmental toxicity.

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CAAT-Europe and knowledge exchange on the progress of the 3Rs and research methods

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The 3Rs field is under dynamic development, especially in the area of replacement approaches in the safety sciences. New technologies and methods are continuously emerging. This requires i) a consolidation and consensus-building process amongst the scientists involved in the research; exchange of information amongst researching scientists and end users of new technology; iii) communication of latest findings and stimulation of further research to young generation scientists; and iv) communication between those producing data based on the new methods and those using such data for regulatory purposes or for providing the legal basis for such applications. CAAT-Europe is one of the organizations that provide communication platforms and information hubs for the above needs. Key activities of CAAT-Europe are i) the teaching of young scientists in 3Rs to motivate and support research careers in this field; ii) the organization of information days on emerging new technologies for industry, regulators and academia; iii) offering of a special workshop format (think tank) that brings together a group of hand-selected specialists to produce a consensus publication on an important topic (such activities have resulted in > 30 review papers from > 200 scientists); iv) information at the level of the European parliament to spread 3Rs background information also to the legislators. An example of such an activity is the MEP-scientist pairing scheme, an event in which members of the European parliament (MEP) were brought together at an event in the parliament with 3Rs scientists from their home countries to promote mutual understanding and information exchange. New ideas, technologies and concepts promoted in this way include Omics technologies, systems toxicology, biological read across, 3D cellular test systems and the concept of adverse outcome pathways or pathways of toxicity. In contrast to the progress and large success of 3Rs in toxicology, there is still a large unmet need for more applications in biomedical research. This is an area that uses 5-8 times more animals than toxicology, and the impact of replacement methods is still very limited. To achieve immediate improvements, refinement and reduction approaches need to be communicated in this area more efficiently, and innovative ideas are required to achieve real replacement on the long run. Stem cell technologies are seen as one promising approach to achieve this goal and future communication efforts will need to take this important area into account.

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B-13 progenitor-derived hepatocytes (B-13/H cells) as a model for lipid storage disorders

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Lipid dysregulation (phospholipidosis and steatosis) is an adverse outcome response in the liver after exposure to toxic drugs and chemicals. Phospholipidosis (PLD) is caused by the accumulation of phospholipids within the cell's lysosome. This can be induced by a class of drugs known as the cationic amphiphilic drugs (CADs). Steatosis is caused by the accumulation of lipid droplets that can occur due to an imbalance of storage and metabolism of triglycerides. An effective *in vitro* model for PLD and steatosis could be used to investigate potential drugs and chemicals that cause these disorders and also study their mechanisms. We hypothesised that B-13 progenitor-derived hepatocytes (B-13/H cells) can provide a simple, cost-effective *in vitro* model for the study of steatosis and drug induced phospholipidosis in liver cells. B-13/H cells were treated with a selection of drugs known to be PLD positive and PLD negative inducers. Using a fluorescent probe the accumulation of phospholipids was quantified. After treatment the cells were fixed and fluorescence was measured. To model steatosis, B-13/H cells were treated with linoleic and oleic acid, fixed and stained for lipid droplets using oil red o. Lipid accumulation was also measured in cells treated with known steatotic drugs. Drugs known to induce PLD, amiodarone, tamoxifen, promazine, chlorpromazine and quinidine showed a dose-depended increase in phospholipid accumulation. This effect was also observed with methapyrilene which had previously not been shown. Oil red o staining of cells treated with 0.25 mM fatty acids showed a primary response of microsteatoic lipid droplet formation whereas those treated with 2 mM showed macrosteatosis as the primary response. Known steatotic drugs were also shown to induce steatosis using this model. Finally, B-13/H cells express the liver x receptor (LXR), a receptor involved in lipogenesis. Treatment with the synthetic LXR agonist T0901317 showed a dose-depended increase in lipid accumulation in B-13/H cells. These results demonstrate that B-13/H cells can be used as a simple, cost-effective model for lipid storage disorders. This model could be used to screen drugs for their ability to induce PLD and steatosis during development, before progression into *in vivo* studies. B-13/H cells could also be used to investigate the mechanism for these storage disorders.

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Evaluating non-animal methods for identifying skin sensitisation hazards: a Bayesian value of information analysis

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Skin sensitisation is a key endpoint for safety evaluations of cosmetics under the European Union Cosmetics Regulation1223/2009. It is mandatory for all substances produced or marketed in volumes larger than 1 tonne per year under the European REACH legislation 2003/15/EC. With REACH supporting *in vivo* testing only "as a last resort" (EECNo1907/2006, article 25) and the marketing ban for finished cosmetic products with ingredients tested in animals [2003/15/EC, 1], attention has been given to developing integrated (i.e. sequential) testing strategies combining *in vitro*, *in silico* and *in chemico* methods [2]. Key challenges to be addressed are which tests to select and how to combine non-animal methods into testing strategies. This study suggests a Bayesian value of information (VOI) approach for developing non-animal testing strategies, which considers information gains from testing, but also expected social gains and costs from adopting regulatory decisions on the use of a substance, and testing costs. The "value" of testing is defined as the expected social net benefit from decision-making on the use of chemicals with additional, though uncertain information from testing. Quantifying the VOI for different types of prediction methods provides insight into their relative performance. As an illustration we apply our model to the case of skin sensitisation hazard assessment of cosmetic ingredients. The VOI is calculated for a set of individual non-animal methods including DPRA, OECD QSAR Toolbox, ARE-Nrf2 luciferase method covered by KeratinoSens™ (OECD, Test No. 442D) and LuSens [3], and hCLAT, seven battery combinations of these methods, and 86 two-test and 360 three-tests sequential strategies consisting of non-animal methods [4]. Their VOIs are compared to the VOI of the local lymph node assay (LLNA) as the animal test [4-6]. We estimate marketing gains and health damage costs caused by allergic contact dermatitis, to approximate social benefits and costs of using cosmetic ingredients. Data about the predictive capacity of the LLNA and the non-animal methods are extracted from [4]. We find that battery and sequential combinations of non-animal methods reveal a higher VOI than the LLNA. In particular, for small prior beliefs (i.e. a chemicals is, prior to testing, assumed to be a non-sensitiser), a battery of DPRA + LuSens reveals the highest VOI. If there are strong beliefs that a chemical is a sensitizer, a sequential combination of the battery DPRA + LuSens, followed by KeratinoSens™ + hCLAT at the second stage and by the OECD QSAR toolbox at the third stage performs best. For given specifications of expected social gains and costs the VOI of the non-animal strategy significantly outperforms the VOI of the LLNA, for the entire range of prior beliefs. This underlines the economic potential of non-animal methods for skin sensitisation assessment. Our results illustrate that a VOI-optimal testing strategy does not necessarily have to follow the order of key events in an AOP. Rather, a sequential combination of methods addressing different key events of the AOP, but also different methods covering the same key event (probably because it reduces the variations of either method) reveals the highest VOI.

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Determination of contact sensitization potential of 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]. 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 either impregnated in 8 mm diameter paper filters or directly applied to the surface of EpiDerm. Acetone : olive oil (4:1) was used as vehicle in all cases. A total of five chemicals from 3 different sources were tested. The testing set included 3 senzitizers, namely 2,4-dinitrochlorobenzene, cinnamaldehyde and isoeugenol/eugenol, and 2 non senzitizers, lactic acid and salicylic acid. Four independent dose – response experiments were conducted in 3 laboratories, 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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Cryopreserved human hepatocytes and IdMOC system for the evaluation of adverse human drug properties

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Human hepatocytes, with human-specific drug metabolizing enzyme activities, represent the "Gold Standard" for *in vitro* evaluation of drug metabolism, drug-drug interactions, and drug toxicity. We have optimized hepatocyte cryopreservation and recovery procedures to retain high viability (approx. 90%), gene expression, drug metabolizing enzyme activities, uptake and efflux transporters, and, most of all, the ability of the thawed hepatocytes to be plateable – to be cultured for multiple days as confluent monolayer cultures. Assays developed with the cryopreserved hepatocytes include long-term metabolism of slowly metabolized compounds, time-dependent P450 inhibition, P450 induction, and *in vitro* hepatotoxicity screening. In collaboration with US FDA, a human hepatocyte assay has been developed to identify drugs that are reported to cause severe liver damages (sDILI drugs) with specificity and sensitivity of approx. 90%. Human hepatocytes have been incorporated into the Integrated Discrete Multiple Organ Co-culture (IdMOC) experimental to allow the evaluation of the roles of hepatic metabolism in non-hepatic toxicity. Cryopreserved human hepatocytes and IdMOC with co-cultures of hepatic and nonhepatic cells represent promising *in vitro* assays for the evaluation of human drug properties that, due to species difference, cannot be readily assessed in nonhuman animals. The presence of inherent drug metabolizing enzymes in hepatocytes and the incorporation of hepatocytes in IdMOC are features allowing these two experimental systems to provide physiologically relevant information which may not be obtained with other *in vitro* assays where hepatic metabolism is absent.

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Cryopreserved human hepatocytes and IdMOC experimental system for the evaluation of human drug properties

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Due to their inherent drug metabolizing capacity, hepatocytes represent the most physiologically relevant experimental system for the evaluation of drug metabolism, drug-drug interactions, and hepatotoxicity. Human hepatocytes are superior to *in vivo* animal system for the evaluation of human-specific drug properties that, due to species differences in drug metabolizing enzyme pathways, pharmacology, and toxicology, cannot be accurately assessed in nonhuman animals. In our laboratory, cryopreservation, thawing and recovery procedures have been optimized for human and animal hepatocytes to retain high viability and functions. Moreover, we are able to routinely cryopreserved hepatocytes to retain plateability – their ability to be cultured as confluent, monolayer cultures. Assays developed with plateable cryopreserved human hepatocytes include long-term metabolism, time-dependent P450 inhibition, P450 induction, and *in vitro* hepatotoxicity. In collaboration with U. S. FDA, an assay has been developed to accurate identify drugs with severe liver toxicity (sDILI drugs). The cryopreserved plateable human hepatocytes have been incorporated into the Integrated Discrete Multiple Organ Co-culture (IdMOC) experimental system with which cells from multiple organs can be co-cultured. Using IdMOC, toxicants to nonhepatic cells can be evaluated in the presence of hepatic metabolism, overcoming a major deficiency of *in vitro* toxicity testing, namely, the lack of hepatic metabolism.

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Testing of differentiation and cytotoxicity to evaluate the safety of a Chinese herbal medicine extracts used for the treatment of atopic dermatitis

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Background: Atopic dermatitis (AD) is a frequent chronic inflammatory skin disease and its prevalence is increasing in the last few decades. It can adversely affect the quality of life, but there is no definitive cure so far and pregnancy often worsens the disease. There is considerable interest in traditional Chinese herbal medicines (CHM) as an alternative treatment. A palatable and well tolerated decoction (Pentaherbs formulation, PHF) has been proven efficacious in improving quality of life and reducing topical corticosteroid use in children with moderate-to-severe AD. However, more scientific data are required on the safety of CHM, particularly for pregnant women and the unborn.

Objectives: The ECVAM-validated embryonic stem cell test (EST) was applied to evaluate and predict the embryotoxicity of PHF and its five individual herbal extracts.

Methods: The mouse embryonic stem cell line (ESC) and the mouse fibroblast cell line (3T3) were used to study possible embryotoxicity. Three endpoints were assessed after 10 days of culture: the inhibition of differentiation into beating cardiomyocytes (ID50ESC), the cytotoxic effects on stem cells (IC50ESC), and the cytotoxic effects on fibroblasts (IC503T3). The quality of the herbal medicines was confirmed by chromatography (TLC and/or HPLC) before performing the EST.

Results: Chemical authentication confirmed the quality of herbal medicines which contained their main active compounds. The differentiation of the contracting cardiomyocytes and the viability of differentiating cells indicated that both the decoction formula PHF and its individual herbal extracts are non-toxic in this mouse cell system.

Conclusions: Our *in vitro* results provide preliminary evidence for the safety of the formula PHF in differentiating embryonic cells. Further studies are needed and will be conducted to support its application as an adjunctive treatment for AD during pregnancy.

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Ending severe suffering: the scientific animal welfare organisation as a catalyst for change

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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 benefit of reducing 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 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 has also been endorsed by the Government, which cited the project in its recent Delivery Plan on animal experiments. We have initiated a number of parallel activities including: A "Road Map" towards ending severe suffering, outlining the key questions and practical considerations that establishments need to address in order to reduce suffering for all animals and to work towards ending severe suffering. We are actively promoting this throughout 2015 and urging scientists to adopt it. A comprehensive web resource for the research community, providing guidance and resources to help end severe suffering. A series of projects to develop new ways to reduce suffering. We form "Expert Working Groups" of scientists, vets, animal technologists, animal welfare experts and representatives from the UK regulatory authority (the Home Office), who work together to focus on a particular area of research and see what can be done to avoid using animals or, if this is currently impossible, to significantly reduce suffering. To date we have produced four reports on reducing suffering in epilepsy, multiple sclerosis, sepsis and rheumatoid arthritis research. We are also urging the UK Government to work towards a policy of no longer licensing any experiments expected to cause animals severe suffering, in line with our dedicated programme to develop practical measures that can make this a reality. This talk will set out how we have been able to work with the scientific community and UK regulator, as well as providing more information on the RSPCA's resources on severe suffering.

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In vitro model of hair follicle for high-through-put *in vitro* screening

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The Microfollicle (MF) is a 3D *in vitro* model of the hair follicle. This culture system is based on co-culture of primary cells from hair with stem cell properties (outer root sheath keratinocytes and dermal papilla cells) and from skin (melanocytes) in 3D, providing a differentiation platform for the keratinocytes via epidermal-mesenchymal cross-talk [1]. In this study, we adapted the MF cultures to a high-throughput format up to 21 days with each well containing a single MF for high-throughput (HTP) screening. MFs start to show hair specific keratin markers as early as one day after initiation of the co-culture. In later time points, late differentiation markers of keratins appear/increase similar to hair follicle development. The Microfollicle enables tracking of the organoids during its development and studying hair follicle biology which is a characteristic system for epithelial-mesenchymal cross-talk. Moreover, as a human *in vitro* model of the hair follicle, HTP MF cultures enable studying acute and chronic effects of substances on hair follicle as a prominent alternative for reducing and eventually replacing animal tests.

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Standardization of the experimental conditions leads to reliable and relevant predictions of the photo-toxic effects *in vitro*

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Assessment of the *in vitro* photo-toxicity belongs to the basic toxicology endpoints for the chemicals absorbing UV light. Pre-validation and validation studies performed with cell lines as well as with reconstructed human 3D models proved high sensitivity and specificity of the investigated assays, however they also pointed out to the high importance of the use of standardized equipment and biological material (i.e. cell lines and 3D tissues). In the current study we have focused on the standardization of the irradiation conditions with the newly purchased equipment for the photo-toxicity testing (solar simulator SOL-500, Hoenle) and on the evaluation of the sensitivity of the biological material (reconstructed human tissue model EpiDerm) towards UV and visible light of the spectra. The poster summarizes the lessons learnt during the process of the equipment calibration and provides comparative data to those generated on the reconstructed human tissue model obtained during the validation studies.

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Modeling of early intestinal infection events of enterotoxigenic *Escherichia coli* using an *in vitro* system with porcine jejunal tissue

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Enterotoxigenic *Escherichia coli* (ETEC) strains are involved in piglet post-weaning diarrhea, a current problem in piglet rearing. Many prophylactic measures such as vaccines or feed additives such as probiotics have been tested in infection experiments with piglets [1,2,3].

In the present study, we tested if effects of ETEC can also be evoked when the strain is added *in vitro* to whole mucosal tissues which might represent a more complex model system compared to cell cultures. Furthermore it was examined if this response could be modulated by prior (*in vivo*) supplementation of the piglets with probiotics.

Material and Methods: Jejunal epithelial tissues from piglets before weaning from a probiotic-supplemented (*Enterococcus faecium* NCIMB 10415) and a control group were taken and mounted into conventional Ussing chambers. The ETEC strain O149:K91:K88 was added at a concentration of 10^8 CFU/ml at the mucosal side of the tissues and barrier and immunological functions were examined by measuring electrophysiological parameters (transepithelial electrical resistance (R_t), potential difference and short circuit current (I_{sc})), as well as gene and protein expression of selected target genes by quantitative PCR and Western blots. Variance analyses and t-tests were performed for statistical evaluation of the data.

Results: The R_t initially increased in the first two hours after ETEC addition, and decreased again later. The rise in Rt coincided with reduced fluorescein fluxes as a marker of paracellular permeability in the ETEC-incubated epithelia. At the end of the experiment mRNA expression of proinflammatory cytokines and of components of the inflammasome as a potential induction pathway were elevated. Expression of the tight junction protein claudin-4 was decreased in mucosal tissues treated with the pathogenic *E. coli*. Coherent effects of the probiotic prefeeding of the pigs could not be observed.

Conclusion: The *in vitro* system can be applied to study the early events of ETEC infection. Addition of ETEC affected barrier function and components of the gut immune system. Further studies are needed to elucidate the response under different incubation conditions.

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Generation of *in vitro* skin models using ORS cells

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Due to growing social and political pressure, the interest in alternatives to animal testing has constantly increased during the past 10 years stimulating the development and validation of new *in vitro* test systems including reconstructed skin models. Additional to toxicological studies and permeability assays, skin models are of high interest for fundamental research to elucidate basic physiological and pathophysiological processes in human skin [1, 2]. As for today, most of the *in vitro* skin models are grown from primary keratinocytes and fibroblasts that were either isolated from excised human skin or from juvenile foreskin following circumcision. In this project, we aimed for the generation of *in vitro* skin models using hair follicle-derived cells. Therefore, different methods to optimize cell isolation and expansion of outer root sheath (ORS) cells from human hair follicles were systematically investigated. The best procedure for isolation of ORS cells was the direct cell outgrowth on a cell culture insert which was co-cultured with a feeder layer of postmitotic human dermal fibroblasts. Following outgrowth, the cells were either further cultivated with feeder cells in specific serum-enriched cell culture medium to obtain hair follicle-derived keratinocytes or using the same culture medium without feeder cells to obtain fibroblasts. Afterwards, the generation of hair follicle-derived fibroblasts and keratinocytes was verified via the fibroblast-specific markers vimentin and desmin and the keratinocyte marker cytokeratin (CK) 14 clearly showing that vimentin and desmin are expressed in hair follicle-derived fibroblasts and in dermal fibroblasts. As expected, these cells were negative for CK14, which was abundantly expressed in hair follicle-derived keratinocytes. Moreover, the expression of collagen type I, IV, TGF-beta, alpha SMA and IL-1 alpha in hair follicle-derived fibroblasts and dermal fibroblasts showed no significant differences. Ultimately, hair follicle-derived keratinocytes and fibroblasts were used to grow full-thickness skin models which were subsequently characterized with regard to epidermal differentiation, skin permeability and skin surface pH. Again, no significant differences compared with skin models grown from skin-derived cells were detected showing the potential of using hair follicle-derived cells for generating *in vitro* skin models.

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Refinement facilitated by the Culture of Care

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Directive 2010/63/EU on the protection of animals used for scientific purposes sets the Three Rs at the heart of all interactions with animals in the field of laboratory animal science. When animal use is unavoidable and ethically justified (no Replacement), compliance with the other two Rs (Reduction and Refinement) must be ensured; the use of the minimum number of animals allowing the statistically robust scientific results to be drawn and minimisation of pain, suffering and distress and improvement of the life time experience of the animal - from the initial concept, and throughout all aspects of the conduct of the project. Application of the three Rs in the daily activities of an animal facility by those involved in the use and care of animals is, however, strongly influenced by the culture of the establishment and the attitudes of the individuals involved. Both elements are largely shaped by the organisation either subconsciously or overtly. In recent years, the importance of ensuring a good culture of care has continued to develop as a high priority by management. The talk summarises the key elements to set up and maintain the right culture of care; a strategic approach with continuous institutional support, appropriate structures and attention to implementation, monitoring and follow-up. With the professional approach to building the right culture of care getting a stronger footing across Europe in its animal facilities, it is time to reach to the next level. Moving from good to brilliant science, and taking Refinement to its true potential, it is time to roll out Culture of Challenge.

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Cytotoxic and immunological effects of engineered nanomaterials

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The aim of this study was to investigate cytotoxicity and immune responses caused by engineered nanomaterials (ENMs): silver (AgNP) and gold (AuNP) nanoparticles, mesoporous silica nanoparticles (MSNP1 and MSNP2), and multi-well carbon nanotubes (MWCNT). Specifically, ENM effects on cell viability and motility (trajectory length and speed), were studied using mouse BALB/c3T3 fibroblasts, human U937 monocytes, and rat NR8383 macrophages. In addition to that, ENM effects on cytokine secretion by NR8383 macrophages were studied. BALB/c 3T3 and NR8383 cells represent normal cell physiology, e.g. NR8383 cells are able to phagocytose particles, and secrete cytokines. Cells were grown under standard culture conditions (37°C, 5.0% CO_2) in 96-well plates and exposed to eight different concentrations of dispersed nanoparticles for 24-48 hours, after which the cytotoxicity assays were performed. Cell viability was assessed using the NAD(P)H-dependent cellular oxidoreductase enzyme assay (WST-1), which is suitable for both suspension and adherent cells. For comparison, ENM effects on BALB/c3T3 fibroblasts were also assessed using the standard neutral red uptake (NRU) cytotoxicity protocol (OECD GD 129). Changes in the motility of ENM-treated cells were investigated using a kinetic live-cell imaging and analysis system. Finally, cytokine and chemokine releases of ENM-treated NR8383 macrophages were studied with defined ELISA kits. Secretions of pro-inflammatory cytokines IL-1beta and TNF alpha, anti-inflammatory cytokine IL-10, and growth factors TGF beta, VEGF and TNF alpha were analyzed. The exposure medium, i.e., dispersion medium, appeared to modulate ENMs to a great extent depending on the dispersion media, ENM's particle size either increased or decreased. AuNP did not affect the viability of any of the cell cultures. AgNP was toxic to all cell cultures studied, whereas the toxicity of MWCNT, MNSP1 and MNSP2 seemed to be more cell-specific: NR8383 macrophages were most sensitive followed by BALB/c 3T3 and U937 cells suggesting potential immune-related toxicity of nanoparticles. Live-cell imaging analysis demonstrated that all ENMs significantly changed the migration length and speed of all cells studied during the 48-h observation period. MSNP1 and MSNP2 inhibited IL-1β secretion, but increased TNF-α secretion. MWCNTs promoted TNF-α secretion. AgNPs tended to increase VEGF secretion at sub-toxic concentrations, but did not modulate the secretion of other cytokines. AuNP, in turn, had a tendency to decrease IL-1β secretion. In conclusion, a substantial interaction between different cell culture media and ENMs suggest that in investigations of NP toxicity, NPs should be individually characterized in each exposure condition, no generalizations can be made. Among the ENM's studied, AuNP's were safest, AgNPs were most toxic. MSNPs and MWCNTs promoted inflammation, whereas AgNPs and AuNPs were anti-inflammatory.

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QsarDB: solution strategy for efficient use of QSAR models

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Researchers community working in the area of quantitative structure-activity relationships [(Q)SAR] is producing around one thousand scientific publications annually (according to Web of Science). This is a very large knowledge base of alternatives to animal testing that unfortunately remains static due to the nature of publishing media. Very few of proposed models find their way to the practical applications because they are very difficult to reuse and this could be the main reason of low consumption of (Q)SAR-s as recently indicated by several reports (one of them from ECHA [1]). The current presentation gives an overview of perspective reuse of published models from physic-chemical properties to human health endpoints. Presentation also looks into web repositories and integrated web modelling environments available for (Q)SAR/(Q)SPR models and discusses the lifecycle of models and how models reach the intended audience from the perspective of model developers and users and how web repositories provide help via reducing the time to decision. In more detail the QSAR DataBank approach for the digital organization and archiving of QSAR model information [2] and QsarDB repository [3] (http://qsardb.org/) for archiving and interactively accessing models is discussed. The repository stores models in an open QsarDB data format that is a generic solution for the electronic organization and archiving of (Q)SAR/(Q)SPR model information according to open standards. At the time of writing this abstract, the QsarDB repository contains 346 unique models for 58 different endpoints and about 17 species in the following mathematical representations: regression models, classification models, decision trees, neural networks, random forests, support vector machines and ensemble (consensus) models.

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The Austrian Catalogue of Criteria. A tool to objectify the harm-benefit analysis within the evaluation of research proposals involving the use of live animals

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The Directive 2010/63/EU requires a harm-benefit analysis as a part of the project evaluation by the competent authority, which has to take ethical considerations into account. In Austria, the Animal Experimentation Act 2012 (TVG 2012) transposes this requirement into national law. As a specific feature, the TVG 2012 demands that applicants have to fill in a catalogue of criteria to objectify and standardize the harm-benefit analysis. A project team of the Messerli Research Institute at the Vetmeduni Vienna developed this Austrian Catalogue of Criteria (ACC) which has to be used within the authorization process on a legal basis. According to the TVG 2012, the ACC has to be published by the end of 2015 and applied to the authorization process six months after publication. The team of the Messerli Research Institute was supported by expert knowledge in a multi-stakeholder process. In two evaluation phases we received input through discussions in numerous workshops with different expert groups and through written opinions and statements. Stakeholders from the fields of science, industry, animal welfare, alternative methods and the competent authorities contributed. Besides the evaluation by several stakeholders, the catalogue of criteria was subjected to a legal review. This legal review was carried out by law experts from different areas such as science, industry, animal welfare and the competent authorities. The ACC is based on scientific criteria in order to objectify and standardize the harm-benefit analysis. It consists of different categories, including specific questions to the applicant. The answers are assessed, counted as factors in the harm-benefit analysis and reviewed, considering the facts and justifications the applicant has to provide. To offer a feasible methodology, several methodological approaches have been integrated, amongst others a scoring and weighing procedure. The ACC will be presented and certain aspects will be discussed.

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EU placentology network for chemicals safety testing in pregnancy – EU-PlaNet

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Current approaches assessing the safety of chemical substances in humans are expensive and time consuming, and may be of limited relevance as a predictor of adverse effects. The human placenta is a critical life-support system that nourishes and protects a rapidly growing fetus. The human placenta is also a unique organ, which differs significantly from placentas of other species both in structure and in function, making it very hard to choose a suitable animal model. This Action aims to address a pressing challenge of providing better advice on the safety of prescription medicines and environmental exposures in pregnancy. The scope of EU-PlaNet ranges from (i) fundamental placental transfer & toxicity mechanisms and (ii) *ex vivo, in vitro* & *in silico* human placenta-based technologies to (iii) standardization and (iv) translation into regulatory, industrial and clinical practice. EU-PlaNet will for the first time offer a concerted approach to test the safety of chemicals for the mother and fetus. This will only be possible by crossing specialism boundaries and direct engagement with end-users, such as pharmaceutical industry and regulatory authorities. EU-PlaNet also aims to create a pool of pan-European standards for the placental test systems and to initiate training and knowledge transfer programs for a new generation of academic and industrial researchers across Europe prepared to tackle the challenge. The Action will explore the potential of new technologies to deliver both substantial savings and reduction in the number of animals used in toxicology testing, while improving the relevance and reliability of the recommendations.

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Human lymphoblastoid cell lines as an *in vitro* tool in preclinical drug evaluation

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The preclinical evaluation of efficacy and safety of novel drugs requires reliable disease and healthy control models that are predictive of human responses while sparing experimental animals. Moreover, animal models often fail to mimic the human pathology, as evident for the many drugs failing in clinical trials after working very well in animal disease models. Human lymphoblastoid cell lines (LCLs), that capture the large human genomic and epigenomic variations, are emerging as a novel tool for predicting drug responses, adverse drug reactions and for addressing inter-individual variability in drug responses. LCLs are generated from peripheral blood lymphocytes by Epstein-Barr virus transformation. The cells retain most of the phenotypic properties of B cells, including the expression of CD19 and CD20 and the production of antibodies. Human LCLs were proven as a predictive *in vitro* model for immunotoxicity screening of xenobiotics. Using LCLs has allowed for reliable differentiation between immunomodulatory and immune-inert compounds based on cell viability and the alteration of cytokine release [1]. The aim of this study was to evaluate LCLs as an *in vitro* tool for determining immunomodulatory effects and selectivity of drug candidates. We are interested in the EP4 receptor as a target in B-cell chronic lymphocytic leukemia, the most prevalent leukaemia in developed countries. This incurable disease is characterized by increased number of B lymphocytes in the microenvironment of constitutive B cell receptor activation and increased levels of cytokines. Thus, a selective EP4 receptor agonist, PgE1-OH, previously shown to induce apoptosis in malignant B cells [2] was chosen as a model drug in the present study. To evaluate the immunomodulatory properties of PgE1-OH, human LCLs were pretreated with PgE1-OH and activated by ionomycin/PMA, which mimics the activation of B cell receptor. Our study shows that PgE1-OH significantly decreased levels of IL-2, IL-10, TNFα and IFNγ compared to untreated controls as evidenced in LCLs from ten individual donors. Second, human LCLs were evaluated as a tool for *in vitro* testing of selectivity of drug candidates. This is of special interest when B-lymphocyte targeting drugs are evaluated, since substantial amount of human blood from healthy donors is required for isolating primary B lymphocytes (e.g. 500 ml whole blood for 20 million B lymphocytes). Our study showed that LCLs enabled *in vitro* selectivity testing, since the EP4 receptor agonist was significantly more cytotoxic to malignant B cells compared to LCLs obtained from healthy individuals. The average EC50 values for PgE1-OH after 24 h were 42 μM for LCLs and 17 μM for malignant B cells obtained (after informed consent) from 51 patients diagnosed with chronic lymphocytic leukemia. Inter-individual differences in response to PgE1-OH were observed in both lymphoblastoid cells and malignant B cells. Moreover, the expression of EP4 receptor determined by flow cytometry was higher on cells obtained from leukemia patients compared with LCLs. Human LCLs enabled *in vitro* evaluation of immunomodulatory properties of novel drug candidate PgE1-OH and determination of selectivity towards malignant B cells. In conclusion, LCLs were identified as a valuable *in vitro* tool for preclinical drug evaluation.

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Systematic literature review on *in vitro* and alternative Developmental Neurotoxicity (DNT) testing methods

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A recent review lists 200 chemicals known to be neurotoxic in human of which 12 chemicals have been identified as developmental neurotoxic. *In vivo* and *in vitro* studies suggest that even more environmental chemicals might affect normal brain development. Therefore, Developmental Neurotoxicity (DNT) represents an emerging issue in future risk assessment. Current DNT testing is specified under the OECD Test Guideline TG426, which refers to *in vivo* studies performed in rat. Due to ethical and economical concerns connected to the TG426 as well as it´s unsuitability for screening large numbers of chemicals there is a need for reliable and predictive alternative methods. The goal of this systematic review was to identify and evaluate testing methods alternative to TG426. The review question was summarized as: "Which test methods or approaches are available to evaluate developmental neurotoxic effects of chemical exposure?" The search, performed within PubMed and Web of Science, retrieved 17272 publications. It employed six search strings designed to retrieve publications on *in vivo* DNT testing methods including alternative non-mammalian models, *in vitro* test methods, *in silico*-methods, read across approaches and combinations of testing methods. One additional search string was designed to retrieve information on blood brain barrier and computational methods. Different screening steps, which were based on pre-defined selection criteria, reduced the amount of relevant articles to 287 *in vitro*, 73 *in vivo*, 90 alternative organisms and 2 *in silico* publications. All relevant data from these 452 selected publications with regard to methodological parameters were collected in data collection sheets. These parameters were further analysed with regard to performance and general suitability to identify chemical-induced adverse effects during neurodevelopment. This review retrieved a variety of methods suitable to identify adverse effects of chemicals during different stages of neurodevelopment. In general the data across all method types is very heterogeneous, with a range of individual cell types, from different brain regions, different time windows of development in the zebra fish model, different exposure paradigms and various compounds tested. This complicates the comparison of test methods and demonstrates the need of an standardization of protocols to allow a more useful evaluation of test methods. Considering the evaluation of a multitude of alternative methods a promising approach for future DNT testing is a strategy covering early and later neurodevelopmental stages, i.e. from stem cell to zebrafish larvae motor behavior, and *in silico* approaches (e.g. estimation of placental barrier permeation). For ultimately gaining regulatory acceptance, definition of biological application domains of human neural stem/progenitor cell based methods, as well as the zebra fish embryo model, by performing *in vitro – in vivo* validation is needed. With such standardized protocols, a test battery needs to be evaluated for its performance parameters (sensitivity and specificity) by analyzing concentration-responses of known DNT positive and negative compounds across different *in vitro* and zebra fish embryo assays. Such data might in the end be used to support international regulatory assessments or compound prioritization, both in favor of a reduction of rodent DNT *in vivo*.

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From pathways to people: applying the skin sensitisation AOP to risk assessment

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Despite recent advances in our mechanistic understanding of the pathways that drive the induction of skin sensitisation (recently documented as an Adverse Outcome Pathway (AOP) [1]) our ability to apply this knowledge to risk assessment remains a key challenge. Our approach aims to simulate the sensitiser-induced immune response (using mathematical modelling) to enable the magnitude of the adverse human immune response to be predicted for a given skin exposure to chemical [2]. Our current model outputs naïve CD8+ T cell activation as a surrogate measure for sensitisation induction in humans. Ordinary differential equations are used to model key events of the AOP: skin penetration (chemical diffusion and partitioning), haptenation of protein nucleophiles and antigen processing and presentation by skin dendritic cells. Biological parameters are taken from the immunological literature with human data used where possible. Bioavailability and chemical-specific parameters are derived from bespoke *in vitro* experiments and from sensitiser-specific literature [3]. The model has been used to simulate a study published previously by Friedmann et al. [4] in which 132 healthy volunteers were exposed to one of five doses of the contact allergen 2,4-dinitrochlorobenzene. As a significant proportion of each dose cohort were sensitised to DNCB within this study, comparison of model simulation results to these clinical data have provided an opportunity to explore the relationship between naïve CD8+ T cell activation and clinical sensitisation. This analysis has enabled selection of an optimal model output parameter (T cell receptor trigger rate) for risk assessment decision-making and demonstrated the inherent difficulty in extrapolating from this cellular event to predict the extent of clinical sensitisation. To address this finding, immune characterisation of allergic contact dermatitis patients is underway to enable mathematical modelling of the sensitiser-induced memory T cell response.

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Implementing the 3Rs: turning words into action

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In 2010 the UK's coalition government pledged to "work to reduce the use of animals in research" during their five year term of office. The resulting Delivery Plan (2014) demonstrates the degree to which collaboration across government departments is necessary, and that such collaboration is likely to produce economic as well as animal welfare benefits. Later, in June 2015, the "Stop Vivisection" Citizen's Initiative – having achieved the necessary 1 million supporting signatures – prompted the European Commission to identify steps needed to reduce and eventually eliminate the use of animals in EU laboratories. Alongside these strategic developments concern for animal welfare has increased, as has recognition of the need to find new ways to develop treatments, test chemicals and stop healthy people from becoming ill but despite that, over 115 million animals still used in research and testing worldwide, often in procedures that cause severe and lasting pain, distress and suffering. Our European chemicals regulation, REACH, still requires outdated animal tests, and billion dollar companies rely on frequently inadequate animal models of human disease. By examining the UK Plan and the Commission's proposed actions, is it possible to elaborate the steps needed to ensure robust implementation of existing 3Rs provisions in the EU, and to propose a global strategy? This presentation aims to identify separate contributions needed from regulators, academia, companies and animal welfare organisations to turn promises into action and create animal-free research opportunities worldwide. In light of the above mentioned initiatives, emphasis will be given to industry choices and possibilities for further action.

Working to reduce the use of animals in research, 2014: http://bit.ly/1jwdKOC

European Commission today set out the actions it intends to take in response to the "Stop Vivisection" European Citizens' Initiative (ECI), 2015: http://bit.ly/1dewr8B

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In vitro hiPSC-based modeling of late-onset disease using progerin-induced aging

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While the use of human induced pluripotent stem cell (hiPSC) technology offers new opportunities for modeling and potentially deriving therapeutic strategies for currently intractable disorders, there is a question as to how well patient-specific hiPSC-derived somatic cells can recapitulate late-onset diseases which normally take decades to develop. While age is a necessary component of disease progression in these cases, it has been reported that the reprogramming process of converting somatic cells to hiPSCs resets the molecular clock of an aged cell back to a young state. Furthermore, the differentiation of hiPSCs to disease-relevant cell types follows developmental timing, yielding immature cell types that often require months to establish robust functional properties. As a result, hiPSC-based models of late-onset disease often do not recapitulate the degenerative aspects of these diseases. In order to overcome this hurdle to hiPSC-based disease modeling, we identified a set of markers that could stratify fibroblasts from young and aged donors, and we reconfirmed the reset of age following reprogramming and re-differentiation to a fibroblast-like fate. We then sought to program cellular age independent of cell fate using ectopic expression of progerin, a protein which accumulates during normal aging and is known to cause the premature aging disorder Hutchinson Gilford progeria syndrome. Progerin triggered aging-related phenotypes in hiPSC-derived fibroblasts, including nuclear morphology abnormalities, nuclear reorganization, and accumulation of DNA damage and reactive oxygen species. Progerin was also capable of inducing phenotypes specific to neuronal aging such as neurite degeneration and neuromelanin accumulation in hiPSC-derived dopaminergic (DA) neurons. When applied to an hiPSC-based model of Parkinson's disease, ectopic progerin expression unmasked disease phenotypes indicative of the late-onset degenerative phase such as accelerated neurite degeneration and AKT pathway dysregulation, as well as genotype-specific responses including mitochondrial swelling in PINK1-mutant hiPSC-derived DA neurons and multilamellar aggregates in PARK2-mutant hiPSC-derived DA neurons. Our more recent work focuses on the application of progerin-induced aging to *in vitro* modeling of age-related macular degeneration (AMD), which results in the loss of central vision later in life due to the progressive dysfunction and degeneration of the retinal pigment epithelium (RPE) and subsequent loss of the underlying photoreceptors. AMD hiPSC-derived RPE treated with progerin display cell type-specific age-related features erased by reprogramming as well as an upregulation of drusen-related transcripts. These novel disease-in-a-dish models demonstrate the requirement of an aging stimulus to more closely mimic dysfunction and degeneration and could represent relevant drug screening platforms for identifying novel therapeutic strategies.

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A new tool for *in vitro* sunscreen quality control

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The effectiveness of sunscreens is characterized by its Sun Protection Factor (SPF). To determine the SPF a world consensus establishes the adoption of *in vivo* tests using healthy human subjects. However the reproducibility of such tests in phase of industrial scale production becomes impractical, since the method requires selection of volunteers, monitoring of specialized practitioners, use of costly equipment and also involves issues of ethical nature. Accordingly, this work aims to develop and validate a new *in vitro* spectrophotometric method for assessing the effectiveness of sunscreens, which can be adopted in industrial routine production as a way to identify quality deviations and ensure the effectiveness of sunscreen product. Briefly, the new method proposed is based on the spectrophotometric absortion on the UV region (280-400 nm) of a thin layer of sunscreen sample applied on the outside of a quartz cuvette which is filled with a standard solution (reference standard) that absorbs throughout the UV region. The area under the curve of absorption of reference standard and sunscreen sample is obtained. The novelty of the proposed technique lies in the presence of a reference standard, that is essential for the development of this method, since acts as an internal standard, having always constant absorption, in order to compensate random, systematic and sample application errors. For the experiment development, it was used two different batches of sunscreen products of the same brand with SPF values of 15, 30, 50 and 60 which were applied manually on the cuvette outside at a concentration of 0.2 mg/cm^2 and, subsequently, the cuvettes were examined by optical microscopy (10X increase) to check the pattern of samples spread. The reference standard used consisted of a quercetin solution added of AlCl3 6%. From the absorbance of each sample spectrum in UV region was calculated the value of the area under the curve (AUC). When the AUC was plotted versus the SPF described on the packaging of sunscreens, it was observed the existence of a linear relationship between the variables with a coefficient correlation of 0.996 making it possible to obtain an equation of line, that allow us to calculate the SPF of sunscreen samples. The method was validated as intra-assay and inter-assay precision and accuracy. The relative standard deviations obtained were lower than 10%. The experimental procedure is simple, but requires technical ability, therefore, subject to greater experimental variation. However, the coefficient of variation obtained was lower than recommended by United States Pharmacopeia for bioanalytical methods that are 15%. The new method presented a high accuracy as follows: 94%, 91%, 92% and 96.3% for the SPF 15, 30, 50 and 60, respectively. The spectrophotometric method developed can be effective to confirm SPF of sunscreen products produced batch to batch, from a calibration curve relating the AUC of SPF versus absorption.

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Examination of human bone tissue regeneration using the chorioallantoic membrane (CAM): an *ex vivo* replacement model for animal research

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The efficacy and safety of novel bone tissue engineering constructs needs to be tested *in vivo* before they can be used clinically. Here we propose the use of the chorioallantoic membrane (CAM) as an experimental platform to explore human bone healing. In particular, this human-avian setup allows the screening of a clinically relevant tissue in the context of blood vessel supply, which is critical for bone regeneration [1], without the need for more invasive procedures on animals. We aim to use this *ex vivo* model to test the hypothesis that a synthetic clay (Laponite) can deliver vascular endothelial growth factor (VEGF), promote vascularization and thereby enhance bone formation [2]. To investigate this, bone grafts were extracted from fresh human femoral heads and engineered as hollow cylinders to resemble a bone injury model. The bone cylinders were perfused with Laponite ± VEGF or without (blank), and then cultured *in vitro* or *ex vivo* (CAM) for 7 days. Micro-computed tomography (μCT) was conducted before and after culture of both sets of bone cylinders to quantify the relative change of bone volume, followed by histological examination. Explant culture showed that bone tissue remained viable following CAM implantation. Immunohistochemical staining showed the presence of avian blood vessels within the human bone marrow and the expression of cathepsin K on the *ex vivo* cultured samples. Multilevel μCT analysis showed a significant increase in low dense bone (new bone deposition) in parallel to a decrease in high dense bone (resorption of old bone) in all treatments. The combination of Laponite-VEGF showed no difference, though Laponite alone treatment resulted in significant bone formation ($p < 0.05$). Ex vivo culture of the bone cylinders demonstrated significant bone volume change compared to the *in vitro* group ($p < 0.005$). This data shows that the CAM assay can be used to support human bone tissue formation. The development of this replacement model can have an important impact in the refinement of animal use in research, not only for bone tissue engineering but many other disciplines

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Modular human biochip-based organoid models in sepsis research

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Sepsis is one of the leading causes of mortality with a hospital mortality rate of 54%. To date mainly mouse sepsis models are used to study the underlying molecular mechanisms. However, recently a controversial debate about the transferability of data obtained in mouse models to human conditions emerged. Although cell-based *in vitro* approaches can be an alternative, conventional cell culture methods hardly reflect cellular cross-communication and neglect essential physiological parameters of the living organism. We therefore develop biochip-embedded organoid models of the liver, gut and brain for use in basic medical research. The organoids are composed of essential human cell types of the respective organs and were tested in the context of sepsis-related organ dysfunction. Dynamic perfusion of biochip-embedded tissues allows an optimal supply with nutrients and oxygen, an efficient removal of catabolic metabolites, and enables a physiological cell polarization and communication within tissues. The modular approach of the biochip design allows a variable microfluidically interconnection of different organoid models in a freely selectable arrangement. Most important, the cellular processes observed under inflammatory conditions *in vitro* closely resemble pathophysiological responses of established mouse models of sepsis as well as clinical observations in human sepsis. Thus biochip-based organoid models are suitable experimental tools to improve study options on pathophysiological mechanisms of sepsis-related multi-organ failure and the development of targeted treatment strategies.

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Hydra, a suitable candidate for risk assessment of nanomaterials

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Most of the data generated in terms of nanoparticle toxicity depends on *in vitro* cultured single cell and these datas are extrapolated to multicellular organisms including human beings. Although co-culture and 3-dimensional culture are the promising technologies that can mimic the miniature of human physiology, yet these cannot be projected to behave like in situ arrangement of cells in an organ/body as the nanoparticles interacts with the biological components such as proteins and cells, and lead to unique biodistribution, clearance, immune response, and metabolism. Hence, until having the most appropriate model to assess nanoparticle toxicity, animal testing at this stage cannot be avoided. In order to understand the whole body response to the nanoparticles animals belonging to the lower levels of taxonomic hierarchy, like *C. elegans* and zebrafish, are in practice for *in vivo* toxicity testing. Following Russell and Burch's 3R's principle of alternatives to animal testing and as projected as TOX21C we hereby propose an invertebrate organism belonging to the Phylum Cnidaria, which does not feel pain and distress, as a suitable model organism for nanotoxicological assessment. Hydra offers tremendous advantage in view of its capacity for regeneration and stemness. It is also a suitable candidate for screening of inorganic substances. Data that will be presented in this poster will demonstrate the effect of a few transition metal oxide nanoparticles (ZnO, CuO, NiO, CoO, FeO, and MnO) on Hydra morphology, behavior, regeneration, growth rate and genotoxicity. It was observed that these nanoparticles have a tremendous effect on morphology, regeneration, and cell proliferation, and inflict genotoxicity. It was also observed that these nanoparticles alter the gene expression profile of stress responsive and apoptotic genes even at sub-lethal levels. Hence, it is suggested that the high sensitivity of hydra towards the inorganic nanoparticles and the bulk particles makes hydra as an amenable system to find the mechanism underlying the toxicity imposed by these hazardous nano-chemicals. To that extent, unraveling the biology of hydra shall be useful in deciphering the toxic manifestations.

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Human stem-cell based functional neuronal *in vitro* disease models

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Human pluripotent stem cells and their neuronal and glial derivatives have raised high hopes in the field of regenerative medicine. Furthermore, these cells are useful in the fields of human development, disease modeling, drug screening, and neurotoxicology. So far, most of the disease modeling has been conducted with *in vitro* and *in vivo* animal cells/models. The invention of human induced pluripotent stem cells has, however, open up a venue to study diseases with genetical background in a disease and a patient specific manner. In addition, the developments in culturing systems have made it possible to create more reductionist models in a dish. We have developed several *in vitro* models using human pluripotent stem cells derived neural cells [1,2,3]. Models are developed in platforms that contain microelectrode arrays (MEA) enabling neuronal network functionality measurements during culturing [4,5]. Also, specific microfluidistic structures are integrated to the platforms when needed. In this lecture, our recent developments in human functional neuronal network model, *in vitro* myelination model and *in vitro* epilepsy model will be discussed.

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A course in alternatives: focus on Replacement and non-animal methods

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A Course in Alternatives opens the door to progress by informing young scientists, researchers and laboratory heads on the latest technologies and methods to perform basic research and to help designing goal-targeted experiments without using animals to respond to precise scientific question of human and scientific relevance. Thanks to an Italian Regional Authory of Tuscany it was possible to create and implement a Course on Alternatives in Italy, with a specific goal, to focus on Replacement Alternatives to animal experiments and non-animals methods to be used in reasearch and education. An overview of the topics and the research enclosed in the Course will be given, together with a practical traing Course on advanced *in vitro* models to provide examples and demonstration of new techniquest and technologies available that can be used also in everyday bench-side and basic research. A brief analisis of funding for Alternatives in Italy, comparing to other countries in EU, as training of scientists on Alternatives, as well as research focussed on develping and using replacement alternatives in research, is given. The number of publications for replacement alternatives will be compared for different countries, analysing the funding for projects in Alternatives for Replacement and in which area of research investments are used and progress is made. An analysis of the funding for Alternatives in different EU countries, National Governments funding and EU funding, private investments, companies, countries and people involved in the projects, what results have been achieved and what more has to be done. The need for Replacement is shown by the limitations and failure of the type of research which uses animals of which recent evidence will be discussed. In order to improve the quality of research and the reliability of the results, Replacement alternatives should be thought and used, pubblications should become idependent from animals' use and should be no longer required to prove a concept with animal models or animal experiments, as it is now ever more clear that using replacement alternatives together with human cells and tissues, or analysing human data, is more reliable and related to the human species than exptrapolating data from a different specie. The implementation of the Good Search Guide for Alternatives by EU provides a quick and reliable method to learn how to design experiments and ensure that all possible steps have been taken to avoid animal use and improve both the quality of reaserch and the progress of science and medicine.

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Animals are not "materials and methods": Why it is important to focus on Replacement

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With this introductory lecture to the session on "Replacement – new approaches" we are going to analyse the reasons why, after 55 years from developing the concept of the 3Rs, it's time now to really focus on Replacement. We are going to analyse the need to focus on Replacement and we will discuss three main reasons why we need this change: animals, humans, progress. Research and development, education and training are evolving towards a continuous progress, as well as the methods available to perform different tasks in research, which involve all the different technologies and experitize to work together towards a new era of science and research, human-based, technology-focused, accurate, measureable, reproducible. We are going to introduce the research and the panel of experts, we will look at the latest methods to grow cells in 3D linked together in a fluidic environment exchanging nutrients and signals between cells from different human organs resembling a living being, arriving to 3D printing of cells and tissues to resemble human tissues and organs, illustrating examples of body-on-a-chip, describing the future of personalised and precision medicine or introducing projects applying mechanistic disease-modeling approaches to support the discovery of innovative new medicines. We are going to provide an overview of the latest developments in Research having in mind the goal of the 3Rs, that is to focus on Replacement, also according to point 10 of the Preamble of the Directive 63/2010/EU: "this Directive represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so. To that end, it seeks to facilitate and promote the advancement of alternative approaches." This is also the goal of this session, opening also to reflections and discussion between scientists on what is needed to achieve this goal and how scientists can actively contribute to the shift towards full Replacement of animals used in Science and Research and towards the development and use of non-animal methods. The focus is no longer just developing these Replacement Alternative methods, but also to communicate effectively the change in progress to the society and to the legislators the need to change and to invest greatly to implement and use what is known, to develop what is in progress and for searching in the mind for creation to have a science once and for all free from animals' use and good for all... animals, humans and progress.

Dr. Candida Nastrucci is also the Scientific Coordinator of the Course on "Replacement Alternatives" at "Azienda Ospedaliera Universitaria Pisana" (AOUP) and University of Pisa, Italy. TheAlternatives.eu@gmail.com

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Stem-cell based *in vitro* models to study human CNS pathophysiology

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Because of their ability to self-renew and differentiate into various tissue types, human stem cells can replicate key aspects of central nervous system pathology and physiology *in vitro*. Thus, stem cell based model have been shown to recapitulate *in vivo* condition and replace or/and reduce significantly animal experimentation. In this presentation, I will present two distinct human stem cell based *in vitro* models: (i) an engineered 3-D neural tissue to study glioblastoma invasion and (ii) patient-specific iPS cells to study the role of an oxidant generating enzyme (NOX2) during *in vitro* neuronal differentiation. In the first project, we have developed a three-dimensional co-culture system using a human stem cell-derived engineered neural tissue and cancer stem cells isolated from glioblastoma patients. This system allowed us to reproduce numerous hallmarks of glioblastoma *in vivo*, such as invasion and formation of secondary foci. Transcriptomic analysis identified that the IFN/STAT-1 signaling pathway was specifically induced in the co-culture system. In addition, significant correlation between the profile of specific gene expression and patient survival was demonstrated. In a second project, we have used induced pluripotent stem cells (iPSC) derived from a patient with chronic granulomatous disease, a genetic disease caused by a mutation in the gene coding for the reactive oxygen species generating enzyme NOX2. We have shown that NOX2 is transiently expressed at an early stage of neural differentiation. Although no morphological differences were seen between normal and NOX2-deficient cells, early markers of neurogenesis such as nestin and BDNF were decreased in NOX2-deficient cells. This observation was confirmed *in vivo* as the neurogenic regions of NOX2 knockout mice show lower expression of main transcription factors regulating early neural differentiation. NOX2 deficient neurogenic regions also present a lower oxidative status and proliferation of neural precursor, suggesting a key role of NOX2-derived oxidants in differentiation and proliferation of neural adult stem cells. Altogether, these studies exemplify the relevance of human stem cells for the study of human CNS in both pathology and physiology.

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Development of best practice in nanosafety assessment via the QualityNano Research Infrastructure

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Quality and relevance of nanosafety studies constitute major challenges to ensure their key role as a supporting tool in sustainable innovation and subsequent competitive economic advantage. In the FP7 QualityNano Research Infrastructure training modules have been organized and best practices in nanosafety research disseminated as part of its networking activities. To this end standard operating procedures (SOPs) for *in vitro* assessment of established biological endpoints have been developed and validated in a multi-step process. By means of round robins in a small group of expert laboratories SOPs have been optimized and verified, to disseminate them to the nanosafety research community. The SOPs have further been used for interlaboratory comparison (ILC) studies among >10 laboratories to evaluate proficiency of the participating laboratories, and to test candidate positive and negative control nanomaterials as assay controls and determine their response ranges. Based on the outcome of ILC studies training needs for research laboratories have been identified and SOPs refined further. The results of these pre-normative activities will finally be proposed for standardization. Such process has been adopted for determination of nanomaterial-induced cytotoxicity using the MTS assay and will be presented here. Similar activities related to other biological assays will be highlighted.

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Cigarette smoke and cigarette smoke condensate induce cell injury and early biomarkers of inflammation in human and rodent Precision-Cut Lung Slices

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Chronic obstructive pulmonary disease (COPD) is a severe disorder of the lung. It is characterized by impaired lung function, chronic inflammation, mucus hypersecretion and emphysema. A major reason for the development of COPD is cigarette smoking. Lipopolysaccharides (LPS) were widely used to mimic COPD. But LPS is active as a single factor and does not entirely reflect the complexity of cigarette smoke. Nowadays, in commonly used *in vivo* models rodents are exposed to cigarette smoke. First symptoms of COPD are developed after at least two month exposure. This early-stage COPD is characterized by inflammation but no emphysema or diminished lung function occurs. A further obstacle of these *in vivo* models is that the human background is missing. Thus, in order to understand the underlying mechanisms the need emerged to develop human relevant COPD models. Hence, exposure of human cells to cigarette smoke are widely used *in vitro* models. *Ex vivo* models like Precision-Cut Lung Slices (PCLS) use the entire viable lung tissue which resembles the lung structure and microenvironment. Currently we establish a model of COPD using PCLS of different species, including human, by exposure of viable lung slices to cigarette smoke (Cs) or cigarette smoke condensate (Csc). Human and rodent PCLS were prepared by filling the lung with agarose solution and slicing into about 300 μm thick tissue slices. PCLS were cultured at Air-Liquid Interface and exposed to Cs using the newly developed P.R.I.T.-Technology. PCLS were also exposed submersely to Csc and LPS. Cytotoxicity was determined by detection of released lactate dehydrogenase, by measurement of metabolic activity using WST-1 assay and by LIVE/DEAD® viability staining. The pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1α were determined by ELISA. A therapeutical intervention of inflammation was performed by using dexamethasone. Csc was collected by passing cigarette smoke through a filter pad and dissolving the particulate fraction in organic solvent DMSO. This particulate fraction of cigarette smoke was used for submerse exposure of human and rodent PCLS using acute and subchronic culture protocols. Csc induced cell death and early markers of inflammation in viable lung tissue after acute and subchronic exposure. The concentration-dependent toxicity after Csc exposure was determined by metabolic activity. EC_{50} values of 85 μg/mL in murine PCLS, 212 μg/mL in rat PCLS and 196 μg/mL in human PCLS were determined. PCLS were also exposed at Air-Liquid Interfaces to Cs using the new P.R.I.T.-Technology. The P.R.I.T. System allows the simultaneous exposure of lung tissue to gas and particle phases of Cs. Thus, Cs induced concentration-dependent tissue damage in lung slices as well as early markers of inflammation. Csc and Cs induce tissue injury and biomarkers of inflammation in rodent and human PCLS. In particular the direct exposure of lung tissue to the entire complex mixture of cigarette smoke closely reflects the *in vivo* situation and is a very promising translational *ex vivo* model.

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Establishment of *in-vitro* models for the study of repeated exposure to toxicants by the respiratory route

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The respiratory epithelium is exposed to environmental pollutants and to drugs for oral inhalation. Both environmental exposure and therapy of obstructive diseases occurs repeatedly over a prolonged time period and could induce long-term effects in the deep lung. Animal studies are usually performed to study chronic toxicity while mode of action studies and acute cytotoxicity are evaluated *in vitro*. Cell lines are preferred to primary cells because they provide greater reproducibility and independence from donor availability. Physiologically relevant culture of respiratory cells has to be performed in air-liquid interface culture and co-culture of main cells of the terminal parts of the bronchial tree, alveolar epithelial cells and macrophages, would be desirable. We aimed to develop models for prolonged (repeated) exposure of respiratory cells in mono- and in co-culture. In one model, cultures of Calu-3 cells as established model for pulmonary permeability were studied for up to 28 days at an air-liquid interface. In the other model A549 human alveolar epithelial cells were pre-cultured for 6 days in monoculture until constant transepithelial electrical resistance (TEER) values were reached. Subsequently, murine RAW247.8, DMBM-2 macrophages or human MonoMac-6 monocytes were added in a cell ratio of 1:10 and both cells co-cultured at an air-liquid interface for up to 28 days. The models were characterized at various time points by TEER and demonstration of secretory granules by anti-MUC5 staining for Calu-3 cells and TEER plus identification of murine macrophages by F4/80 staining and human macrophages by CD15 staining for the co-culture model. Particles (1.8 μg/cm^2 carboxylated polystyrene particles (CPS) in nominal size of 20 nm and 200 nm) were applied once a week and particle uptake, cytotoxicity, and induction of proinflammatory response were chosen as parameters for biological effects. Calu-3 could be cultured over 28 days as monolayers with stable TEER values. The co-culture models were only partly successful. Very few MonoMac-6 cells remained on the A549 monolayer, while RAW247.8 and DMBM-2 cells showed a tendency to overgrow the A549 cells. Uptake of CPS in the cells showed a linear time-dependent increase. At the low doses used in this study, no disruption of barrier function, decrease of viability and increase of IL-6 and IL-8 secretion was observed. While the Calu-3 model could be used for the assessment of cellular effect over a prolonged period of time, combinations of phagocytes and A549 cells could be co-cultured only for a limited time span.

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Harm-benefit analysis of animal experimentation: a qualitative study of ethics committee practice in six European countries

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Harm-benefit analysis is now a required key element of the procedure for evaluating animal experimentation in the legal framework in Europe and elsewhere. We have previously discussed key challenges to including a harm-benefit analysis in the evaluation of animal research [1]. Thus we have argued that there is lack of clarity and potential disagreement over what to include in the benefit analysis (focusing more narrowly on knowledge gain or including societal contribution) and over how to value different research purposes. It is also unclear to what extent harms are actually weighed against benefits in the actual decision-making. In order to understand how these challenges are dealt with in practice, we have conducted semi-structured interviews with members of Animal Ethics Committees across Europe. After mapping the ethics review systems of the European countries, six case countries were selected for the interviews: Estonia, Holland, Ireland, Portugal, Sweden and Switzerland. Four interviews were conducted in each of these countries. The profile of the chosen interviewees took into account age, gender and expertise and all interviewees had at least one year of committee experience. Interviewees were presented with four hypothetical cases of animal experiments in which factors influencing harm (severity/invasiveness of animal procedures), benefit (purpose, expected outcome) and other aspects of ethical assessment (animal species) were varied. Interviewees were asked to elaborate on how harm and benefit would be assessed, as well as on the final decision-making. Both interviewees' own assessment and their view of how their committee as a whole would handle the same case were addressed. The results, which are presently being analysed, will provide insight into how the main decision-makers over animal experiments assess and weigh harms and benefits in the context of ethical deliberation.

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Ethics review of animal experiments in Europe under Directive 2010/63/EU

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The fundamentally revised and expanded European legislation (Directive 2010/63//EU) protecting animals used in research now covers evaluation and authorization procedures, and includes aspects that were not included in the previous Directive (86/609/EU). These aspects (e.g. predicted benefit, 3Rs compliance, severity, harm-benefit analysis) correspond to what is typically covered by an "ethics review", although this term is not used in the Directive. How and by whom projects are to be evaluated was left to individual Member States (MS) to determine. As part of the ANIMPACT project (http://www.animpact.eu), we are exploring the ethics evaluation and authorization process within the European Union. As of March 2015, we have confirmed information for 19 MS. The available information shows considerable diversity between MS as regards the approach to evaluation and authorization. In many MS there is a combination of several approaches. The two most common approaches are i) that the projects are only evaluated and authorized at a national level (the evaluation is conducted by a national committee and the authorization provided by the national competent authority) and ii) that the projects are evaluated at institutional, local or regional level (by a committee) combined with an evaluation at a national level (by a committee and/or officers) with the authorization being provided also at a national level. Committee size and composition also vary. Most committees include scientific expertise and veterinary expertise, whereas expertise in law, ethics and alternatives to animal experiments is less common. Committee members from outside the scientific and technical community are most often representatives of special interest groups (mainly animal welfare associations), whereas genuine lay members are rare. In the presentation, we will discuss potential underlying reasons for MS opting for these different approaches, and the consequences for the project evaluation process.

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Versatile, polymer-based devices for multi-parameter *in vitro* toxicology

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A paradigm shift is needed to convince funding agencies, regulators and the general scientific community at large of the potential for *in vitro* toxicology monitoring. To do this, viable alternatives must be provided and validated, that focus not only on the cell type used, and the environment of the cell but also the monitoring system used. The ultimate goal is to generate a system that is truly predictive of *in vivo* toxicology. Electronic methods for monitoring live cells are emerging as powerful alternatives to traditional optical, or end-point assay techniques for *in vitro* toxicology. One technology which has been adapted for use in a number of commercially available medium-to-high-throughput screening systems, is electrical impedance spectroscopy (EIS). However, a reliance on traditional electronic materials results in relatively high-cost products, that are difficult to integrate with optical imaging techniques used to verify or construe electrical signals into cellular events. Using organic electronic materials as an alternative to traditional electronic materials, we have developed a series of devices that allow highly-dynamic and sensitive monitoring of toxicology of barrier tissue [1]. Flexibility of the materials in terms of design has allowed us to solve a number of issues common to other EIS techniques including incompatibility with high-resolution imaging [2], sub-second temporal resolution [3,4], and difficulty to monitor more complex tissues, including 3D cysts/spheroids [5]. In this presentation I will also discuss the development of the organic electrochemical transistor (OECT) in combination with high-resolution imaging, demonstrating unambiguous correlation of electrical signals with cellular events.

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Identification of transcriptome signatures and biomarkers specific for migration-inhibiting potential developmental toxicants in human neural crest cells

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Disturbance of the neural crest cell (NCC) migration process by toxicants is known to lead to severe malformations in model organisms, and several factors (e.g. genetics and chemicals) have already been identified as causes for neural crest- related developmental defects. The Migration of Neural Crest cell (MINC) assay was set up as first human stem cell-based method that is able to detect the functional effects of chemicals on one of the key events of nervous system development, the neural crest cell migration [1]. Its performance characteristics allowed the MINC assay to be included in the screening test battery of the European project ESNATS (Embryonic Stem cell-based Novel Alternative Testing Strategies). This project allowed the testing of a wide array of substances comprising environmental pollutants and modern pharmaceutical substances and it lead to the identification of several migration-impairing toxicants [2]. In this study, we compared transcriptome profiles of different MINC-positive compounds, including drugs (geldanamycin), environmental chemicals (triadimefon, arsenic trioxide and the flame retardant PBDE-99) and known developmental toxicants (valproic acid and trichostatin A), in order to investigate which processes are mainly targeted by the different migration inhibitory drugs. We used an unbiased system-wide transcriptome analysis by micro-array to gain insight into altered gene expression patterns transcriptome profiles were compared and enriched GOs and KEGG pathways were classified and contrasted, showing the presence of different processes targeted by the MINC-positive compounds. Furthermore, we used different approaches to identify suitable biomarker candidates. The first method is based on the comparison and the overlap of the transcriptome profiles among the different toxicants the second approach is based on a novel system of scoring, which assigns different weight to each gene, according to its characteristics of "measurability", "statistical power" and "biological relevance". The found candidate biomarker genes will contribute to optimize the biomarker identification strategy for new developmental toxicant identification and characterization.

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A 3D *in vitro* iPSC derived mini-brain model to study Parkinson's disease

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Research in Parkinson's disease is experiencing an upswing at the moment, on one hand due to a lack of curative drugs for the large number of patients. Drug testing is nearly exclusively performed *in vivo* in the so-called MPP, methamphetamine, 6-hydroxydopamine and rotenone models requiring tens of thousands of animals. Human neurons, which would be most relevant, are not usually available and existing cell lines e.g. neuroblastoma cells are only a very poor substitute. During our project funded by NCATS, NIH (1U18TR000547) we have developed a human induced pluripotent stem cell (iPSC) 3D model recapitulating many aspect of the human brain. The model can now be for the first time applied for the study of Parkinson's disease with respect to mechanisms of degeneration and mechanisms and efficacy of drugs. The main objective of this project is to make use of our developed 3D human brain model to study Parkinson's disease. The 3D model has shown to recapitulate early *in vivo* human neurodevelopment by showing the emergence of different kinds of neurons and glial cells, induction of genes that play important roles in neurodevelopment as well as presence of electrical activity. Human 3D aggregates stain positively for different neuronal types, astrocytes and oligodendrocytes markers after 2, 4 and 8 weeks of differentiation showing a maturation in the cells during the differentiation process. In addition, real-time PCR analyses show higher expression of various neuronal differentiation markers in 3D cultures compared to 2D culture, showing a faster maturation in the 3D system. We have used Rotenone, a pesticide known to induce neurotoxicity by inhibition of mitochondrial complex I, and one of the compounds used for Parkinson disease studies, in order to test the relevance of the model. The model shows increased ROS production and decreased mitochondrial function after exposure to Rotenone. In addition, the model shows a decrease in sensitivity to Rotenone exposures with increasing maturation. Notably, such human brain models will represent a versatile tool to study CNS physiology and pathology, and neurological disorders such as Parkinson.

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Understanding chemical allergen potency: role of NLRP-12 and BLIMP-1 in the induction of IL-18 in keratinocytes

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Keratinocytes play a key role in all phases of skin sensitization. We have recently identified interleukin-18 (IL-18) production in keratinocytes as a potentially useful endpoint for determination of contact sensitization potential of low molecular weight chemicals. A total of 33 chemicals were tested, with an overall accuracy of 97%. The aim of this study was to identify genes involved in p-phenylediamine (PPD)-induced NLRP3 inflammasome activation and consequently in IL-18 production. When activated, the inflammasome components assemble and self-oligomerize, leading to caspase-1 activation and maturation of pro-IL-18 into bioactive cytokine. The involvement of this complex was demonstrated by the use of the pan-caspase inhibitor ZVAD-FMK, which significantly reduced allergen-induced IL-18 production in human keratinocyte cell line NCTC2544. For gene expression analysis, cells were treated for 6 h with PPD, RNA was extracted and examined with the Inflammasome PCR array PAHS-097S. One gene was selected based on high induction: NLRP12 (Nod-like receptor P12). The NLRP12 promoter contains a Blimp-1 (B-lymphocyte-induced maturation protein-1)/PRDM1 binding site. From the literature, it is known in monocytes/macrophages that Blimp-1 reduces NLRP12 activity and expression. To confirm the gene identified and to investigate its relationship with Blimp-1, cells were exposed for different times (3, 6 and 24 h) to contact allergens of different potency, namely PPD and 2,4-dinitrochlorobenzene (DNCB) and eugenol. We found that all allergens tested induced with a different kinetic and extend both genes as assessed by Real Time-PCR. NLRP12 and Blimp-1 expression appeared to be inversely correlated. We are currently investigating, by silencing experiments, the role of these two genes and proteins in allergen-induced IL-18 production.

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BoNT activity determination by stimulus-dependent release of neuro-secretory vesicle-targeted luciferase from neuronal cell lines

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Botulinum toxin is a bacterial toxin that inhibits neurotransmitter release from neurons and thereby causes a flaccid paralysis. It is used as drug to treat a number of serious ailments and, more frequently, for cosmetic medical interventions. Botulinum toxin for pharmacological applications is isolated from bacterial cultures. Due to partial denaturation of the protein, the specific activity of these preparations shows large variations. Because of its extreme potential toxicity, pharmacological preparations must be carefully tested for their activity. For the current gold standard, the mouse lethality assay, several hundred thousand mice are killed per year. Alternative methods have been developed that suffer from one or more of the following deficits: *In vitro* enzyme assays test only the activity of the catalytic subunit of the toxin. Enzymatic and cell based immunological assays are specific for just one of the different serotypes. The current study takes a completely different approach that overcomes these limitations: Neuronal cell lines were stably transfected with plasmids coding for luciferases of different species, which were N-terminally tagged with leader sequences that redirect the luciferase into neuro-secretory vesicles. From these vesicles, luciferases were released upon depolarization of the cells. The depolarization-dependent release was efficiently inhibited by of botulinum toxin in a concentration range (1 to 100 pM) that is used in pharmacological preparations. The new assay might thus be an alternative to the mouse lethality assay and the immunological assays already in use.

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Alzheimer's disease research in the 21st century: the shift towards a new paradigm

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Animal models of Alzheimer's disease (AD) have been extensively utilized in the last few decades in an effort to elucidate the pathophysiological mechanisms of this disease and to test novel therapeutic approaches. However, research success has not effectively translated into therapeutic success for human patients. We investigated the reasons for this translational discrepancy. Our analysis revealed that translational failure is due – at least in part – to the overuse of animal models that cannot accurately recapitulate human AD etiopathogenesis or drug responses and the inadequate use of human-based investigation methods. Here we present the challenges and opportunities in AD research and propose how we can mitigate this translational barrier by employing human-based methods to elucidate disease processes occurring at multiple levels of complexity (from gene expression to protein, cellular, tissue/organ to individual and population level). Novel human-based cellular and computational models are already being applied in toxicology and regulatory testing, and the adoption and the widespread implementation of such tools in AD research will undoubtedly facilitate human-relevant data acquisition. Additionally, clinical studies focused on nutritional and lifestyle intervention strategies to reduce and/or prevent early symptoms of AD represent another relevant and important way to elucidate AD pathogenesis and treatment options in a human-based setting. Taken together, it is clear that a paradigm shift towards human-based research is the best way to tackle the ever-increasing prevalence of AD in the 21st century.

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A model for predicting human liver toxicity, based on molecular fragments

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The identification of the potential for a compound to cause hepatotoxicity is of special interest for human health risk assessment. Since the prediction of liver injury trough the identification of structural alerts (SAs) could allow to early identify toxic substances increasing the protection level of human health, the purpose of this study is to build a Structure-Activity Relationship (SAR) model for evaluating hepatotoxicity. For modeling we used data coming from U.S. Food and Drug Administration (US FDA) Spontaneous Reporting System (SRS) (490 non proprietary data) and data published in Fourches et al., 2010 (951 compounds). The two lists of substances were merged and checked for agreement and duplicates and inorganic substances were removed. We finally obtained a dataset containing 950 organic compounds that we randomly split into training set (760) and test set (190). For extracting SAs related to hepatotoxicity and non-hepatotoxicity, we used SARpy, a software that automatically identify and extracts chemical fragments related to a specific activity based on statistical calculations (e.g. likelihood ratio, LR). Every SA was carefully checked and in some cases generalized. SAs with low reliability (LR < 4.5 and percentage of TP < 75%) were deleted and not used for building the model. Moreover some of the SAs were selected on the basis of information available in the literature on their potential ability to cause hepatotoxicity. Considering the complexity of this endpoint the model gave satisfactory performance. Into the training set the accuracy was 85%, specificity 81%, sensitivity 89% and Matthews correlation coefficient (MCC) 0.73. Into the test set the accuracy was 62%, specificity 37%, sensitivity 85% and MCC 0.26. For some of the SAs used for modeling, it was also possible to find a mechanistic explanation. Since this model was build using human data, it might be applied without any need of extrapolation from other species. The prompt identification of molecules with high potential to affect liver may help in drug discovery and identification of harmful substances for humans. Acknowledgements: we acknowledge the financial support of the European Commission and Cosmetics Europe, for the project ToxBank.

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Evaluation of developmental neurotoxicity triggered by N-methyl-D-aspartate receptor (NMDAR) antagonists based on the Adverse Outcome Pathway (AOP) concept

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An Adverse Outcome Pathway (AOP) concept portrays existing knowledge of the linkage between the Molecular Initiating Event (MIE), and an Adverse Outcome (AO), connected via a cascade of Key Events (KE) and the relationships between them (KER). Therefore, AOP organizes mechanistic knowledge at various levels of biological organization to facilitate its integration and evaluation for regulatory application. In the area of developmental neurotoxicity comprehensive understanding of toxicity pathways leading from chemical exposure to an adverse outcome is sparse. In this study, the AOP concept has been applied to organise existing knowledge related to the binding of antagonists to NMDARs (Molecular Initiating Event) in neurons during synaptogenesis in hippocampus and cortex leading to deficit of cognitive function (Adverse Outcome). It is well documented that learning and memory processes rely on physiological functioning of NMDAR [1]. Activation of NMDARs results in long-term potentiation (LTP), which is related to increased synaptic strength, plasticity and memory [2]. It also enhances brain derived neurotrophic factor (BDNF) release, which promotes neuronal survival, differentiation and synaptogenesis [2,3]. Consequently, the blockage of NMDAR by chemical substances during synaptogenesis (critical process for brain development) disrupts neuronal network formation resulting in the impairment of learning and memory processes [4]. NMDAR has been identified as a target for a number of well-established developmental neurotoxicants such as lead, toluene or ethanol. A qualitative evaluation of the key events triggered by exposure to lead at the cellular, tissue, organ and organism level will be presented including reduction of intracellular calcium levels, reduced BDNF release, decreased neuronal differentiation, decreased synaptogenesis and neuronal network formation and function leading to learning and memory deficit (AO). Based on the identified data gaps various alternative approaches will be applied (including *in vitro* experiments) to further elucidate the key events and if possible, to establish the quantitative relationships between them.

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A dynamic multi-organ-chip as a tool for effect prediction of hepatic metabolism on drug toxicity *in vitro*

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Most drugs are metabolized in liver, which may lead to their activation or inactivation by liver enzymes. As a result, the parent compound pharmacokinetics and pharmacodynamics might be affected and the liver is damaged by active metabolites. Preclinical drug safety studies on animals are carried out. *In vivo* studies are expensive and sometimes fail to predict the effect of the drug in human due to the species-specificity. A reliable *in vitro* system that provides the prediction of hepatotoxicity and the effects of hepatic metabolism on target organs is thus needed in pharmaceutical industry for rapid drug screening. In this study, multi-organ-chip "Homunculus" was presented. This microfluidic platform consisted of two cell culture compartments interconnected with microchannels in a closed circuit with an on-chip pump that provided a dynamic perfusion at different speeds of culture medium through these compartments for mimick *in vivo* conditions. Human hepatoma HepaRG cell line was chosen as a liver model due to its capacity to acquire mature hepatocyte phenotype upon differentiation [1]. Spheroids consisting of 5000 differentiated HepaRG cells with diameter of 200 microns were formed using hanging drop method [2]. Structural integrity of this 3D model consisting of polar and zonal located liver cells was confirmed by immunocytochemical method using antibodies to ZO-1, MRP2, CYP3A4, CK19 and F-actin. At first, the effect of microfluidic culture conditions on HepaRG spheroid functionality was examined. As shown by RT-qPCR, the culturing of cells at optimal speed of culture medium resulted in up-regulating the gene expression of phase I, II and III enzymes (CYP2B6, CYP3A4, CYP2E1, SULT1A1, ABCB1, ABCC2, ABCB11), as well as the genes involved in cell protection against reactive oxygen species (NRF2, GSR), which probably reflected the cells adaptation to microcirculation of the culture medium and mediated the increase in synthesis of albumin and glucose without reducing HepaRG cells viability. Moreover, the treatment of HepaRG spheroids cultured in the microfluidic system with 5 mM prodrugs, cyclophosphamide (IC_{50} 13.053 ±1.40 mM) or acetaminophen (IC_{50} 14.942 ±1.19 mM), resulted in an increased formation of their metabolites by cytochrome P450 enzymes, compared to the culture in static conditions, as determined by mass spectrometry. Then metabolically active 3D HepaRG cells were co-cultured with 2D HaCat in the chip "Homunculus" and the effect of this co-culture exposure to cyclophosphamide in serum-free medium was studied. The human keratinocyte were chosen here due to the lack of xenobiotic metabolism and as target model of human epidermis, which is commonly used in cytotoxicity testing [3]. It was found that 3.6 mM cyclophosphamide had not affected HaCat cell viability after 48 h treatment in the absence of liver cells, whereas co-culturing with HepaRG spheroids had resulted in HaCat death above 40% (test with neutral red), that can be explained by the formation of the cytotoxic metabolite by hepatocytes. Thus, this work demonstrates that the usage of dynamic multi-organ chip "Homunculus" may be a promising tool for drug toxicity prediction *in vitro* taking into account their metabolism in liver cells (Russian Ministry of Education and Science grant № RFMEFI57914X0018).

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The Lush Prize – rewarding innovative animal-free science around the world

Rebecca Ram

Lush Prize, Manchester, United Kingdom

Each year, the Lush Prize awards £250,000 (300,000€) to initiatives working to end the use of animals in toxicology testing for consumer products and ingredients.There are six categories of award: ScienceTraining Young Researcher Lobbying and Public Awareness. The sixth category is the Lush Black Box Prize which offers, in any one year, the full £250,000 for a key breakthrough in human toxicity pathways research. Many regulations and prizes are directed towards the broader idea of the 3Rs: reduction, refinement, and replacement of the use of animals. The Lush Prize, as a project driven by animal ethics, seeks only to support projects working on the complete replacement of animal tests. At £250,000 every year, the Lush Prize is the largest annual financial reward in its field.The Lush Prize also commissions unique research into issues relevant to each prize category. Nominations open for the Lush Prize open in April each year. An individual can nominate their own work or that of another organisation or individual. Judging is carried out by a panel of experts from around the world – scientists, politicians and campaigners. Winners participate in a conference, can have their work published in a special edition of the peer-reviewed journal ATLA (see 2013 edition) and attend a fantastic awards ceremony in London. Full details of the prize categories and how to nominate are available on the Lush Prize website http://www.lushprize.org

The Lush Prize: A partnership between Lush Fresh Handmade Cosmetics and Ethical Consumer Research Association to support animal-free toxicology.

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Intra- and interlaboratory validation of LuSens: a reporter gene-cell line to detect keratinocyte activation by skin sensitizers

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Several *in vitro* methods address the steps leading to skin sensitization as defined by the adverse outcome pathway. KeratinoSens™ has been validated in the EU to address the cellular event of keratinocyte activation. Herein, we report on the me-too validation of the LuSens assay, a simple bioassay that uses a human keratinocyte cell line harboring a reporter gene construct composed of the rat antioxidant response element (ARE) of the gene of the NADPH:quinone oxidoreductase 1 and the luciferase gene. In-house validation with 74 substances showed predictivity of 82% in comparison to human data. LuSens is, however, intended to be used in a battery of *in vitro* methods which results in even higher predictivities. To meet European validation criteria, a study was conducted with 5 partners from US, Germany and Switzerland. The study was divided into two phases, to assess 1) transferability of the method and 2) reproducibility and reliability. Phase I showed a good transferability to naïve labs and within laboratory reproducibility, leading to correct prediction of 80%. Phase II was performed with 20 coded test substances (current performance standards of the ARE OECD Draft TG), and is under evaluation. Our data show a remarkable reproducibility of 100% within the testing labs and a good concordance of the data (above 80%) towards the human *in vivo* data. The study demonstrates the transferability and reliability of LuSens for detecting skin sensitizers.

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Assessment of the predictivity of neuronal networks coped to microelectrode arrays for identification of neurotoxicants

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A challenging aspect to assure the safety of a product is the assessment of its neurotoxic hazard potential. Currently, only *in vivo* methods are regulatorily accepted and so far, no *in vitro* model has been fully validated. With the advance in technology and the ability to maintain primary neuronal models for prolonged periods, a promising test system emerged, combining the use of microelectrode arrays (MEAs) and *in vitro* culture of 2D neuronal networks (NN). Here we report on the in-house validation of the NN MEA assay using a set of 70 compounds of different chemical classes with known neurotoxic and non-neurotoxic potential with the aim to use it for screening of compounds under development and in the future for its potential application in the regulatory framework. The results demonstrate that when evaluating the mean firing rate of the NN, the assay presents a predictivity of 75%, which is comparable to previous reports using a different set of test substances. In order to increase the predictivity rate of the assay, we also report on the integration of live-dead staining to analyze cell viability and the evaluation of further electrical parameters to identify if there are changes that cannot be reflected in the analysis of the mean firing rate.

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Metabolomics *in vitro*: a new approach for systemic toxicity – first applications for mode of action identification and chemical grouping

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The use of alternatives to animal testing has considerably increased in the last years but is still mostly limited to topical applications. Metabolomics *in vitro* is a novel approach to identify systemic toxicity by determining modes of action and (dis) similarities in the profiles of chemicals. *In vitro* metabolomics allows for the acquisition of quantitative information about the multi-parametric metabolic response of the cellular systems in normal and patho-physiological conditions. Our data demonstrate a highly stable and reproducible method. The purpose of this study was to investigate a) if different toxicity modes of action can be identified by means of metabolome changes and b) if toxicological equivalence between pure enantiomers and their corresponding racemates can be demonstrated using this technology. We therefore exposed HepG2 cells at two concentrations for 48 h to different, hepatotoxic test substances and determined the metabolome profile. The results obtained indicate on one hand that test substances with different liver toxicity are able to generate differential changes in the metabolome profile and on the other hand, no biologically relevant difference was observed between enantiomers and their racemates, an effect that has been also proved *in vivo*. The present results are a proof of concept for the use of this technology for toxicology. We thus demonstrate straightforward applicability and conclude that *in vitro* metabolomics is (more than) a promising tool to address systemic toxicity.

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Metabolomics as a Read Across Tool

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Over the last 10 years, BASF has developed the MetaMap® Tox database with toxicity and metabolome data of >550 reference compounds obtained from rat studies. Plasma metabolome analysis was performed after 7, 14 and 28 days. Based on the metabolome data for the reference compounds, we have established >120 specific patterns which are predictive for certain toxicities. We have shown that the metabolome data can be used to predict the toxicological/ biological outcome induced by the respective compounds. Furthermore, during the course of our project we have used more than ten exact repeats to show reproducibility and reliability of the metabolome analysis (Kamp et al., 2012). REACH is currently one of the main drivers for safety testing in Europe. This testing is still primarily based on animal experimentation. However, one way to waive animal testing is read-across, a measure to assess hazard of a compound based on data from similar compounds. Today, similarity of compounds is answered based on their chemical structure. In this talk, we will present evidence of *in vivo* metabolomics analysis of case studies supporting the thesis that structural similarity can lead to toxicological similarity. Moreover, we will also show case studies in which compounds are toxicologically similar although structurally dissimilar (e.g., different peroxisome proliferators, such as fibrates, phthalates, etc.) and cases in which they are toxicologically dissimilar but structurally similar (e.g. 2-AAF and 4-AAF). These case studies underline that a read-across purely based on chemical structure could also produce false negative or false positive results and that metabolome data can provide the biological anchor to base similarity.

In addition, our current efforts are also oriented in the application of metabolomics to *in vitro* systems. As a proof of concept, we have been able to demonstrate toxicological equivalence between pure enantiomers and their corresponding racemates, an effect that has been proved *in vivo*. The present results suggest the potential use of this tool to achieve chemical grouping.

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Chip-based co-culture of neurospheres and liver spheroids for long-term substance testing

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Present *in vitro* and animal tests for drug development do not reliable predict the human outcomes of tested drugs or substances because they are failing to emulate the organ complexity of the human body, leading to high attrition rates in clinical studies. The phylogenetic distance between humans and laboratory animals is enormous, this affects the transferability of animal data on the efficacy of neuroprotective drugs. Therefore, many neuroprotective treatments that have shown promise in animals have not been successful when transferred to humans. We present a multi-organ chip capable of maintaining 3D tissues derived from various cell sources in a combined media circuit which bridges the gap in systemic and human tests. A peristaltic on-chip micro-pump reproducibly operates a PDMS-embedded microcirculation system, emulating the systemic arrangement of organs within the human body. In this study we used the multi-organ chip for a co-culture of human artificial liver microtissues, consisting of HepaRG cells and primary HHSteC, and human NT-2 neurospheres. Daily lactate dehydrogenase activity measurements of the medium and immunofluorescence end-point staining proved the viability of the tissues and the maintenance of differentiated cellular phenotypes. Moreover, the lactate production and glucose consumption values of the tissues cultured indicated that a stable steady-state was achieved after 6 days of co-cultivation. The neurospheres remained differentiated neurons over the two-week cultivation in the multi-organ chip, proven by qPCR and immunofluorescence of the neuronal markers βIII-tubulin and microtubule-associated protein-2. Liver equivalent differentiation was also restored as shown by the expression of cytokeratin 8/18, vimentin, cytochrome P450 3A4 and MRP-2. Additionally, a two-week toxicity assay with a repeated substance exposure to 2,5 hexanedione in two different concentrations induced high apoptosis within the neurospheres and liver microtissues, as shown by a strong increase of lactate dehydrogenase release in the medium and by TUNEL / KI67 staining. The principal finding of the exposure of the co-culture to 2,5-hexanedione was that not only two different toxicity doses profiles could be discriminated, but also that the co-cultures were more sensitive to the substance compared to the sum of the respective single-tissue cultures in the multi-organ-chip. Hence, we provide a promising tool for dynamic co-culture of different human organoids to better predict the outcome of tested drugs in clinical studies.

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Working together to develop and implement a "culture of care"

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Establishing, promoting and maintaining a good "culture of care" is a fundamental requirement if legal, ethical and animal welfare obligations, along with wider responsibilities towards employees and the public, are to be met. Evidence that the "right" culture is in place is through a combination of good and focused management communication 3Rs and animal welfare. Ultimately, to achieve high standards of animal welfare and science, a wide range of factors have to come together to provide the right framework within an organisation. Having the right attitudes, values and people, with everyone engaged and positively contributing, knowing what is required of them and doing the right thing without prompting! This presentation will focus on the desired approach of working in dialogue and collaboration with all active stakeholders, the sharing of best practices and experiences in developing a standard for culture and care.

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Avian encephalomyelitis live vaccines – alternative methods for batch virus titration

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Avian encephalomyelitis virus (AEV) infections affect poultry worldwide and cause severe neurological symptoms including ataxia, paralysis and tremors [1,2]. The first vaccine was developed in the 1960s to prevent the spread of the disease [3]. For AE vaccines, the European Pharmacopoeia provides a monograph including the determination of the virus titer which has to be performed for batch release on each batch of the final product. This titration can be performed either in embryonated SPF-eggs followed by the hatching of chicks ("hatchout test") or in a suitable cell culture [4]. The hatchout test involves the infection of embryos in ovo, incubation of the eggs until hatching, and the examination of the hatched chicks for neurological signs for a specific period of time. Unfortunately, no suitable cell titration method has yet been established for the AEV vaccine. Thus the hatchout test is the method used among the manufactures, although it has several disadvantages: Including the need to use animals and the subjective nature of the test evaluation, due to the visual examination of the subjects. This project aims to refine or even replace the hatchout test with a more specific and sensitive method in accordance with the 3Rs principle. The proposed refinement is by using an ELISA for the determination of the final virus titer. For the titer determination susceptible 6-day-old chicken embryos (eggs) are inoculated with different dilutions of an AEV vaccine strain. After an incubation period of 12 days and killing of the eggs by low temperature, the embryonic brains are tested for the presence of AE antigen with an AE-antigen ELISA. To investigate the suitability of the refined titration assay, the variability, sensitivity and accuracy will be compared to the hatchout test by performing both methods in parallel. Preliminary results of the comparative analysis suggest that the use of the specific AE-ELISA could enhance the objectivity of the virus titration. Only a low range of variation was observed in the results. Due to the nature of animal experiments a high degree of variance within the different dilution groups of the hatchout test could be detected, even though the final titer was quite stable. Although both test methods show an acceptable low range of variation of the final virus titer the alternative test method could reduce the number of used animals and the test duration. The assay could also greatly assist in the standardization of the batch release testing for AEV vaccines.

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Brain melanoma metastasis cell line and clones – a model system for *in vitro* research

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The incidence of melanoma is significantly increasing and is responsible for 80% of all skin cancer deaths. Worldwide, the incidence of melanoma is approximately 200,000, leading to about 46,000 deaths. Metastases to the brain characterized by their aggressiveness and delicate location are common in 60% of melanoma stage IV patients representing a challenge for successful treatment with many patients succumbing to the disease. Cell lines provide a rational means to better understand tumour biology, development and probe for new therapeutics. No commercial cell line from melanoma brain metastases is currently available. Here we present a cell line isolated from a brain metastasis. To recapitulate the heterogeneity of brain melanoma metastases we separated two different clones from the established cell line by FACS cell sorting and were able to create cell lines from the clones. The cell lines are phenotypically and genetically characterized by morphology, growth and migration behaviour, immunohistochemistry, surface marker expression, microarray-based Comparative Genomic Hybridization and electron microscopy. Identification of the three cell lines was carried out by short tandem repeat analyses. Tumour tissue available from the brain metastasis showed the expression of melanoma markers Melan A, Tyrosinase, HMB45 and S100. During cell cultivation all cell lines lost these markers, whereby currently only immunhistochemical tests have been performed. Each of the cell lines were characterized by a genetic profile showing typical melanoma marker for example multiple chromosomal gains on chromosome 7, 8 and losses on chromosome 10. One clone showed a dominant CD271 staining suggestive of the stem cell character of this cell line. In terms of electron microscopy differences within the cell lines in regard to endoplasmatic reticulum, vesicles and lipid droplets were observed. As a first application the effect of human lactoferricin derivates has been tested on the different lines and different IC_{50} concentrations were detected. The cell line MUG-Mel1 and their clones MUG-Mel1 C8 and MUG-Mel1 D5 add to the armamentarium of already existing melanoma cell lines but more importantly are to our knowledge the first cell lines isolated from a brain metastasis offering a single opportunity to researchers for study brain metastases in more detail and avoid or reduce thereby animal experiments.

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Pro- and pre-hapten mechanisms in skin sensitization

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Many skin sensitisers act as directly reactive haptens (usually by electrophilic chemical mechanisms), but a significant minority are not directly reactive and act via conversion to reactive derivatives. Chemicals that are metabolically converted to reactive haptens are known as pro-haptens, and chemicals that are abiotically activated are known as pre-haptens. This presentation will discuss recent advances in defining structural alerts for pro- and pre-haptens, and quantitative modelling of their sensitising potency.

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Reconstituted human epidermis models and IL-18 levels for identification and potency ranking of skin sensitizers: identification of critical steps and harmonization of existing protocols for the purpose of validation

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Background: IL-18 production by keratinocytes is a potentially useful endpoint for determination of contact sensitization potential of low molecular weight chemicals [1]. Potency classification of skin sensitizers relates to the irritant potential of the chemical in a reconstituted human epidermis (RHE) model (dos Santos et al., 2011). Gibbs et al. (2013) successfully integrated the IL-18 endpoint in various established RHE models, currently used to assess chemical substances for their potential to trigger irritation and corrosion. The protocol allows for the identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability. The observed accuracy ranges from 80 to 95%, while the preliminary potency data on a subset of compounds for which human data are available suggest a superior correlation of the test with human data over LLNA data. Aim of this study: (i) To identify the most critical steps in the protocol by Gibbs et al. [2], and (ii) to establish a standard protocol for validation of the RHE IL-18 potency test for identification and classification of skin sensitizers, applicable on any RHE model.

Materials and methods: The protocol was transferred to eight laboratories using either CellSystems epiCS® (N = 3), MatTek EpiDerm™ (N = 4) or SkinEthic™ RHE (N = 1). Cell viability was assessed using the MTT assay (dos Santos et al., 2011). IL-18 levels were quantified using either an in house ELISA (MBL IL-18 specific capturing and detection antibodies in combination with streptavidin-conjugated poly-horseradish peroxidase from either Immunotoools or Linaris, or an IL-18 ELISA kit (MBL or RayBiotech). The chemical substances used for training, technology transfer and implementation include: 1-chloro-2,4-dinitrobenzene (DNCB), eugenol, cinnamaldehyde, salicylic acid and lactic acid.

Results: The training and technology transfer of the protocol were successful as the participants in this study produced the same prediction as the test developers providing the training. At subtoxic levels the SkinEthic™ RHE model produced basal IL-18 levels that were consistently > 10x higher than the basal levels observed with the epiCS® and MatTek EpiDerm™ models. This did not significantly affect stimulation in IL-18 levels triggered by the vehicle controls (< 2x). Considerable variation (< 5x) in cell viability and IL-18 stimulation was observed after exposure to sensitizers. These variations correlate with the RHE models and the ELISA used to determine IL-18 levels. Statistical analysis of the data obtained for the five test substances tested under the different experimental conditions has resulted in a acceptance criteria (90% Confidence Interval) check list that is incorporate in the updated protocol of the RHE IL-18 potency test for skin sensitization.

Conclusion: This study has demonstrated that the RHE IL-18 potency test for skin sensitization is easily transferred from the test developer to a naive laboratory. It also identified critical issues that need to be controlled if quantitative data have to be compared (e.g. for relative potency determination). The consortium is currently preparing an extensive validation of the updated protocol. The planned validation study compares the three tissue models for reliability, predictive capacity and limitations in terms of chemical applicability domain.

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Differentiated human skin-derived stem cells as a novel cell source for *in vitro* screening of drug-induced liver injury (DILI)

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Drug-induced liver injury (DILI) is one of the major causes of preclinical drug withdrawal. Recent advances in stem cell technology made it possible to create realistic human-based alternatives for replacing the currently used *in vitro* screening models for DILI, mostly consisting of rodent/human primary hepatocytes and hepatic cell lines of cancerous origin. Stem cells represent a virtually inexhaustible cell source and have the ability to differentiate in multiple cell types. In this study, human skin-derived precursors are evaluated as an alternative cell source for the *in vitro* prediction of DILI. These cells can be easily isolated from human (fore)skin. Using the previously developed sequential differentiation protocol that mimicks liver embryogenesis, they have the capacity to differentiate into hepatocyte-like cells. When exposed to acetaminophen, a compound well-known for inducing acute liver failure in patients, cell responses at the mRNA and functional level show similar hepatotoxicity characteristics as observed in patients and are even more pronounced than in exposed primary human hepatocytes in culture. Furthermore, it could be shown that exposure of the skin-derived hepatic cells to the steatotic compounds sodium valproate and amiodarone induced the production of microvesicular lipid droplets and the enrichment of genes typical for hepatic steatosis. The relevance of these *in vitro* responses was confirmed by comparing the results to the gene expression profiles of liver samples of patients suffering from steatosis. In conclusion, these results provide a first indication that human skin-derived precursors, differentiated to hepatocyte-like cells, may represent a suitable human-relevant preclinical model for *in vitro* testing of hepatotoxic compounds, mitigating as such the necessity of using animal tissues and scarcely available human hepatocytes.

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New tools to assist in the application of the read-across approach

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Read-across is one of the alternative methods most commonly used by registrants under the framework of the REACH regulation to avoid experimental testing [1]. While QSAR models usually involve the use of a number of chemicals, in the read across approach the estimation of properties might be based solely on a reduced number (even a single one) of similar compounds. With this approach, the definition of similarity is not only a structural one but might be related to other characteristics of the compound (e.g. common degradation products, common mode of action, etc ...). Since the metrics to identify similar compounds may be different one possible concern is related to the selection bias that can be applied in choosing the proper analogues. This is for instance one piece of information evaluated in the Read-Across Assessment Framework (RAAF) [2] to establish the reliability of a read-across approach applied to fulfil a REACH compliant property. To help in overcoming this issue, we recently developed a new tool to assist in the read-across procedure called ToxRead [3], freely downloadable from the web (http://www.toxgare.eu). In this software compounds similar to the target (from a structural point of view) are displayed together with relevant features associated to the property under evaluation. So far the software deals with two properties: mutagenicity (in terms of bacterial reverse test) and bioconcentration factor in fish. Within a LIFE funded project called CALEIDOS (http://www.caleidos-life.eu) we proposed an exercise involving about 50 participants to assess the variability of the outcome of the read across approach on a small group of compounds for three endpoints (mutagenicity in Ames test, BCF and fish acute toxicity). We will present some examples related to analysis of the similar compounds selected by the different participants to analyse the selection bias above mentioned.

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Holistic approach to Risk Assessment of chemicals for REACH purposes

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REACH Regulation (Reg. 1907/2006) was the first act to ask for full risk characterisation of all marketed chemicals, which are approximately 80,000. The effort that is mandatory to Companies to accomplish to the REACH requirements is very challenging. In fact, all substances must be registered by submitting to ECHA, the European Chemical Agency, a dossier containing full physical chemical characterisation toxicological and ecotoxicological profile, environmental fate and risk management measures for safe use. The need for toxicological tests is increasing with the tonnage band and includes demanding tests like carcinogenicity and repeated dose toxicity tests for substances manufactured or imported in quantity > 1000 t/y. When REACH was published, it was estimated that millions of animals would have been sacrificed if the provisions were applied literally [1]. Thankfully that was not the case. In fact, REACH was the first Regulation which officially accepted non standard methods, including testing strategies with non-validated methods, opportunities for read across and grouping, computer modeling, and so on. Last but not least, the toxicological and ecotoxicological assessment is modulated according to the real exposure to humans or the environment and long term tests can be waived if it is possible to demonstrate that the risk is fully controlled. This is a great opportunity that should be fully exploited with the support of a rigorous scientific approach. A proper holistic approach to the problem may have several advantages. Risk assessment should be performed by considering the chemical properties globally. The proper toxicological profile starts from the chemical analysis of the substance that is necessary to find similarities with other molecules and to start a chemical reactivity prediction with the aid of a computer modeling system. Analysis of the physical chemical properties is necessary to understand the possible distribution of the substance and to support Quantitative Substance Activity Relationship (SAR). Gathering all existing information, including historical data of worker exposure, will contribute to depict the preliminary biological behavior of the substance. Most of the time, this is not enough to get to a conclusion but sufficient to build an *in vitro* testing strategies that can back the assessment. The initial information is helpful also to select the test with the most suitable applicability domain and to reduce the cost for the new experiments. Eliminating the requirement for new *in vivo* tests is not always feasible, even though many time possible, in particular in the area for skin/eye irritation and skin sensitisation. For other toxicological assays, *in vitro* tests represent a valid technique to justify the read across and grouping strategies. REACH was the first Regulation which officially accepted non standard methods. The guidelines for testing strategies are now followed by other regulations in the EU, like that one on biocides or plant protection products. Outside the EU, something similar is accepted in most of the OECD countries.

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MIEs – the gateways to pathway prediction

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Consumer and environmental safety decisions are based on exposure and hazard data interpreted using risk assessment approaches. The Adverse Outcome Pathway (AOP) conceptual framework [1] has been presented as a logical sequence of events and processes within biological systems which can be used to understand adverse effects and refine the current risk assessment practice. Through this framework, current risk assessment practice can be refined through developing sound scientific and mechanistic understanding, shifting focus away from traditional toxicological apical endpoints to an increased understanding of a chemical's interactions and effects at a molecular level. A critical step in the framework is the molecular initiating event (MIE) which is defined as the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway [2]. Fundamentally, MIEs can be considered as molecular interactions occurring in a dynamic and complex matrix system [3] and as such the knowledge developed in one species can potentially be applied to other species in a unified risk assessment approach (e.g. human to species of environmental significance and vice-versa). MIE knowledge will enable prediction of the subsequent biological pathways which may be initiated or perturbed by an exogenous chemical. Identifying potential adverse pathways initiated by chemicals can be achieved by using the MIE as the anchor. Identification of key MIEs can be considered from either an exposure or chemical driven approach, or alternatively via the biological signature created by the effect of the chemical. Using either tactic, we are able to gain confidence in the presence or absence of off-target effects through predictive chemistry, informatics and *in vitro* screening. Subsequently, the MIE can also be used as a focus for more investigative studies to characterise perturbed pathways in a quantitative sense for safety risk assessment decision making. The challenges and opportunities of MIE led mechanistic safety assessments will be discussed.

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Six organ chip for substance toxicity testing using Homunculus platform

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Homunculus is a multi-organ, toxicity testing platform designed for use in pharmaceutical industry, ecology monitoring and in the personalized medicine. Homunculus platform consists of a flexible, microfluidic control unit suitable for long-term non-stop investigations or acute studies and a cell chip. Basic concept is co-cultivating different types of cells, representing human organs, in closed environment with culture media circulation. Here we report development of six-organ biochip with multiple, separate cell compartments, each the size of a standard well from a 96-well plate. Cell compartments are connected with channels and culture media perfused using on chip micro pump. Media exchange and test substance addition could be done automatic without opening the circuit. Used culture media is stored in separate glass tube and different metabolomics and proteomics methods could be used. Viability and functional activity of cells could be monitored online during cultivation and different end-point analysis method is established to evaluate toxicity of the substance. Using a "Human-on-chip" approach, Homunculus gives researchers the unique opportunity to investigate human cell models responses *in vitro* for development of new testing strategies.

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Reconstructed human skin pioneers the implementation of diversity into preclinical testing *in vitro*

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Several OECD approved methods for toxicological testing utilize reconstructed human epidermis and reconstructed human skin. In addition, reconstructed organs may be used for preclinical testing of new drugs as well as in fundamental research. Given that these organotypic models prove their disease relevance and reflect diversity factors like age and sex, the generation of highly valid data for human patients will become possible. Here we describe approaches to generate models for aged skin (AS), atopic dermatitis (AD), and cutaneous squamous cell carcinoma (cSCC). Normal human keratinocytes and human fibroblasts, either isolated from juvenile prepuce or from outer root sheath cells of the hair follicle, depict the backbone of all models. The AS models use UV-induced senescence in keratinocytes or fibroblasts from donors older than 60 years, respectively. UV-induced ageing causes the expression of the senescence marker ß-galactosidase, alters epidermal morphology, and increases the permeability for OECD standard compounds three-fold and of nanoparticles up to ten-fold. The AD model uses filaggrin knockdown keratinocytes and the exposure to Th2 cytokines. Here we observe the features of atopic dermatitis, e.g. altered epidermal morphology because of deviant differentiation, disturbed order of the stratum corneum lipids, and enhanced sensitivity for irritants. cSCC models generated by seeding tumour cells onto the developing epidermal compartment of the full-thickness model emulates hallmarks of the *in vivo* tumour morphology. The cSCC impairs the skin barrier of the organotypic model as demonstrated by enhanced permeability for caffeine and nanoparticles. Latest Fluorescence Lifetime Imaging Microscopy measurements proved even an accumulation of the nanoparticles in the tumour nests. Moreover, the organotypic cSCC model shows the clinical effects of ingenol mebutate. Taken together, the reconstruction of diseased human skin – and possibly other organs, too – bears not only a huge potential for the replacement of animal studies but paves the way to diversity implementation. This allows a much deeper insight into the relevance of various impact factors than provided by animal studies and may reduce the failure rate of investigational new drugs in clinical trials [1].

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The Berlin-Brandenburg Research Platform BB3R and integrated graduate school

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Funded by the German Government the Berlin-Brandenburg Research Platform BB3R with integrated graduate education has started in April 2014. Joint research of scientists from FU Berlin, Potsdam University, Charité Berlin, TU Berlin, BfR, and Zuse-Institute Berlin focuses on gaining substantial progress in the fields of alternative and humane testing and in strengthening the national 3R expertise. BB3R aims to accomplish the following goals:

- Establishment of alternative methods for preclinical drug development and basic research facilitation of research collaborations and sustainable research activities in the region Berlin-Brandenburg. In addition, cooperation with national and international partners is built.
- For the expansion of regional research activities three junior research groups are established, successful candidates will be qualified for management positions in professional areas related to the 3Rs
- Sustainable establishment of the BB3R graduate school for structured training of graduate students who complete a specific mandatory course program on alternative test methods to animal experimentation and related fields in addition to the research project
- Creation of a pool of 3R experts for advice and assistance
- Increasing the awareness of the society for 3R-related issues
- The research platform BB3R along with the associated graduate school will close substantial knowledge gaps in the fields of 3Rs and alternatives to animal experimentation in the years to come.

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Strategies for combining skin and vasculature in a multi-organ-chip platform

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Human skin is a complex tissue composed of various types of cells. Most skin equivalents currently commercially available are based on static culture systems emulating human epidermis or a combination of epidermis and dermis. None of the existing systems contain important elements, such as vasculature, skin appendices or an immune system [1]. However, *in vitro* tests ultimately aiming for the replacement of animal models in drug development require an almost perfect fit with the human physiological microenvironment. In particular, the presence of a vasculature is a prerequisite for sufficient supplementation of the skin and it's appendices with required nutrients and oxygen. For our multi-organ-chip (MOC) platform we, thus, envisage to model a microvasculature-driven cutaneous homeostasis of our skin equivalents [2]. In this work, we combine skin equivalents with vasculature in the two-organ variant (2OC). The 2OC's circuit contains only 600 μl of medium. A built-in micropump provides a constant pulsatile flow and ensures oxygen and nutrient supply. Thus, no external reservoirs need to be attached that would otherwise dilute the enriched medium. Human dermal microvascular endothelial cells (HDMECs), isolated from human foreskin, were injected into the microfluidic channel system. After even cell distribution inside the circuit, the device was incubated at 37°C under static conditions for 3 h to allow the cells to attach to the channel walls. A continuous pulsatile flow was applied that provided an optimal shear environment. After 3 days of mono-cultivation, the skin equivalent was added for another 7 to 14 days. Skin equivalents were build up prior to the MOC experiment under static conditions. Full thickness skin equivalents (ftSE) were constructed by growing keratinocytes on Matriderm® punches colonized with skin fibroblasts. FtSE developed *in vivo*-like epidermal architecture with regular differentiation and marker expression. Mediated by the shear stress the HDMECs demonstrated physiological-like elongation and orientation with the direction of flow. The cells remained viable over the co-cultivation period. The skin equivalents cultivated in the perfused 2OC system together with the endothelial cells showed remarkable consistency and vitality. Comparisons of specific markers under different cultivation conditions will be presented. Further, the current status of the development and remaining hurdles will be discussed. The results clearly demonstrate the capability of the MOC system as a useful tool for long-term co-culture and a potential platform to replace animal models.

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40 years of Animalfree Research. Results and lessons learned from a quality assessment

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Animalfree Research was founded in 1976 as a non-profit organization with the explicit goal to fund and enforce the development and implementation of 3R-relevant methods. In 2016, we are celebrating our 40th birthday, which we found a good opportunity to perform a critical evaluation of our achievements. At the time of presentation, many results are still preliminary. We feel though, that general insights from this work may be helpful for other 3R – Organizations who might be facing the challenge of justifying their activities to the public and politics. For example, we found that success in 3R-relevant work has to be defined in a fundamentally different way than it is usually done. Success is not only measured as scientific output (papers and citations), but even more vitally in improvements in animal welfare. Unlike purely scientific work, the challenge for 3R-relevant projects does not end at the moment of successful publication, but, to the contrary, impacts on animal welfare actually start to develop at this time point. To measure the impact on animal welfare (as opposed to scientific impact) quantitatively has proven impossible, for several reasons. Although this may appear disappointing at first sight, it is nevertheless a vital insight that "counting the saved mice" cannot be a demand imposed on institutions dedicated to the 3Rs. We nevertheless have identified personalized questionnaires as a very valuable approach to provide us with qualitative data on whether our work has contributed to laboratory animal welfare.

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Automation of production and quality control of a reconstructed human epidermis

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Changes in the European and worldwide regulations, ethical concerns and scientific reasons lead to a rising demand for alternatives to animal testing. Thus, there is a need for a cost-effective, reproducible bulk production of tissue models. However, the current manual production is not able to adequately supply the needed quantity of tissue equivalents. Based on that need, the Fraunhofer-Gesellschaft developed a production plant, called "Tissue Factory", which is able to produce reconstructed human epidermis (RHE) of constant high quality. An essential quality criterion of RHE is a high barrier function, which is determined by ET-50 tests. Currently, these assays are conducted manually, which is labor intensive and binds trained personnel. The aim of this study was the automation of production and quality control of RHE to increase reproducibility and accuracy of measurements and to reduce the error rate in manual processes. The RHE is comprised of primary human epidermal keratinocytes which differentiate to a multi-layered epidermis with a well-formed basal layer and a dense stratum corneum. After the manual cell extraction and expansion the "Tissue Factory" is able to do all subsequent steps fully automated. The barrier function of RHE was assessed by transferring the manual process of an ET-50 assay to a dual-arm robotic system. ET-50 assay was performed manually and automatically under same experimental conditions. After a topical application of Triton X-100 for 1.5, 3, 4.5 and 6 hours, viability was calculated for every exposure period. An exponential decay curve was determined for each process and ET-50 values were calculated from the respective regression equations. The production plant ensures a standardized, and reproducible manufacturing with a monthly output of up to 2,000-5,000 epidermal equivalents. To determine the quality of the automatically produced RHE histology, barrier function and viability between the manually and the automatically produced models were compared. The automatically produced RHE matched all quality criteria and no significant differences between the automatically and manually produced OS-REp were detectable. The ET-50 tests were performed simultaneously. For the manual conducted test, the ET-50 value was 5.58 hours and for the automated test 5.29 hours. To determine the discrepancy between data and regression equation, the residual sum of squares (RSS) was calculated. With a RSS value of 382.3 for the manual process, approximation of measured data by an exponential function was better than for the automated procedure (RSS: 1049.2). The results show that, the dual-arm robotic system can perform ET-50 tests. In addition, the automated process was established time and cost effectively as most process steps were conducted using standard lab equipment. This study demonstrates that a transfer of the manual to an automated production as well as the automated testing of the quality control was successful. Furthermore, dual-arm robotic systems are a promising tool for the automation of bioengineering processes, which is a vital perquisite for future high throughput testing. Especially in the field of *in vitro* testing employing tissue models, such systems have a great potential to automate downstream assays such as substance testing.

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Omnisphero: a novel computational approach for high content image analyses (HCA) of organoid neurosphere cultures *in vitro*

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Neurodevelopmental toxicants have shown to be a serious threat for society, clearly demonstrating the need for developmental neurotoxicity (DNT) testing for chemicals. *In vivo* testing according to the current guidelines is extremely time- and cost-intensive. Therefore, we established an alternative *in vitro* method, the neurosphere assay, as a screening tool for DNT. Neurospheres are three dimensional (3D) cell clusters consisting of neural progenitor cells (NPCs), which are able to mimic basic processes of brain development (proliferation, migration, differentiation and apoptosis). Safety and efficacy testing of chemicals with 3D neurospheres by employing high content image analyses (HCA) requires advanced scanning and evaluation processes which is due to characteristic features of the culture: 1) A 3D sphere core, which leads to unfocused images, 2) a variable cell density within the migration area, 3) a heterogonous cell population of neurons and glia cells and 4) sphere-specific endpoints. Therefore, the goal of this project was to generate mathematical algorithms for HCA that are able to deal with these challenges. Our software called "Omnisphero" assesses radial migration, quantifies differentiated neurons, measures neurite outgrowth and assesses distance-dependent density distributions of neurons within the neurosphere migration zone. We implemented a new feature in "Omnisphero", in which the user defines a neuron by manual assignment rather than by defining an object over parameter settings. In contrast to laborious parameter settings, this feature facilitates rapid and accurate image analyses. In an initial step the neurosphere-specific endpoint "radial cell migration" is assessed using the Array Scan technology identified coordinates of all Hoechst stained cell nuclei to identify the sphere core as a single object and determines migration distance as an average of the distances between the rim of the sphere core and all nuclei with the furthest migration distances in all directions. Subsequently, neuronal differentiation is evaluated within this migration area using the user defined coordinates to assess the detection power (DP, 80-85%) and false positive (FP, 10-15%)-rate of its own algorithms outperforming foreign automated evaluations (Thermo Scientific DP: 50%, FP-rate: 40%). This low DP and high FP rate in evaluating differentiated neurospheres is due to the application domain of the "Neuronal Profiling", which was initially designed for pure neuronal cultures with low cell densities. Additionally, preliminary results indicate an improved quality for the assessment of neurite outgrowth and branching points using "Omnisphero". Another self-written algorithm quantifies positioning of individual cell types within the identified migration area leading to a distance-dependent density distribution. For analyses of neural migration also *in vivo*, we developed another software called "BrdeLuxe" to assess positioning of BrdU (Bromodeoxyuridine)-labeled cells in cortical brain slices. These techniques provide the opportunity to compare *in vitro* and *in vivo* results for effects on cell migration by using the Array Scan VTI. In conclusion, HCA of neurospheres is a promising technique for medium throughput screening to be used in safety and efficacy testing in the future – with additional features also for *in vivo* evaluations.

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A novel assay for evaluating wound healing in a full-thickness *in vitro* human skin model

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Cutaneous wound healing involves interactions between dermal fibroblasts and epidermal keratinocytes as well as cell-extracellular matrix interactions. The current study describes wound healing experiments conducted in a full thickness *in vitro* human skin model (EpiDermFT). This model exhibits stratified epidermal components and a fully developed basement membrane and resembles *in vivo* skin in regard to both morphology and barrier function. Small epidermal only wounds were induced in the model using a 3 mm punch biopsy and subsequently evaluated at various recovery time points by two methods. Historically, EpiDermFT has been used to evaluate re-epithelization of the wound by: a) manually bisecting the tissues through the center of the wound, b) staining with hematoxylin and eosin, and c) quantifying migration from the wound origin. Accurate bisection of the wound is difficult and often leads to variability in assay results. Here we describe a novel method of visualizing wound re-epithelization *in situ* simplifying analysis and reducing introduction of variables inherent in tissue processing that can confound data. Following wounding, tissues were fixed and immunostained with markers of epidermal differentiation as well as a marker of fibroblasts allowing simultaneous visualization of migrating keratinocytes (keratin 14), differentiated suprabasal cells (involucrin), and dermal fibroblasts (vimentin) within the wound. Histological and immunohistochemical analysis showed keratinocyte migration at 2 days following wounding. In both methods, wounded tissues cultured without growth factors (2% human serum) had a reduced healing rate in which keratinocytes did not cover the entire wound within a 6 day timeframe. In contrast, wounded tissues cultured with growth factors demonstrated a dramatic increase in healing rate as keratinocyte migration completely covered the wounded area by day 6. In conclusion, this novel method of evaluating re-epithelization by utilizing immunohistochemical markers of differentiation is a quicker and more reproducible method of analyzing wound healing.

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An *in vitro* reconstructed psoriasis tissue model for evaluation of drug therapeutics

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Psoriasis is an inflammatory skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation affecting 1-3% of the global population. Availability of an *in vitro* psoriatic tissue model will facilitate drug discovery. In the current study, normal human primary keratinocytes and psoriatic fibroblasts were harvested and cultured to form a highly differentiated 3D tissue model. The tissue model was characterized for histological features, gene expression, and cytokine release patterns that are associated with psoriatic phenotype. Results showed that psoriatic fibroblasts can induce psoriatic phenotype *in vitro* with overexpression of HBD-2, psoriasin, elafin, and ENA-78, similar to the *in vivo* situation. Cytokine analysis showed increased release of IL-6 (7 fold), IL-8 (5.5 fold), and GRO-α (3.8 fold) compared to control tissues. Confocal microscopic evaluation revealed: 1) hyperproliferation of basal epithelial cells (Ki67 staining), 2) increased psoriasin, elafin, and CK16, and 3) reduced levels of filaggrin. Topical treatment of the tissue model with three over-the-counter psoriatic drugs decreased HBD-2, psoriasin, elafin, and ENA-78 gene expression. In conclusion, the *in vitro* psoriatic tissue model is anticipated to be a valuable tool to accelerate safety and efficacy studies of candidate therapeutics.

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A human *ex vivo* model to investigate cigarette smoke-induced lung tissue damage

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Background: Various *in vitro* models using e.g. immortalized cell lines as well as *in vivo* animal models mimicking hallmarks of lung diseases are commonly used in basic respiratory research. Their main objectives are the elucidation of disease pathomechanisms and the testing of novel therapeutic approaches that cannot directly be assessed in patients. In order to bridge the gap between these *in vitro* / *in vivo* models and the human situation, proof-of-concept studies on primary human biomaterial are necessary. Based on this, an *ex vivo* organ model was used to investigate the toxic aspects of cigarette smoke-induced tissue damage occurring in chronic obstructive pulmonary disease (COPD).

Methods: Lung tissue specimens were obtained from surgical materials of patients, who underwent pneumectomy or lobectomy due to cancer at the LungenClinic Grosshansdorf, Germany. All procedures were performed according to permission of the local ethics committee at the University of Lübeck (Approval number: 15-163A). Under gross morphologic examination, only parts of the lung tissue without inflammation, neoplasia, and pleura were selected and dissected with a size of 4 x 10 mm using a biopsy punch. Cigarette-smoke extract (CSE) was obtained using 3R4F research cigarettes by drawing smoke of one cigarette slowly through a water pump into a tube containing 10 mL of DMEM-F12 (= 10% CSE). Stimulation was performed under serum-free conditions. Tissue specimens were stimulated with increasing concentrations of CSE (0.1-10%) for either 18 h or 1 h followed by 17 hr CSE-free post-incubation. Subsequently, cytotoxicity (LDH), cytokine secretion (IL-6, IL-8), and protein content were measured in the supernatant. In order to preserve morphology and nucleic acids the HOPE® fixation technique was used.

Results: CSE exposure for 1 h followed by 17 hr post-incubation revealed no cytotoxic effect and no increase in IL-6 and IL-8 secretion in comparison to sham-treated lung tissue specimens. Contrary, a longer exposure period caused a strong IL-6 and IL-8 release, which was, however, absent at the highest CSE concentration. Moreover, 10% CSE for 18h induced a significant cytotoxic effect which might be causal for the lack of cytokine release.

Conclusion: The *ex vivo* model of human lung tissue represents a suitable approach to investigate cigarette smoke induced inflammatory effects. Further studies using inhibitors of carcinogenesis and inflammation are ongoing.

Funding: This work was funded by the German Center for Lung Research (DZL). The BioMaterialBank Nord is funded in part by the DZL, site Airway Research Center North (ARCN), and is member of popgen 2.0 network (P2N) which is supported by a grant from the German Ministry for Education and Research (01EY1103).

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Beyond Europe: accelerating regulatory uptake and use of 3R best practices globally

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There is general agreement among regulators and companies that where replacement alternatives are available, testing on animals is scientifically unnecessary and ethically indefensible. Yet despite the ever-expanding toolbox of validated and internationally codified *in vitro* test guidelines, *in silico* models, and non-testing strategies, regulatory data requirements in many countries and product sectors are falling increasingly out of date with these recognized 3R best practices. Even in the EU, there is as yet no formal process for ensuring timely updating of regulations to keep pace with scientific progress, or to lay the science-policy groundwork for the "Tox21" paradigm shift already under way. Humane Society International, through its expansive network of regional offices, works in cooperation with regulators and industry, sector by sector and country by country, toward timely updating of toxicological data requirements and assessment approaches to minimize animal use and optimize regulatory alignment and science-based decision making. This lecture will explore progress being made toward stakeholder education, training, and ultimately adoption of 3R best practices in Brazil, China, India, South Korea and beyond.

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Use of *ex vivo* organotypic lung tissue in translational research of respiratory injury and inflammation diseases

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Translational science has remarkably changed the general perception of 3D organotypic tissue models such as precision-cut lung slices (PCLS), parenchymal strips, isolated vessels and airways. These models are considered to be of importance since the cellular anatomy of the respiratory tract comprises many extensively varying cell types with different functions. Moreover, according to the 3R concept, more and more 3D models are used in tiered approaches – testing first *in vitro* and *ex vivo* before first *in vivo* – which provides supporting data early in drug development. Precision-cut lung slices for example are now widely used for basic research and pre-clinical efficacy and toxicity testing of (biological) compounds. Such tissue sections of the lung contain for example epithelial cells, fibroblast, smooth muscle cells, nerve fibres, and immune cells such as antigen-presenting cells and T-Cells. Cells are still viable and interact with each other, by this reflecting the highly specialized function of the lung. In the context of our mechanistic understanding of airway biology and pathology it is important to use appropriate *in vitro* models with highest impact on the real life situation. By exploring these mechanisms in advanced tissue and cell culture models with chemical and biological agents such as pharmaceuticals, immune active proteins, antibodies, cytokines, and drugs we take into account that individual cell types may respond differently to the same mediator, drug or substance. Therefore, lung tissue is exposed *ex vivo* to these substances and examined for phenotyping of cellular changes, respiratory toxicity, broncho- and vasoconstriction and -dilation, immune responses, and tumor invasion. By this, different features of respiratory diseases such as acute lung injury, asthma, COPD, fibrosis, infection and tumor can be investigated – by using tissue of different species, including human. We found that the tissue response is highly comparable with the *in vivo* response and can be used for the prediction of toxicological endpoints and adverse health outcomes such as organ injury, respiratory sensitization and inflammation. The presentation will give an overview about the current use of lung tissue in inhalation toxicology but also state their use for drug research.

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Micellar liquid chromatography as an alternative for *in vivo* and expensive *in vitro* tests commonly used in prediction of human intestinal absorption

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Oral administration is one of the most important and abundant routes of drug administration, therefore estimation of intestinal absorption is crucial for pharmaceutical compounds, especially for drugs with poor solubility [1]. That is why it is important to determine the extent of drug absorption for new drug entities (NDE) in preformulation studies. This study proposes a novel, simple, economic, accurate and precise way to predict intestinal absorption using a modified chromatographic method. Introduction: Animal testing is the standard method currently used to predict the extent and rate of intestinal absorption [2,3], i.e. through *in vivo* testing, for pharmaceutical compounds. In this study a form of chromatography, known as MLC, has been applied to predict passive intestinal absorption with a selection of model compounds through measurement and calculation of the partition coefficient, Pmw. In this method bile salts were used as a mobile phase (instead of the more standard head and tail surfactants) to provide an environment more closely simulating the human intestinal environment. Methods and Reagents: Two bile salts were considered separately, namely sodium deoxycholate (NaDC) and sodium taurodeoxycholate (NaTDC) no buffers or organic modifiers were added during the study to simulate intestinal conditions as closely as possible. Low concentrations of 13 model drugs were prepared in the corresponding mobile phase concentration to avoid saturation of the bile salt mobile phase. The samples were injected into the chromatographic system with capacity factors obtained by analysis of the retention data recorded for the model drugs used. Modelling of Human intestinal absorption (% HIA), intestinal permeability of Caco-2 and PAMPA was performed by multiple linear regression. The models obtained confirmed the ability of MLC to predict human intestinal absorption (% HIA), Caco-2 and PAMPA intestinal permeability coefficients. Conclusion: The use of NaTDC in MLC was found to be a better method in the prediction of Human intestinal absorption while NaDC was found to be better for prediction of PAMPA and Caco-2 intestinal permeability coefficients although both bile salts provided a superior prediction for both the *in vivo* (% HIA) and *in vitro* (Caco-2 and PAMPA) methods for intestinal absorption determination compared with current methods. Therefore, it was concluded that the MLC method was found to be a successful tool in prediction of human intestinal absorption (% HIA), Caco-2 and PAMPA intestinal permeability. Since MLC is a more economic [4], time saving [5], eco-friendly [6] method that does not include the use of animals, it can be considered to be a superior method over all available alternative options for prediction of intestinal absorption.

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Training as a tool to implement of 3R in India

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The use of live animals in research from an ethical point of view has been debated amongst researchers and animal welfare societies. The use of live animals in research facilities needs adequate hands on training and competent skills for both scientists and technical staff [1]. This is well known and recognized among researchers and veterinarians because of its importance for maintaining the quality of scientific results and for safeguarding the welfare of the animals used. Current Indian law is the Prevention of Cruelty to Animal Act [2] which is a safeguard for animals used in research as well for other captive animals in the country. The use of animals from an ethical point of view in research facilities requires a certain level of competence which is not usually defined by laws and regulations. Hence, a program like a Course on Laboratory Animal Science is a step forward to train, teach and prepare scientists and technical staff to carry out research using laboratory animals in a responsible way. In order to facilitate such training program, a FELASA Category "C" based course was organized in collaboration with Utrecht University, The Netherlands, funded by Laboratory Animals ltd., U.K and the local Indian governing authority CPCSEA (Committee for the Purpose of Control and Supervision of Experiments of Animals). In India, presently there are limited training courses in laboratory animal science (e.g. in South India, organized by TANUVAS- Tamil Nadu Veterinary and Animal Sciences University) for a number of reasons, including differences in the animal welfare law/regulations implementation at research level. In order to establish a course implementing 3R's [3] in laboratory animal science, CSIR- Institute of Genomics and Integrative Biology has taken the lead in this process. At CSIR-Institute of Genomics and Integrative Biology, this course was established for most of the PhD participants and staff working or going to work in future with animals as they have to complete this course and a written exam in order to work independently and competently with experimental animals. The overall aim of this course is to help and guide the participants and staff in their approach towards use of animals with greater knowledge and insight so that welfare will also be a point of consideration within their experiments. For that reason it is necessary to gain knowledge and experience on their own under the supervision of skilled professionals. The methods of teaching and practical training used during the program are interactive lectures with videos and discussions for better understanding, problem-solving exercises in small groups such as a critical review of an article on animal based research and designing an animal experiment protocol based on the gained knowledge, during this two weeks course, demonstrations of experimental techniques and hands on practical's, with most commonly used species like mice, rats, rabbits and guinea pigs, hamsters. The primary aim was to determine whether the participants' attitude towards the use of animals in experimental work has been changed and whether the acquired knowledge would be beneficial for their future experiments.

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Do scientists have an adequate overview of global 3R resources?

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The 3R concept is now accepted worldwide as a means of improving laboratory animal welfare, often with the added bonus of producing better research data. Many countries and regions such as the EU require in their legislation that the 3Rs are addressed. Although the 3R concept was launched in the 1950s, it took over 20 years before the scientific community made concerted efforts to ensure their use. This period coincided with a similar rapid evolution within the area of Laboratory Animal Science. Although we now have vastly more knowledge about animal welfare and 3R-alternatives, these subjects have still by no means been fully explored. There is an enormous need for further research in the area, coupled with dissemination of 3R-knowledge to ensure best possible practice. This need for communication is hindered in part by a general lack of crosstalk between scientific disciplines: relatively few scientists participate in meetings outside their own specific areas of work. Those using 3R-techniques should also facilitate information retrieval by highlighting them in their publications, which improves the chances of relevant indexing by database providers. It is therefore of vital importance for implementation of the 3Rs that the latest research findings and current best practice within laboratory animal science and welfare are readily available to all scientists. This includes those performing *in vitro* studies, who are indirectly responsible for the welfare of those animals from which material is harvested. The efforts which are being made worldwide to improve global cooperation and dissemination of 3R information will be discussed. The latest databases and collections of 3R resources will be described. Areas in which improvements can still be made will be highlighted.

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Scientific and animal welfare issues surrounding the new CRISPR/Cas-9 approach to produce transgenic animals and advanced *in vitro* disease models

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While the new CRISPR/Cas-9 technology reduces the level of animal wastage resulting from the production of each new strain, any long-term contribution to reduction will be offset by the overall increase in the numbers of transgenic animals likely to result from its widespread usage. Likewise, the contribution to refinement of using a more-precise technique, thereby minimizing the occurrence of unwanted genetic effects, will be countered by a probable substantial increase in the production of transgenic strains of increasingly sentient species. Therefore regulatory and legislative bodies should approve GM studies only in extremely exceptional circumstances. In the meantime, we urge that further consideration be given to monitoring and exploring in greater detail, the welfare consequences for all species of every available method of transgenesis, and to the wide application of the most promising of these, such as CRISPR/Cas-9, for increasing the diversity of cell lines for use in *in vitro* studies. Recent examples serve to illustrate some of the many pitfalls and complexities in developing and using transgenic animals as disease models, and they stress the need to consider more than just the coding genome as a target for transgenesis. In addition, where the involvement of regulatory genes has been taken into account, it is always possible that successful therapeutic interventions in the respective mice may not work in the clinic, due to species differences in regulatory control mechanisms, although the general consensus is that regulatory gene mechanisms show a high degree of conservation. The time is surely ripe for the pharmaceutical industry and the major funding bodies to re-assess their "obsession" with genetically modifying animals, by holding an inquest into the reasons for the slow progress in developing effective drugs against major diseases that still cause suffering directly to many people each year, and indirectly, to many thousands of laboratory animals. In the meantime, the use of CRISPR/Cas-9 to generate new transgenic cells in culture has to be encouraged and funded both for scientific and animal welfare reasons.

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CRISPR/Cas-induced structural variants

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Structural variations (SVs) contribute to the variability of our genome and are often associated with genetic disease. The study of SVs in model systems was hampered until now by labor-intensive genetic targeting procedures and multiple mouse crossing steps that could take years. We present the use of CRISPR/Cas for the fast (10 weeks) and efficient generation of SVs in mice. We specifically produced deletions, inversions, and also duplications at different genomic loci ranging from 1.1 kb to 1.6 Mb with efficiencies up to 42%. After PCR-based selection, clones were successfully used to create mice via aggregation. To test the practicability of the method, we reproduced a human 500 kb disease- associated deletion and were able to recapitulate the human phenotype in mice. Furthermore, we evaluated the regulatory potential of a large genomic interval by deleting a 1.5 Mb fragment. The method presented permits rapid *in vivo* modeling of genomic rearrangements and opens new possibilities to study the role of regulatory sequences of the genome. The possibility to manipulate and characterize ES cell *in vitro* with CRISPR/Cas and then create mice via ES cell aggregation makes extensive breeding unnecessary and thereby reduced animal numbers.

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Modeling Parkinson's disease using forward and reverse genetics in human iPS cells

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Traditionally, human genetics and disease modeling was based on indirect studies in mice as mammalian model. The mouse model provides a powerful tool to conclude on genotype/phenotype relations, since it was possible only in mouse embryonic stem cells to specifically introduce new genotypes. This paradigm has been changed with the invention of new techniques, such as somatic cell reprogramming and the use of designer nucleases, such as the CRISPR/Cas9 system. The reprogramming technology enables to derive induced pluripotent stem (iPS) cells from somatic cells of healthy subjects and patients suffering from any disease [1] and [2]. iPS cells are able be further differentiated into any cell type of interest. The CRISPR/Cas9 system is a simple and powerful tool to manipulate genomes of any organisms. Using this technology, specific DNA double-strand breaks (DSB) can be induced at any locus of interest. DSB are processed by endogenous DNA repair mechanisms either by the nonhomologous end-joining (NHEJ) pathway or by homology directed repair (HDR). NHEJ causes small insertions or deletions that can be used to generate loss-of-function mutations. The HDR pathway allows engineering targeted knock-ins, for example of precise point mutations or of reporter genes. By combining somatic cell reprogramming with designer nuclease technologies, such as the CRISPR/Cas9 system, human genotype/phenotype relations can now be investigated in human iPS cells or differentiated cell types, derived from iPS cells. Thus, gene engineering in human iPS cells enables researchers to model complex human diseases in a human system. Our group focuses on the modeling of Parkinson's disease (PD). PD is a neurodegenerative disease, characterized by the loss of neurons in the central nervous system, particularly the dopaminergic neurons of the substantia nigra. The CRISPR/Cas9 system allows us on the one hand, to introduce one or even multiple risk alleles (gene knock-out or knock-in of single base pair mutations) into iPS cells with healthy genetic backgrounds. On the other hand, we pursue gene correction in iPS cells derived from familial Parkinson's patient's fibroblasts. Furthermore, the nuclease technology can be used to engineer reporter lines. We already successfully generated tyrosine hydroxylase (TH) reporter cell lines, assisting us to easily monitor the differentiation of iPS cells to dopaminergic neurons. Furthermore we aim to generate reporter cell lines that facilitate the analysis of the genotype/phenotype relations. Although restricted to the level of cellular analysis, this system enables for the first time to bridge the gap between experimental mouse models and the simple, noninvasive observation of primary human cells.

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Improving animal welfare through competence: the EUPRIM-Net education and training programs

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Advancement of scientific knowledge through biological and biomedical research is critical for our understanding of human and animal physiology and consequently for medical progress. Where no alternatives exist, this research necessitates the use of non-human primates (NHPs) currently and for the foreseeable future. The EU-funded research infrastructure project EUPRIM-Net brings together the unique facilities and solid experience of ten European publicly-funded primate centers, in order to meet the highest ethical standards in using these sentient animals while ensuring top-level research. To ensure that NHPs receive adequate care and are handled correctly, all personnel involved in the work with primates have to receive appropriate education and training. Personnel should have a sound understanding of primate biology, their needs and requirements and knowledge in best practice in care, husbandry and handling procedures. In an effort to pool essential knowledge, state-of-the-art techniques and newest developments and to disseminate it to all staff working with NHPs, EUPRIM-Net has developed a range of education and training programs for a broad international auditorium. The EUPRIM-Net courses aim on education, training and the professional development of scientific as well as husbandry personnel, but also students or veterinaries involved in the work with primates (http://www.euprim-net.eu/network/courses.htm). Accordingly, the courses cover a great variety of topics, including general primate biology and behavior, medical aspects, husbandry and ethics as well as interpersonal skills to communicate state-of-the-art primate-based research. More recently a new course series has been developed which topics and content are more strongly linked to the most recent FELASA education guideline and their specific learning outcomes. This course series provides a standardized fundamental education and training and aims in particular on an audience, which is less experienced in the work with NHPs. To ensure the best and most effective learning progress topics and content have been separated into an e-learning component and an on-site component. While the e-learning component covers the important theoretical frameworks (e.g. legal aspects), the on-site component focuses on more practical based content. Besides the different EUPRIM-Net courses, a seminar group has been formed, consisting of veterinarians, ethologists, and animal trainers (http://www.euprim-net.eu/network/ABM-seminars.html). Together, they have created eight lectures on topics regarding Animal Behaviour Management (ABM), ranging from breeding laboratory primates to environmental enrichment and positive reinforcement training. They offer to visit primate facilities and present the lectures with the option to customize lectures and provide on-site consultation and guidance according to the needs of the facility. Animal welfare in the day-to-day routine depends strongly on the knowledge of the people who work with the animals. The EUPRIM-Net education programs provide this knowledge to people working with primates, helping to ensure both top-level research and state-of-the-art animal welfare.

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VPA Read across: development of predictive biomarkers by using toxicity data of structurally similar compounds

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The common model for safety evaluation/human health risk assessment is currently repeated dose toxicity (RDT) testing in rodents. RDT studies require numerous animals and the capacity for this conventional testing is limited. The development of alternative toxicity tests is, thus, of great interest. However, this goal remains challenging as complex *in vivo* processes like absorption, distribution, metabolism, excretion (ADME) and different mechanisms of toxicity need to be addressed by a network of reliable test systems. In this project we evaluated -omics readouts to identify predictive biomarkers by using a read across approach. Main goal is the prediction of the type of toxicity, and the toxicological potency, the concentrations of a compound at which adverse effects *in vivo* are observed. Preliminary experiments with data base selected biomarkers in HepG2 cells and primary human hepatocytes demonstrate that, with a few exceptions, for most of the compounds it was possible to predict blood concentrations in humans which are associated with hepatotoxic effects. The current study focusses on the identification of biomarkers of toxicity in rat hepatocytes and on their capacity to predict toxicity for structurally similar compounds. Model compound for the study is valproic acid (VPA), a drug which induces microvesicular steatosis in liver in human patients at therapeutic doses. VPA also induced lipidosis, lipid accumulation or vacuolization of hepatocytes in *in vivo* RDT studies in rodents. Gene array data from *in vitro* experiments in rat and human hepatocytes and from rat *in vivo* studies demonstrate its strong effect on expression changes especially on genes involved in energy and lipid metabolism. Based on the most frequently deregulated genes we selected 11 candidate biomarker genes representing biological functions such as metabolism of xenobiotics, deregulation of energy and lipid metabolism, cellular stress response, transport, cell cycle, structure dynamics and migration and development and differentiation. These candidate biomarker genes were tested in the context of a read across approach. Six branched and unbranched carboxylic acids were identified showing a high structural similarity to the lead compound VPR. RDT studies were available for all six structural analogues e.g. from the RepDose database (http://www.fraunhofer-repdose.de). Three compounds, namely 2-ethyl hexanoic aid, 2-ethylhexanol and dioctyl adipate cause steatotic changes *in vivo* and are therefore classified as "active". Hexanoic acid, propionic acid as well as 2-ethylbutyric acid were found to be negative *in vivo* up to the highest tested doses and are classified as "inactive". In *in vitro* experiments, five different concentrations and untreated controls were tested for all seven compounds and the expression of the selected biomarkers was analyzed. Their ability to qualitatively/quantitatively predict steatosis and/or liver toxicity is discussed. The comparison of biomarker expression after exposure of target cells to structurally analogues provides insight in how far toxicity data from well characterized chemicals might help to predict toxicity for structurally similar data poor compounds. Simultaneously, comparing the concentration at which biomarker expression is induced with concentrations where histopathological effects are observed *in vivo* can be used as a tool to predict the concentration at which toxicity occurs *in vivo*.

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Non-animal testing: it's within REACH

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When the EU passed the REACH legislation, this testing programme intended to ensure that animals were used only as a last resort. Yet, reports show that more than 800,000 animals have been used thus far in REACH tests [1], and millions more are expected to be used in the coming years. To minimise animal testing, REACH contains a number of specific measures and general provisions designed to establish and enforce the last resort principle. For example, non-testing methods, such as read-across, weight-of-evidence approaches, (Quantitative) Structure Activity Relationships ([Q]SARs), and non-animal testing methods, must be used wherever possible. Integrated Approaches to Testing and Assessment (IATA) provide opportunities for minimising the use of animals and to promote the efficient use of resources. Ideally, IATA should have a rational, knowledge-driven design that includes both *in vitro* and computational methods based on well-described Adverse Outcome Pathways (AOPs). Integration and interpretation of non-standard information generated for key events within an AOP can be used to provide an assessment of a toxicity endpoint, which is more predictive of the effects on human health than testing on animals. IATA are now available, or in development, for many of the REACH Annex VII and VIII endpoints, including skin sensitisation, and skin irritation and corrosion [2]. However, updates to the REACH Annexes and Test Method Regulation lag behind in the latest developments in toxicology assessment, resulting in animals being used in testing that is not required by law. Furthermore, the European Ombudsman has determined that the European Chemicals Agency (ECHA), the agency responsible for overseeing REACH, is not fully applying its authority to check that companies minimise animal experiments, as required by law, and should begin to do so [3]. Therefore, measures, separate and apart from regulatory oversight, must be taken to ensure that registrants understand how to use non-animal methods in order to satisfy REACH requirements. To assist registrants in preparing for the 2018 REACH registration deadline, the PETA International Science Consortium Ltd. (PISC) teamed up with the online news service Chemical Watch to organise a seven-part webinar series [4] focusing on Quantitative Structure Activity Relationships and read-across, five Annex VII and VIII endpoints (skin irritation and corrosion, serious eye damage and irritation, skin sensitisation and mammalian, and aquatic acute toxicity) as well as the regulatory processes involved in acceptance of non-animal tests. To assess the success of the webinar series, attendees completed an online survey about the webinars and their attitudes towards using non-animal methods for REACH. This presentation will describe how registrants can be effectively educated about minimising animal use under REACH. However, the perceived regulatory barriers may not be overcome by registrant education alone. Regulatory authorities must address these concerns to ensure that the number of animals used for the REACH 2018 deadline are minimised.

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Mimicking the cyclic mechanical stress of the lung parenchyma in a breathing lung-on-chip

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Current *in-vitro* models of the lung parenchyma poorly reproduce the complex microenvironment of the alveoli. To investigate the effects of the mechanical stress generated by the breathing movements, we developed an advanced *in-vitro* model, called lung-on-chip, mimicking the thin air-blood barrier, including the 3D cyclic stretching. This lung-on-chip is equipped with a thin, porous and flexible membrane, on each side of which lung cells are cultured. The cyclic stress of this bioartificial air-blood barrier is induced by the movements of a second membrane, mimicking the diaphragm and its contractions. In addition to the reproduction of these *in-vivo* features, the lung-on-chip is designed to be easy to handle and compatible with multipipetting systems. A co-culture of lung epithelial cells and endothelial cells was carried out to reproduce the three-dimensional air-blood barrier. The effect of the cyclic stretch on the permeability of the lung epithelial barrier was investigated. Bronchial epithelial cells (16HBE14o-) were cultured upon confluency on the flexible and porous membrane. Then the epithelial barrier was either kept in static conditions or was cyclically stretched (10% linear strain, 0.2 Hz) over 19 h. Afterwards a permeability assay was performed in static and dynamic conditions, using large (RITC-Dextran) and small molecules (FITC-Sodium). The cyclic stretch significantly increased the transport of the small hydrophilic molecules, whereas it did not significantly affect the transport of the larger molecules [1]. These results are in line with an experimental permeability study done in humans [2] that revealed an increased clearance of small hydrophilic solutes upon lung volume increase. Further we investigated the effect of cyclic stretch on primary human pulmonary alveolar epithelial cells (pHPAEC), derived from patients undergoing lung resections. The primary cells were cultured on the fibronectin coated, flexible membrane upon confluency and either cyclically stretched or kept in static conditions for 48 h. The supernatant was collected every 24 h and analysed for differences in metabolic activity and IL-8 secretion. After 24 h, a tendency for a higher metabolic activity as well as IL-8 secretion of the pHPAEC, if the cells are cyclically stretched was observed. Then, after another 24 h the metabolic activity of the pHPAEC was significantly higher. The same was true for the secretion of IL-8 [1]. The presented bioinspired lung-on-chip enables to investigate the effects of the breathing motions on the lung alveolar barrier. Our results demonstrate that the cyclic stress significantly affects the properties of the lung alveolar barrier and the metabolic activity of primary human lung alveolar epithelial cells.

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Rat early reactions on the empty and enriched environment

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Rat is a social animal which is filogenetically adapted to the diversified environment. Many studies confirm the importance of the enriched environment and the social contact that has positive effect on behavioral pattern and cognitive function. In our study the aim was to detect the early reaction of the rats to the empty and enriched environment. 50 x 50 x 50 cm open field arena (OFA) was used for the observation. The bottom of the arena was divided in 9 equal squares. 53 male and 8 female Wistar rats were used in different weights. Every animal participated in three trials (1.T 2.T 3.T), each trial took 10 minutes. The horizontal and vertical movement, the crossing of central square and the number and length of grooming were detected in every trial. In 1.T an animal was placed in the empty OFA. In 2.T there was another well known rat in the OFA and the social interaction was detected as well. In 3.T the rat was alone again but a plastic rodent house, a paper roll and a handful paper bedding material was placed in the OFA. The behavior of male versus female and active versus inactive male rats was compared. Female vs. male: Basically females had more horizontal and vertical movement and the difference was significant except in 3.T. Central square was avoided by both sexes in most cases. The number of grooming was higher in the female and there was statistical difference in 1.T. The length of the grooming was short and in most of the cases there was no difference between males and females. There was statistical difference between the social interactions only in the first half of the period. Environment enrichment cleared away the activity difference. Active vs. inactive male rats: There was significant difference in the 1.T. In 2.T and 3.T the inactive rats moved more horizontally and vertically. There was no statistical difference in the number of grooming and hardly any difference in the grooming time/grooming. There was not statistical difference in social interaction. There were significant differences in the house interactions between the active and inactive rats. The statistical difference disappeared mostly in the 3.T. The animals dealt with the new objects so the horizontal and vertical movement reduced. The environment enrichment had not effect on the number of grooming. Social interaction is important especially in the first half of the trial. The animals mostly were interested in the house in the last trial. In 63/2010 EU Direction in Annex III the social contact and the importance of environment enrichment are settled. The number of grooming reduced, the length of the grooming increased in the enriched environment and co-presence of another animal which is the sign of the reduction of mild-stress. The enriched environment decreased the animals' neophobic response, which was signed by the elevated number of central crossing. Environment enrichment and social interaction is important in the early reaction of the rats. They can reduce the anxiolitic reaction and result in less "unneccesary" movement.

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An *in vitro* model for hepatocyte-like cell differentiation from Wharton's jelly derived-mesenchymal stem cells by cell-base aggregates

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Wharton's jelly-derived cells have been detected to display a wide multilineage differentiation potential, showing some similarities to both embryonic (ESC) and mesenchymal stem cells (MSCs). The present study investigated the differentiation potential of human Umbilical Cord Mesenchymal Stem Cells (UCMSCs) into hepatic lineage through embryoid body-like aggregates formation in the presence of IGF-I. Human MSCs isolated from the umbilical cord were plated in 20 µl micro drops. A two-steps differentiation protocol was used and the cell aggregates were exposed to the media supplemented with IGF, HGF, oncostatin M, and dexamethasone for 21 days. Immunoperoxidase and immuno-fluorescence were performed for cyrokeratins 18, 19 and albumin. Functional assays were done by periodic acid Schiff (PAS) and indocyanine green. The expression of cytokeratin 19 was shown to be higher in the cells derived from 3D spheroids compared to those cultured in conventional protocol. They showed a polygonal shape after being exposed to hepatogenic media. Immunostaining demonstrated the expression of cytokeratin-18, 19 and albumin by the differentiated cells. Besides, PAS staining revealed glycogen storage in differentiated cells. Also, a greater number of large size differentiated cells were found at the periphery of the expanded cell aggregates. We established a protocol for UCMSCs differentiation into hepatocytes and these cells were morphologically and functionally similar to hepatocytes. Thus, Hepatocyte differentiation may be facilitated by UCMSCs aggregate formation before administration of the differentiation protocols.

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Localization and internal dose of CeO_2 nanoparticles in an airway epithelium cell model in comparison to A549 lung cells

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Inhalation is currently considered being the major pathway of exposure against nanoparticles in humans with regard to health concern. While animal testing still appears to be the "gold standard" in toxicology, the large variety of newly developed nanomaterials on the one hand and the necessity to further reduce animal testing on the other gives paramount importance to the development of alternative *in vitro* test methods that could mimic *in vivo* test conditions. A crucial prerequisite for such experiments is the definition and application of the appropriate dose and size of the nanomaterials. Therefore, lung burden data recorded in a 28-day study on subacute inhalation toxicity of cerium dioxide (CeO_2) in rats [1] were used for the calculation of the actual dose, which was applied *in vitro* to a 3D airway epithelium model (MucilAir®). We investigated the possibility to assess cell membrane changes after nanoparticle contact as well as uptake and internal dose using imaging mass spectrometry (TOF-SIMS). This analytical technique is conventionally used in surface characterization of nanostructured materials [2]. However, the removal of successive layers can also deliver a 3D image of the chemical composition of whole cells [3]. We tried to correlate the activation or inhibition of toxicological signaling pathways after CeO_2 nanoparticle contact with the cell membrane phospholipid pattern to identify marker compounds being altered under these conditions. To elucidate the internal dose for the 3D airway epithelium, CeO_2 nanoparticles (NM212, JRC, size of 30 nm) were apically administered to the MucilAir® model. The MucilAir® cells secrete mucus that forms a layer on top of the surface and that subsequently develops functional cilia to generate conditions similar to those present in the human bronchioalveolar tract. For comparison we used A549 cells, which neither produce mucus nor develop cilia on their cell surface. Dispersion of nanoparticles was carried out according to an established protocol developed by the EU project NanoGenoTox. For this purpose nanoparticles were suspended in a 0.05% bovine serum albumin solution (% m/v) and subsequently ultrasonicated. The quality of dispersion was verified with dynamic light scattering (DLS). Inductively coupled plasma mass spectrometry (ICP-MS) was used for determining the CeO_2 dose administered. Due to its high sensitivity and very broad linear measurement range this analytical technique is suitable for detection of small amounts of inorganic material. Together with TOF-SIMS it also can provide information on how much CeO_2 enters the cells and on the portion that stays on the cell surface. As expected, we could demonstrate that tissue characteristics, such as mucus layers or functional cilia, play an important role for the bioavailability of nanoparticles in cells or tissues. Such protective cellular features may prevent nanoparticles from entering the cells and creating toxic responses.

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Barriers to the implementation of refinements in animal laboratories

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Many laboratories that house animals used in research and testing operate a normal office hours culture, with most members of staff, including those involved with the care of the animals, working only during the hours of 9 am to 5 pm. There is also typically reduced staff during weekends and bank holidays. Whilst some laboratories have over-night monitoring by care staff, our experience is that many do not. Security issues are left in the hands of security firms and electronic monitoring systems are relied on to detect equipment failures. The implications of a 9 to 5 culture on animal welfare are surprisingly varied and are, in our view, typically underestimated. Investigations by Cruelty Free International over the last ten years have consistently found animal welfare issues arising directly from the 9 to 5 culture of care. There are three ways in which the 9 to 5 culture can impact on animal welfare. Firstly, failure to have care persons on site does not prevent the occurrence of rare, unpredictable and yet serious welfare issues. These can include failure of water systems that can cause flooding of cages, or over heating of animals in the case of hot weather or deaths of animals not expected to be ill. Whilst some facilities employ electronic monitoring systems or ad hoc security checks, this is not widespread and cannot detect or prevent all of these rare but serious events. Secondly, our experience is that overnight care is rarely provided even for animals that are known to be unwell, for example following procedures. Some laboratories have a policy of not doing surgeries on Friday (as there is no or limited care at weekends) or in the afternoon (as there is no or limited care overnight) to mitigate some of the risk. However, animals are still typically left unattended overnight having had surgery in the morning on other days. Monitoring and delivery of pain relief or humane endpoints is typically not available from 5 pm until 9 am the next day and our experience is that deaths or deterioration of animals does occur overnight. Thirdly, the 9 to 5 culture can have more subtle impacts on animal welfare. For example, the need to starve animals before procedures such as gavage and surgery can result in animals being withheld food from 5 pm until after the procedure then next day (typically between 18-24 hours). The impact of this on animal welfare has been recognised in the literature but not systematically resolved. Rodents have fast metabolisms and need only be starved for 6 hours prior to procedures to ensure empty stomachs. Animals can suffer physically (weight loss is common) as well as psychologically which can lead to fighting. It is our belief that not enough consideration has been made of these issues and the need to address more 24 hour care in animal facilities.

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Non-technical summaries- a review of EU member states efforts so far

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Under the new Directive 2010/63/EC Member States have to ensure that non-technical summaries of authorised projects involving animals are made public. Article 43 asks that this summary include information on the objectives of the project including the predicted harm and benefits and the number and types of animals to be used. Summaries should also demonstrate compliance with the 3Rs. Summaries should be anonymous and not identify individuals or places that use animals. The intention was that NTS would provide some increase in the transparency of animal research in each Member State, over and above that provided by the annual statistics. In 2012 the Commission worked with Member States to provide guidance including a template for the summaries that allows this information to be included (EC, 2013). Although the Directive had to be transposed by 10 November 2012 and the provisions therein adopted by 01 January 2013, there was no specific timescale given by which the NTS had to be published, nor how frequently and how soon after the project had been authorised. In June 2015 the European Coalition to End Animal Experiments (ECEAE), which has members in most EU countries, surveyed all EU member states to identify which ones were by then publishing their NTS. By June 2015 not all member states had started publishing NTS and some, for example, the UK, were publishing very slowly. In the UK projects authorised in 2013 were still being published in 2015. Here we summarise the status of publication of NTS across Member States and compare best practices both in terms of publication rate, transparency and accessibility. We also include our observations on the quality of the NTS and the extent to which, within their limited length, they adhere to the requirements of Article 43.

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The need for proactive rehoming policies in the EU

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The new EU Directive 2010/63/EC explicitly encourages rehoming of animals from laboratories. Specifically recital 26 says, "animals such as dogs and cats should be allowed to be rehomed in families as there is a high level of public concern as to the fate of such animals". Whilst there is a will from the European people that rehoming should occur, there is currently a lack of guidance including documented experiences to show how it can be successfully achieved. Furthermore it is the experience of Cruelty Free International that rehoming does not occur as a matter of course in most facilities, even those that use cats and dogs. Apathy, nervousness of interacting with the public and a belief that such animals cannot lead normal lives outside the laboratory seems to be playing a large role in blocking this process within institutions. Article 27(1e) of the Directive however gives the Animal Welfare Body in each establishment the task to "advise on rehoming schemes, including the appropriate socialisation of the animals to be rehomed." Some Member States have, or are in the process of, issuing guidance on rehoming to assist establishments in this. To assist the UK authorities Cruelty Free International produced an expert report on the considerations that need to be made when rehoming animals. This report included case studies from 14 families homing a total of 17 dogs. Consistent with studies in the literature, the case studies showed unanimous support for rehoming. In all cases the homing was seen as extremely positive for the dog and the family. Dogs were typically reported to enjoy their new lives, be affectionate and enjoy the outdoors. Adjustment periods were reported to be relatively short, from "days" to a "few months" with two exceptions who took several months to adjust. There were no reports of aggression to humans or other dogs. Whilst some families reported initial "clingy" behaviour, only one reported classical separation anxiety. In fact, most behavioural problems appeared to resolve during or shortly after the initial adjustment period with the exception of exaggerated startle reactions to noises and sudden movements. It is important to recognise that dogs in laboratories are still dogs and can lead normal lives in family homes. Concerns about failure to adapt or the stress of the process itself seem unfounded. Member States need to make more efforts to encourage particularly breeding facilities to not only properly socialise their dogs but to set up more proactive homing schemes.

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Virtual physiology: computer laboratories as alternatives in life-science education

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First versions of the Virtual Physiology teaching series were developed in the mid-nineties of last century. Since then, these computer simulations of classical experiments in neurophysiology (e.g. SimNerv), cardiac physiology (SimHeart) or the physiology of skeletal (SimMuscle) and smooth muscle tissue (SimVessel) have been used to supplement or replace experiments with animal tissue at hundreds of universities and schools worldwide. An overhaul suitable for use on current operating systems is overdue. The Marburg Neurodynamics group, using the advice of experts from other research groups and with the help of external companies, has overtaken the reprogramming of the Virtual Physiology series as resolution independent versions for use on current operating systems that can now downloaded for free from http://www.virtual-physiology.com as fully functioning demo versions. Virtual Physiology II currently comprises five updated computer laboratories: SimHeart and SimVessel feature physiological and pharmacological experiments with the isolated, perfused heart in the Langendorff-setup and with isolated smooth muscle strips of blood vessels (aorta) and the stomach (antrum) to examine the muscle contractions after application of physiological transmitters (Acetylcholine), hormones (Adrenaline) and diverse drugs like the competitive receptor antagonists (Atropine, Phentolamine, Propranolol) as well as non-competitive modulators of Ca^{2+}-currents and Ca^{2+}-concentrations (Verapamil, g-Strophantin). An additional "drug laboratory" can be used practicing the correct preparation of the requested dilutions. SimMuscle and SimNerv offer highly realistic experimental set-ups on the computer screen to record contractions of the frog's gastrocnemic muscle (e.g. single twitches vs. tetanic contractions, muscle fatigue, curves of isometric and isotonic maxima) or compound action potentials from the frog's sciatic nerve (dependence on electrode positions, refractory period, anode break potentials, etc.), respectively. The fifth teaching tool, SimNeuron, additionally allows performing widely used voltage- and current-clamp experiments in an easy to overlook lab design to examine, for example, the threshold of action potential generation and its dependencies on stimulus strength and duration. It is also possible to measure the current-voltage curves to determine physiologically relevant parameters like maximum conductances, reversal potentials etc. and to examine the effect of a blockade of Na^+-or K^+-currents by TTX or TEA.

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Integration of the epidemiological data with the experimental toxicity studies on pesticides use of the AOP framework for the definition of biological plausibility

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Epidemiology studies represent one of the complementary data to analyse risk and should be evaluated together with well-designed toxicological *in vivo* and mechanistic (*in vitro* or *in silico*) studies as part of the overall weight of evidence for regulatory purposes. Therefore, there is indeed a clear regulatory need to incorporate epidemiology findings into risk assessment and this is understandable for multiple reasons: a) human data are compelling and trigger important considerations on the risk perception, which are frequently reported in the media b) many epidemiological studies deal with pesticides and their integration (why and how) or exclusion in the risk assessment process should be legitimate c) there is an increased demand of integrating human data with toxicological data. Approaches elucidating mechanisms or pathways of toxicity are needed rather than rely only on the standard regulatory requirements. In this top-down context, epidemiology findings may potentially be used for validation of these approaches. In the context of risk assessment, they can trigger alternative approaches to investigate the biological plausibility when the human data are not corroborated by experimental animal data. Integration of the epidemiological data with the experimental toxicity studies on pesticides use of the AOP framework for the definition of biological plausibility. Epidemiology studies on pesticides represent one of the complementary data to analyse risk and should be evaluated together with well-designed toxicological *in vivo* and mechanistic (*in vitro* or *in silico*) studies as part of the overall weight of evidence for regulatory purposes. Therefore, there is indeed a clear regulatory need to incorporate epidemiology findings into risk assessment and this is understandable for multiple reasons: a) human data are compelling and trigger important considerations on the risk perception, which are frequently reported in the media b) many epidemiological studies deal with pesticides and their integration (why and how) or exclusion in the risk assessment process should be legitimate c) there is an increased demand of integrating human data with toxicological data. Approaches elucidating mechanisms or pathways of toxicity are needed rather than rely only on the standard regulatory requirements. In this top-down context, epidemiology findings may potentially be used for the validation of these approaches. In the context of risk assessment, they can trigger alternative approaches to investigate the biological plausibility when the human data are not corroborated by experimental animal data. The Adverse Outcome Pathway (AOP) approach could help in organizing what we know and this knowledge can be used to assess biological plausibility and to implement the overall risk assessment of pesticides by making use of all the available information on a pesticide active substance. Furthermore, the AOP framework is increasingly considered as a systematic and transparent tool for organizing, reviewing and interpreting complex information from different sources. We intended to apply the AOP approach to investigate and possibly provide an objective and transparent way for delineating the biological plausibility supporting the possible link between exposure to pesticides and Parkinson's disease and childhood leukaemia.

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The role of the animal welfare officer on the 3R´s principle in laboratory animal science in Germany

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Heads of institutions, where experiments are conducted on vertebrates, shall appoint one or more animal welfare officers and notify each appointment to the competent authority. The notification shall also indicate the position and powers of the animal welfare officer. The institution must support the animal welfare officer in the performance of his tasks and inform him of every planned experiment so that he can carry out his duties without restriction. Only persons who have completed university studies in veterinary medicine and specialized in laboratory animal science may be appointed as animal welfare officers. The task of an animal welfare officer is to take care that each person subject to the regulations of the animal welfare act, to advice institutions and persons to implement the 3 R´s in laboratory animal science by using statistical methods in study design to reduce the number of laboratory animals as much as possible. Furthermore, animal welfare officer shall be obliged to ensure that the provisions, conditions and requirements shall be observed in the interest of animal welfare; to advise the institution and the staff involved in animal experiments and the keeping of laboratory animals; to give a statement on each application for authorization to conduct an experiment on animals and to work towards the development and introduction of procedures and means for avoiding or reducing experiments on animals inside the institutions. Overall, the officer ensures animal welfare within the institution.

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The activation of macrophages in co-culture initialised by metal oxide nanoparticles

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Human studies have shown that NPs can pass into blood circulation and can be found in inner organs such as liver, kidney and colon. Such exposure might initialise the inflammation process mediated by chemokine and cytokine signaling pathway. The activation of phagocyting cells is generaly associated to bacterial infection but is suddenly followed after exposition of exogenous xenobiotics. Current study aimed to look on the effects taking place in epithelial cell or fibroblast and macrophage co-culture after a single exposition of exogenous MetO nanoparticles. The co-culture of human epithelial cell Caco2 or murine fibroblasts Balb/c 3T3 and murine macrophages NR8383 was established and exposed to Sb_2O_3, Al_2O_3, Co_3O_4, CuO and ZnO nanoparticles (NP) in subacute toxic concentrations after two days. The high proliferation rate of macrophages compared to non exposed co-culture emerged already after 3 hours in the case of ZnO and Sb_2O_3 followed by Co_3O_4 and CuO NP. Within 24 hour the phagocytosis of co-cultured fibroblasts by macrophages exposed to Sb_2O_3 (C = 25 ppm) and ZnO (C = 10 ppm) had detected. The results of Caco2 and NR8383 co-culture confirmed the cell specific sensitivity to xenobiotics initialised phagocytosis where the exposure to CuO NP left no Caco2 cells in the co-culture at concentration 25 ppm and EC_{50} was the lowest at time point 24 hours. The monoculture or co-culture without exposition served as reference. Interestingly in case of CuO NP the NPs engulfing macrophages seemed to increase the EC_{50} of fibroblasts. The study is considered to show that the inflammation deriving properties of exogenous particle result in fast proliferation of macrophages leading in some cases to phagocytosis of co-cultured cells and could be used to detect the inflammagenicity of substance. Acknowledgements: Supported by the Centre of Excellence "Natural sciences and sustainable development" of Tallinn University.

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HTS for screening of selective muscarinic cholinergic receptors antagonists

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In the middle of 70-s the experimental data of different subtypes (M1 M2,M3 etc.) of M-cholinoreceptors distinguishable by their structure, localization in the organism, affinity to some ligands called selective and by some another properties were obtained. It is well known that some selective M1-ChR antagonists (cholinolytics) are widely use for treatment of organophospates (OP) poisonings. The acute toxicity of OP in mammals is primarily due to their irreversible inhibition of acetylcholinesterase (AChE) and abnormal accumulation of acetylcholine (ACh) at nerve synapses within the central nervous system and at the myoneural junctions. Current medical protection against the toxicity of OP consists of a regimen of MChR antagonists (cholinolytics) like atropine which acts by blocking the effect of ACh. At present the results of the experiments *in vitro* with the radioligand analysis of cholinolytics interaction with M-ChR serves usually as a basis for the conclusion on selective action but these data does not always give a possibility to predict of their pharmacological activity *in vivo*. In the experiments on the rats we have developed methodological approach to the evaluation of the selectivity of muscarinic cholinergic receptors (M-ChR) antagonists action in the whole organism conditions. According the results obtained during investigation the protective effect of M cholinolytics during acute poisonings of organophosphates (DDVP, DFP etc.) depends on M1 subtype ChR occupation. The efficiency of antagonists in inhibition of tremor reaction caused by M-ChR agonist arecoline administration associates with interaction of M2 subtype of ChR. It was established by the method of linear regression, that there was a high degree of correlation (r = 0.99) for different M cholinolytics between the ratios of ED_{50} of M antagonists in the tests with arecoline and organophosphates and the ratios of dissociation constants of antagonists complexes with M-ChR from the homogenates of rat's cerebral cortex and heart containing M1 and M2 ChR subtypes respectively. Thus, the ratio of ED_{50} arecoline/ED_{50} DDVP is serve as a measure of the selectivity of drugs action. Pharmacological analysis of *Daphnia magna* cholinergic system with the use of anticholinesterase compounds, and M-ChR agonists and antagonists was carried out. On the experiments to *Daphnia magna* the effects of some non selective, mainly M1 and M2 ChR antagonists on the toxicity of DDVP and arecoline were studied. There was a strong correlation between the ED_{50} of antagonists in the tests with arecoline and DDVP in the experiments on rats and the EC_{50} of antagonists in experiments on *Daphnia magna*. For the first time in the experiments on *Daphnia magna* it was shown that a ratio of the average effective concentrations (EC_{50}) of M antagonists in the tests with arecoline and organophosphates also may be used as a measure of the selectivity of M-ChR antagonists action. The principal similarity in action of muscarinic antagonists to *Daphnia magna* and rats allow to recommend the Daphnia for screening of selective muscarinic chlinireceptors antagonists.

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Refinement strategies in experimental autoimmune encephalomyelitis (EAE) in mice

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Experimental autoimmune encephalomyelitis (EAE) is an animal model for autoimmune disorders of the central nervous system (CNS) and especially used in multiple sclerosis (MS) research. It was established in different species. This talk will focus on mice. Scientific research has proven EAE to be a valuable tool to study pathogenesis of MS as well as to identify potential therapeutics. Nevertheless the complexity of the disease in focus (MS) and the model used (EAE) gives rise to discussions about the translational success of the animal model. Models of EAE are as manifold as MS in humans: they reproduce different histophathological, immunological and neurobiological key features of MS. Classical EAE is induced actively by CNS-peptides, complete Freud's adjuvant (CFA) and pertussis toxin or passively by transfer of stimulated immune cells [1]. This leads to destruction of the CNS resulting in ascending paralysis beginning at the tail. Depending on various factors (e.g. chosen mouse strain or sex, peptide, concentration of CFA) the severity and progression type of the disease can vary. In the last years genetically modified mice were developed to mimic different features of autoimmune CNS disorders. The variety of models and the severity of distress for the mice are big challenges for scientist, animal welfare officers and animal caretakers. Carefully choosing the right model for the scientific topic to answer is the first challenge for scientists. Different and incomparable scoring systems for severity assessment as well as unpublished data about experimental details make it difficult to compare single studies and improve keeping and caretaking strategies for the mice. Critically following ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [2] thereby optimizing the information with respect to design, conduct and analysis of the published experiments with animals. This will help to make different individual studies more comparable. But scientists as well as journals are not yet following these reporting standards [3] although this could raise the quality of the scientific results and it would also have a positive impact on the number of animals used in these studies. There is a big chance for reducing animal numbers and refining experimental conditions. Cooperation of scientists, animal welfare officers and animal caretakers especially in animal models with severe distress is of extreme importance. EAE is known to be very sensitive to many external factors like hygienic status or time point of weaning [4]. Standardizing animal care, keeping conditions and experimental groups has strong influences on animal numbers, distress and scientific outcome. Although no healing strategy for MS could be found yet, EAE seems to be one of the most promising models for research. Following strict refinement strategies will improve both: live of mice in EAE experiments and scientific results.

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Characterization of transporter activities in fresh isolated primary hepatocytes of different species by using fluorecent substrates

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Primary mammalian hepatocytes are used for several *in vitro* applications like testing of drug metabolism, toxicity and transporter assays. A comprehensive characterisation of plated primary hepatocytes is of high interest for researchers involved in ADME studies. Beside CYP inducibility we have therefore focused our work on transporter activities in fresh isolated human and Cynomolgus monkey hepatocytes. Previous studies have shown that radiolabeled substances can be used to characterize transporter activities in fresh and cryopreserved hepatocytes. Our current work is focused on the use of fluorescent substances in assays than can be performed in all standard laboratories. Human and monkey hepatocytes were incubated in serum free media. After cell isolation, a time and concentration dependent uptake of FMTX (Fluoresceine Methotrexate) was measured in presence or absence of Rifampicin as inhibitor. FMTX was evaluated as a suitable transporter substrate for primary hepatocytes. Hepatocytes from both species showed a saturable time-dependent uptake of FMTX within 30 minutes. The intracellular accumulation of FMTX was inhibited by Rifampicin to approx. 70%. In addition, a concentration-dependent uptake assay for FMTX was performed for 2 and 5 min. Here, the substance transport was impaired by Rifampicin only to a minor extent. Other fluorescent substances like Fluoresceine did not show a transporter-mediated uptake. In conclusion, fluorescent compounds may be used as an unhazardous alternative to radiolabeled chemicals for investigating transporter activities in primary hepatocytes.

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Are xenobiotic metabolizing enzyme activities in cells relevant for skin sensitization?

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Since pro-haptens may be metabolically activated in the skin, information on xenobiotic metabolizing enzyme (XME) activities in cell lines used for testing of sensitization *in vitro* is of special interest. We have proven metabolic activity of e.g. N-acetyltransferase 1 (NAT1) and esterase in keratinocytic (KeratinoSens® and LuSens) and dendritic (U937 und THP-1) cells. Aldehyde dehydrogenase (ALDH) activities were found in keratinocytic (KeratinoSens® and LuSens) cells. Activities of the investigated cytochrome P450-dependent alkylresorufin O-dealkylases, flavin-containing monooxygenase, alcohol dehydrogenase as well as UDP glucuronosyl transferase activities were below detection in all investigated cell lines. In the meantime, the mentioned cell lines are, beside the abiotic direct peptide reactivity assay (DPRA), routinely used to predict specified events of the adverse outcome pathway (AOP) of skin sensitization. Since the abiotic DPRA showed a higher predictivity than the above mentioned cell-based assays for a total of 213 compounds, we investigated the molecular structures of reaction products of 18 putative pre- and pro-haptens by LC/MS techniques to clarify the reaction mechanism leading to true positive results in this assay. In the set of putative pro-haptens, a covalent peptide adduct led to a positive DPRA result for cinnamic aldehyde, whereas oxidation products like dipeptide formations or the oxidation of the peptide-based sulfhydryl group led to positive results for benzo[a]pyren or 5-amino-2-methylphenol, respectively. In contrast, covalent peptide adducts were identified for 10 putative pre-haptens, indicating the DPRA to be suitable for compounds requiring abiotic oxidation to get activated.

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Outlook on currently available non-animal test methods for skin sensitization testing

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Skin sensitization is a key endpoint in both hazard and risk assessments. The key steps of the pathways involved are well characterized and can be structured into an adverse outcome pathway (AOP) for skin sensitization. One single non-animal test method will not be sufficient to fully address this AOP and in many cases the use of a testing strategy including a combination of non-animal tests methods will be necessary. A number of methods are now fully developed and validated. In order to facilitate acceptance of these methods by both the regulatory and scientific communities, results of 1 *in chemico* assay (DPRA), 4 *in vitro* assays (KeratinoSens™ , LuSens, h-CLAT, (m)MUSST), 2 *in silico* tools (OECD QSAR Toolbox, TIMES SS) as well as for a the simple "2 out of 3" testing strategy for a total of 213 compounds have been compiled and qualitatively compared to both animal and human data, where available. The dataset was also used to define different mechanistic domains by probable protein-binding mechanisms. The *in chemico* and *in vitro* assays exhibited good predictivities when compared to local lymph node assay (LLNA) data and even better predictivities when compared to human data, whereas the *in silico* tools predicted LLNA data with a higher accuracy than human data. The "2 out of 3" testing strategy achieved accuracies of 90% or 79% when compared to human or LLNA data, respectively and thereby even slightly exceeded that of the LLNA (82%).

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Application of *in chemico* and *in silico* methods to address protein-binding, the molecular initiating event of skin sensitization

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The molecular initiating event (MIE) leading to skin sensitization is the covalent binding of a hapten to a dermal protein. The protein reactivity of a compound can be assessed *in chemico* with the direct peptide reactivity assay (DPRA, OECD TG 442C) or *in silico* by the use of the QSAR Toolbox and TIMES SS. In this study, the utility of these methods to predict skin sensitization potential and potency was investigated for a total of 213 compounds by comparing the *in chemico* and *in silico* results to *in vivo* skin sensitization data (local lymph node assay (LLNA) and human data). Compared to human data, the DPRA showed an overall accuracy of 84%, whereas the QSAR Toolbox and TIMES SS provided accuracies of only 69 and 67%, respectively, when considering molecular structures of parent compounds only. Several compounds (pre- and pro-haptens) require spontaneous (autoxidation) or enzymatic transformation to form haptens capable of binding proteins. The *in chemico* DPRA covers spontaneous, but not enzymatic transformations, whereas the *in silico* tools lack both transformations. The *in silico* models can, however, simulate these transformations which increased the accuracies of the QSAR Toolbox and TIMES SS to 81 and 83%, respectively, matching the performance of the *in chemico* DPRA. This comparison of methods indicates that *in silico* tools may have the potential to address the MIE of skin sensitization as effectively as *in chemico* methods when the activation of pre-/pro-haptens is included in the *in silico* study. In addition to using the *in chemico* and *in silico* methods to determine the skin sensitization potential, these methods were also applied to potency predictions, but did only prove to be useful to distinguish sensitizing substances of GHS sub-categories 1A and 1B in specific mechanistic domains.

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Assessing skin sensitization hazard in mice and men using non-animal test methods

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A Decision-making framework for the grouping and testing of nanomaterials developed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" is presented. This so-called DF4nanoGrouping consists of 3 tiers to assign nanomaterials to 4 main groups, to perform sub-grouping within the main groups and to determine and refine specific information needs. The four main groups distinguish between (1) soluble nanomaterials, (2) biopersistent high aspect ratio nanomaterials, (3) passive and (4) active nanomaterials. The DF4nanoGrouping covers all relevant aspects of a nanomaterial's life cycle and biological pathways, i.e. intrinsic material and system-dependent properties, biopersistence, uptake and biodistribution, cellular and apical toxic effects. Use, release and route of exposure may be applied as "qualifiers" to determine if, e.g. nanomaterials cannot be released from a product matrix, which may justify the waiving of testing. The DF4nanoGrouping allows grouping nanomaterials by their specific mode-of-action that results in an apical toxic effect. This is eventually directed by a nanomaterial's intrinsic properties. However, the exact correlation of intrinsic material properties and apical toxic effect is not yet established. Therefore, the DF4nanoGrouping uses the "functionality" of nanomaterials for grouping rather than relying on intrinsic material properties alone. Functionalities include system-dependent material properties, *in vitro* effects and release and exposure. The DF4nanoGrouping is a hazard and risk assessment tool that contributes to the sustainable development of nanotechnological products. It ensures that no studies are performed that do not provide crucial data thereby saving animals and resources.

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Cost-effective low-serum cell culture media for expression and purification of molecular motor

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Conventional use of *E. coli*, yeast and insect cells as protein expression and purification systems is limited and not compatible with the large repertoire of mammalian proteins, especially when working with motor proteins such as myosins. Mammalian cell culture expression systems are able to introduce proper protein folding and post-translational modifications, ensuring the greatest probability of producing fully functional human proteins. Here we present the establishment of a large-scale mammalian cell culture expression and purification system. We based the system on the fast growing, serum-free adapted HEK293SF-3F6 cell line which can reach high cell densities. HEK293SF-3F6 cells were initially utilized for recombinant protein production using commercially available serum free media (SFM). The commercial SFM media is cost ineffective for large scale protein production needed for biophysical characterization of molecular motors, which is especially valid due to their low purification yield. To overcome this obstacle, we developed several low-serum cell culture media formulations based on specific soy peptones and yeast extract. Our developed culture media is able to support the growth of HEK293SF-3F6 cells in suspension with similar efficiency as commercial SFM, however at less than 1/5 of the price. Moreover, we can completely replace fetal bovine serum with commercially available human male (AB) serum without any difference on cell growth and viability. Thus, we are able to generate "humanized" animal free cell culture media with great potential in human therapeutic research. Our results also indicate that specific soy peptone-yeast combinations can completely replace serum. This cell growth platform has been coupled with Promega HaloTag purification system, allowing us to purify ~1 mg recombinant Homo sapiens myosin 19 from 1 L of cell culture. We compared the activity of the protein purified from cells grown in either commercial media or in our own formulated media. Actin activated steady state ATPase parameters (KATPase & kcat) of myosin 19 motor are comparable regardless of the media used for cell growth, strongly suggesting that our media can substitute the commercial SFM media. Based on our experience, and knowledge, the high costs of the current commercially available serum free media represents the major obstacle for their wide use, limiting the vision of The Three Rs. The burden of cost ineffectiveness of commercially available serum free media is shared by the industry. Several companies have been also developing their own serum free culture media such as M11V3 developed by Novartis. Our developed low-serum medium is a cost-effective alternative to commercially available, serum-free formulations designed for suspension cell cultures. We foresee that our results will promote and encourage further increase in use of low-serum and serum free cell culture technology and open the door to many unattainable expressions of proteins essential for therapeutic purposes and hence for human health in the long run.

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The Interspecies Database and the Humane Endpoints website: essential tools to implement the 3Rs

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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. Databases and informative websites on specific topics facilitate retrieval of specific 3Rs-related information for scientists, but also for the institutional Animal Welfare bodies and the Competent Authorities for project evaluation. These databases and websites save time-consuming searches, facilitate completeness and contribute to the 3Rs. To be successful, they should be easily found, accessed, managed and updated. In addition, relevant data should be easily retrieved. Designing, building and populating a database is a time and money consuming activity. Often, financial support can only be obtained at the start. Challenges arise when databases and information sources have to be maintained and updated on a regular basis. These are continuous activities that are essential for the success, usability and sustainability of the websites and databases. Continuous financial support is therefore crucial, in particular when the information sources are established as open access. Some websites and databases are supported by the government or larger organisations. Others are dependent on external resources and have to spend substantial time on fundraising. The 3Rs-Centre Utrecht Life Sciences (ULS) has recently started the 3Rs-database programme. Currently, the programme has adopted the Interspecies Database and the Humane Endpoints website. 1. The Interspecies Database (http://www.interspeciesinfo.com) provides insight into physiological, anatomical and biochemical parameters of different animal species and humans. By using the database, researchers can make a smarter design of animal experiments in terms of choice of an animal model. This could lead to a reduction in the number of experimental animals. Some users have indicated a reduction of 5,500 laboratory animals annually. 2. The Humane Endpoints website (http://www.humane-endpoints.info) gives guidance on how to apply humane endpoints. It is an interactive tool to make animal experimentation more humane by offering information, videos and photographs of clinical signs in rodent species. In addition, the website provides training modules that are successfully used in several laboratory animal sciences courses worldwide. Both information sources were originally established with support from the government, but lack continuous financial support. After adoption by the 3Rs-Centre ULS database programme, both websites have undergone a substantial makeover. They have a new look-and-feel, are more user friendly and will be further updated soon. The aim is to expand the 3Rs-database programme with other 3Rs information sources (especially on replacement methods). To guarantee a sustainable future for both websites and increase their usage, the 3Rs-database programme is inviting partners who are willing to cooperate and support its activities to further contribute to animal welfare with these and new 3Rs-websites.

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Human intervertebral disc as an efficient source for annulus fibrosus and nucleus pulpous cell isolation

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Introduction

In vitro organ culture systems are becoming essential as a replacement option for laboratory animals. To study possible mechanisms of intervertebral disc (IVD) degeneration, live disc cells are highly appealing. Most intervertebral disc cells are isolated from animal tissue. In order to translate preclinical results into clinical practice, the human tissues and cells of human origin are preferred, as animal cells do not reflect the properties of human tissue. We established a relatively quick and easy protocol for isolation of nucleus pulposus (NP) and annulus fibrosus (AF) cells out of the IVD fragments with a high yield and low risk for contamination.

Materials and methods

Human intervertebral disc fragments were obtained following discectomies. In sterile conditions, disc fragments were collected. The tissue was cut, grinded and partially digested with trypsine. After sequential centrifugation and separation, sediment was harvested and cells were seeded in suspension, which was supplemented with special media containing high nutrient level. Characterization was made and sub-isolation of nucleus NP and AF cells then followed.

Results

In appropriate laboratory conditions, the isolated cells retained viability and proliferated quickly. After 6 to 8 hours, attachment was observed and proliferation of the isolated cells followed after 12 hours. The time to confluence was 96 hours. Cell proliferation, apoptosis and cell senescence were examined after 16 days in culture. Both NP and AF cell cultures were stabile. Under standard culture conditions, cell proliferation and cluster formation was observed. Cell viability was 90%. The number of apoptotic cells and enucleated cells was positively correlated to cell seeding density.

Conclusions

The reported cell isolation process is simple, quick and economical. It allows establishing a viable long-term organ culture. The availability of such system will permit the study of cell properties, biochemical aspects, the potential of therapeutic candidates for human discs as well as toxicology studies in a well-controlled environment. Thus, this study has implications for both our understanding of degenerative disc disease and also cell-based therapy using isolated cells.

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Phenotypic and biomarker evaluation of zebrafish larvae as alternative model to predict mammalian hepatotoxicity

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Hepatotoxicity is one of the causes of drug attrition. Zebrafish assays show promise to assess hepatotoxicity, though subjective phenotypic scoring and time are drawbacks. The objective of this study was to develop a method with zebrafish larvae as an ethical acceptable alternative "whole-organism approach" to evaluate the potential of compounds to induce hepatotoxicity and predict human health effects. In this study, liver toxicity in zebrafish larvae was assessed using gene expression as biomarker approach, complementary to phenotypic analysis with the purpose to contribute to mechanistic understanding and improved human hepatotoxicity prediction. Results: Morphological effects of 5 hepatotoxic (acetaminophen, amiodarone, coumarin, methapyrilene, and myclobutanil) and 1 negative compound (saccharin) were assessed after 48 h exposure in zebrafish larvae at 5 days to define sublethal concentrations for gene expression experiments. Analytical methods based on liquid chromatography-mass spectrometry were optimized to measure stability of selected compounds in exposure medium and their internal concentration in larvae. Detection of hepatocyte markers (CP, CYP3A65, GC, and TF) were accomplished by in situ hybridisation after 48 h exposure of larvae to coumarin and myclobutanil and confirmed by real-time RT-qPCR. Experiments showed decreased expression of all markers. Next, other liver-specific biomarkers (i.e. FABP10a and NR1H4) and general apoptosis (i.e. CASP-3A and TP53) or a cytochrome P450-related (CYP2K19) and oxidoreductase activity-related (ZGC163022) genes, were screened for the 6 compounds. Links between basic mechanisms of liver injury induced by compounds and results of biomarker responses will be described in the context of hepatotoxicity prediction.

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In house validation of the bovine corneal opacity and permeability assay and EpiOcular™ Eye Irritation Test

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A number of alternatives to the rabbit eye irritation test (Draize), based on the principles of reduction, refinement and replacement, are in use. However, no universally applicable, validated non-animal alternative is currently available. Technical proficiency in the Bovine Corneal Opacity and Permeability [1] (BCOP) assay and EpiOcular™ Eye Irritation Test [2] (EIT) have been demonstrated at Charles River, Edinburgh, by correctly predicting the ocular irritation outcome of the respective recommended test chemical batteries [3,4]. The results are provided in the following tables. BCOP Assay UN GHS Classification No. of Correctly Identified Chemicals Category 1 5 of 5 No prediction can be made 3 of 3 No Category 5 of 5 EpiOcular™ EIT UN GHS Classification No. of Correctly Identified Chemicals Category 1/2 8 of 8 No Category 7 of 7 While neither method alone is capable of fully classifying substances as United Nations Global Harmonised System (UN GHS) Category 1 (serious eye damage), Category 2 (eye irritation) and substances which do not require classification, a strategic combination of the two tests in a tiered approach may be able to replace the Draize test. Technical proficiency in the Bovine Corneal Opacity and Permeability1 (BCOP) assay and EpiOcular™ Eye Irritation Test2 (EIT) have been demonstrated at Charles River, Edinburgh, permitting the availability of both assays for routine use at this site.

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Evaluation of the scientific validity of animal experiments in Switzerland

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Accumulating evidence indicates that experimental design and conduct of animal research are often poor, which threatens the scientific validity of the research and may lead to translational failure. Measures to reduce bias (e.g. blinding, randomization, sample size calculation) and rigorous reporting (e.g. ARRIVE guidelines) have been proposed to improve the internal validity of animal research. However, the relationships between the quality of experimental design, experimental conduct, and reporting have remained elusive. To investigate these relationships for animal research conducted in Switzerland, and to identify factors influencing internal validity, we scored all applications for licenses to perform animal experiments (Form A) authorized in 2008, 2010 and 2012 (n = 1277) and a sample of publications (n = 50) originating from this research, for criteria of internal validity. To control for accuracy in completing Form A, we assessed a separate set of accuracy criteria. Criteria of internal validity were rarely mentioned in applications: blinded outcome assessment was mentioned in 3.2% of applications, randomization in 12.6%, allocation concealment in 17.6%, and only 8% formally justified sample size (power analysis). By contrast, the accuracy of Form A was high (77%). However, a weak positive correlation between accuracy and internal validity indicated that researchers who completed Form A more accurately also provided more information on criteria of internal validity. In publications derived from this research, reporting of blinding (10.6%), randomization (17.1%) and allocation concealment (22.6%) was slightly better compared to applications, although none of the publications mentioned sample size calculation. Furthermore, a weak positive correlation between the internal validity score of applications and publications indicated that better reporting at the planning stage may improve reporting in publications. Using a generalized linear model, we assessed factors influencing the internal validity score in applications (e.g. species, authorization year) and publications (e.g. impact factor, endorsement of ARRIVE guidelines). Although the scores were generally low (range 0-0.86, grand mean = 0.104 ±0.14), scores of applications involving sensitive species (e.g. primates, dogs, cats, rabbits) (mean = 0.216 ±0.18) or farm animals (mean= 0.149 ±0.18) were higher compared to the grand mean. Internal validity scores tended to be higher for applications authorized in 2012 (mean = 0.12 ±0.16) compared to other years (2010: mean = 0.091 ±0.13 2008: mean = 0.096 ±0.13). In publications the internal validity score decreased with increasing journal impact factors (OR = 0.81, 95% CI 0.71-0.91). Whether the journal had endorsed the ARRIVE guidelines had no effect. Our results indicate that the harm-benefit analyses in the authorization of animal experiments in Switzerland are based on trust rather than evidence about scientific validity. By means of online survey and personal interviews we will now investigate how these findings relate to the true quality of experimental design and conduct at the bench, which should allow us to identify problems underlying poor quality of experimental design, conduct, and reporting, and propose targeted solutions.

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The effect of fetal liver extract and 3D culture on hepatogenic capability of human Wharton's Jelly mesenchymal stem cells co-culturing with endothelial cells

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Background: Extracellular matrix components and the growth factors are considered as a cell culture support that impact stem cell differentiation. The homologous a cellular matrix that obtained from healthy organ is biocompatible and consists of adhesion molecules and growth factors used in tissue engineering. e.g. collagen and Matrigel. The functions and stability of the hepatocyte phenotype can be improved during the differentiation in 3D multicellular aggregates compared to in monolayer cultures. In thist study we want to find the effect of fetal liver extract and 3D culture on hepatogenic capability of HWJMSCs co-culturing with endothelial cells.

Material and methods: MSCs derived from Wharton's jelly explants were characterized by detecting the surface CD markers and capability to differentiate toward osteoblast and adipocyte. HWJMSCs were co-cultured with endothelial cells in the Matrigel/Collagen scaffold for 21 days in the presence of fetal liver extract. Lectin histochemistry was used to detect endothelial cell (Lectin UEA) and biliary epithelial cell (Lectin PNA) in bile ducts. The functional assays were performed by Periodic Acid Schiff (PAS) staining.

Results: Some cells arranged into the structure with tubular shape in matrigel/collagen scaffold with or without extract. The sections were stained with PNA and UEA lectins. PNA can detect the bile duct epithelium and UEA reacted with endothelial cell in matrigel/collagen scaffold and could be observed in both cultures with or without extract. Some UEA-positive cells were formed a tubular structure. The cells which are surrounded a luminal space were slightly PAS positive.

Conclusion: Matrigel/collagen mixture can be considered as an efficient scaffold that play a critical role in differentiation of cells toward hepatocyte-like cell, biliary epithelial cell and formation of a tubular shape structures in the presence of the fetal liver extract.

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Vascular-like network enhances structural and functional maturation of human pluripotent stem cell derived cardiomyocytes in cardiovascular construct

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A relevant *in vitro* model should mimic the structure and essential functional properties of the native tissue. Widely used human pluripotent stem cell derived cardiomyocyte monocultures have been shown to present immature morphology and fetal-like electrophysiological properties that may effect on the outcome of the study. In this study, we developed a multicellular *in vitro* cardiovascular construct modeling human heart tissue for cardiac safety and efficacy testing. In the cardiovascular construct, human pluripotent stem cell-derived cardiomyocytes (CM) were cultured with vascular-like network formed by human foreskin fibroblasts and human umbilical vein endothelial cells (HUVEC). Vascular-like network serves as a supporting and interactive platform for the cardiovascular construct. In the study, CM morphology, orientation, electrophysiological properties and drug responses of the cardiovascular construct were characterized and compared to CM in monoculture. CM in cardiovascular construct showed an oblong morphology and aligned with the vascular-like network thus resembling more mature phenotype compared to CM in monoculture. Physiologically normal responses to E-4031 and adrenalin were detected in MEA and in calcium imaging. Genetic studies confirmed the ongoing structural and functional maturation of the CM in cardiovascular construct. In conclusion, the maturation of pluripotent stem cell derived cardiomyocytes can be markedly improved in culture with vascular-like network. Our results suggest that the cardiovascular construct presents more mature *in vitro* cardiac model compared to CM monoculture and could therefore serve as an advanced test system for cardiac safety and efficacy assessment.

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Numbers do not lie? Why the lives of "surplus animals" need to count, too

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The ever-increasing use of animals for scientific purposes is alarming per se. However, what so far has been kept in the dark in the majority of EU member states is the number of animals that are bred but never used for testing or experiments – the so-called "surplus animals". Even though breeders are required by Directive 2010/63/EU to keep track of the number of all animals that are bred and killed, those numbers are at present not included in the yearly statistical reports of Germany and the EU. Estimates are amounting to millions of animals that are bred but then killed because they have the "wrong" sex, age or do not express desired genetic changes. In our view statistical reports on the number of animals used for scientific purposes are intended to give an overview of the extent of the use of animals to enable stakeholders to identify measures how to reduce the number of animals or replace their use. In addition, statistical reports are also an important transparency tool. It is not acceptable that information on surplus animals cannot be obtained via the statistical reports and that the public is thus unaware of the problem. In our study, we analysed the current situation concerning surplus animals and developed measures to solve the problem of breeding and killing surplus animals. Due to Directive 2010/63/EU and the associated recommendations by the European Commission (EC), the requirements for statistical reporting will change starting with the numbers that will be published in 2015. A positive change is the now mandatory reporting of the number of animals that are used to establish and sustain genetically modified animal strains. Unfortunately, there still will not be a requirement to report every animal that is bred but not used for scientific purposes. The killing of surplus animals is not only causing unfathomable harm to those animals when they are killed but is also acting in legal limbo. In the German Animal Welfare Act, the killing of animals requires a reasonable cause. It is our opinion that in the case of surplus animals no such reasonable cause is given and therefore it is a breach of the German Animal Welfare Act. However, to this day this practice is tolerated by competent authorities. We are not aware of any case in Germany where charges were pressed due to the killing of surplus animals. To tackle the issue and to identify and develop effective countermeasures it is essential to have insight into the total number of surplus animals. In addition, with the Directive's goal of increasing transparency there is also a legal obligation to obtain, analyse and publish this information. We therefore demand complete reporting of all animals bred and killed for scientific purposes and to put countermeasures into action that prevent the breeding and killing of surplus animals.

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Influence of CD4+ T cell migration into a filaggrin deficient skin model in terms of skin barrier proteins and barrier function

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Loss-of-function mutations in the filaggrin gene (FLG) are a major predisposing factor for atopic dermatitis (AD), but also immunologic mechanisms are involved in its pathophysiology. The release of cytokines such as IL-4, IL-13, IL-31, TNF-alpha and cellular crosstalk between keratinocytes and lymphocytes are important promoters for the manifestation and maintenance of AD. Moreover, thymic stromal lymphopoietin (TSLP) is highly expressed by keratinocytes in lesional skin of AD patients and stimulates the differentiation of naïve CD4+ cells into Th2 cells which contribute to the induction of allergic inflammation [1]. Hence, to study the effects of CD4+ T cell migration caused by a lack of FLG in more detail, a FLG knock down skin model was established [2, 3]. After T cell supplementation, the expression of TSLP and skin barrier proteins as well as the barrier function of the skin models was investigated. Normal (FLG+) and FLG deficient (FLG-) skin models were generated according to previously published procedures [2]. Naïve CD4+ T cells were generated from human peripheral blood mononuclear cells by negative selection, activated with anti-CD3/CD28-beads and thereafter, added to the skin models for 2 days at day 12 of tissue cultivation. Subsequently, the skin models were analyzed for protein expression of FLG, involucrin (IVL) and TSLP using immunofluorescence staining. To analyze the influence of Th2 cytokines on T cell migration and differentiation, the skin models were pre-incubated with IL-4 and IL-13 (30 ng/ml) for 2 days before T cell supplementation. Skin absorption studies were performed with the radioactive labeled standard compound testosterone to assess the skin barrier function. Interestingly, FLG deficiency alone triggered the migration of CD4+ T cells into the dermal equivalent of the skin models. In contrast, no immune cell migration was observed in FLG+ but the stimulation with IL-4 and IL-13 stimulated migration of T cells into FLG+ and FLG-. Immunofluorescence staining showed significantly increased TSLP levels in FLG-, FLG- supplemented with CD4+ cells and in FLG+ supplemented with CD4+ cells, although in the latter no immune cell migration was detected. A compensatory upregulation of IVL in FLG- was observed, which was leveled out after T cell contact. Furthermore, only due to the presence of CD4+ T cells underneath the construct, without direct contact, the expression of FLG and IVL in FLG+ were reduced. The influence of T cells led to a reduced barrier function of the skin models. Our data show, that an increase of TSLP due to the lack of FLG plays a potential role for the migration of T cells into dermal tissue. As there are no dendritic cells presenting in the skin model, our data suggest a direct link between TSLP and stimulation of T cells without involvement of dendritic cells. Furthermore, this study clearly underlines the potential of reconstructed skin models for studies *in vitro*. Acknowledgements Financial support by the foundation SET (Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing) is gratefully acknowledged.

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Weighted Gene Co-Expression Network Analysis to Characterize Drug Induced Cardiotoxicity

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Drug-induced cardiotoxicity remains a major concern in preclinical and clinical research and development of drug, as well as contributing to drug withdrawal from market. Mapping chemical injuries to organ-specific damage via accurate and reliable biomarkers may provide a basis for highly specific and robust testing. Organ gene expression data of drug-induced cardiotoxicity are less in publication. Therefore, analyzing gene expression characteristics of drug-induced cardiac toxicity, and further screening the potential cardiotoxic biomarkers are vital important. Here, we used database DrugMatrix to implement the weighted gene co-expression network analysis and intent to identify the groups of co-expressed genes modules specific to injury endpoints in the heart. We identified 2 modules significantly associated with 7 diverse histopathological injury endpoints among 14 gene co-expression modules constructed. Using functional annotation and enrichment analysis, we further showed that specific module genes mainly mapped to (1) cell life cycle and (2) inflammation/immune response. Among the top 10 hub genes in the identified modules, only UNC93B1、CYBB、CYBA、Aif1 have been previously reported implicated in heart disease, whereas the rest have not been documented association with heart disease, and perhaps they can be used as putative biomarkers of cardiotoxicity. Protein Nox2 is encoded by CYBB gene, which is a superoxide generating enzyme that produces reactive oxygen species (ROS). Using our animal data that were generated before, we have preliminary demonstrated that Nox2 expression levels were significantly associated with ISO-induced cardiotoxicity. In conclusion, these findings indicate that network modeling provides systematic analysis of drug-induced cardiotoxicity, and identify 2 gene co-expression modules strongly associated with drug-induced heart injuries based on network approach, further analysis of these modules and hub genes may contribute to identify potential biomarkers for cardiotoxicity.

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Generation of three-dimensional cortical tissues – an innovative cell culture model for toxicity testing, drug development and basic research

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Fundamental principles of life in whole organism are based on highly complex interactions of many biological systems. Until today, the biology is not fully understood and animal experiments are still needed for example in the field of basic biomedical research to understand the development of diseases, for product and chemical safety testing or the development of new drugs and therapies. To totally resign from animal experiments is the final goal. However it is a big challenge to develop alternative methods for all of these various issues and methodological approaches which are capable to replace all animal experiments. We therefore pursue a more realistic strategy aiming at the development of new cell culture systems which are able to model distinct processes of a living organism *in vitro*. Currently our efforts are focused on the generation of three-dimensional cortical tissues from mouse embryonic stem cells to mimic fundamental processes of brain development *in vitro* for toxicity testing and to provide a tool for basic research. Here we show that mouse embryonic stem cells recapitulate *in vitro* major developmental stages of cortical development leading to the expression of cortical and neural markers as well as the generation of stratified cortical epithelia of distinct layers.

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Adressing inflammatory parameters of the intestinal mucosa: from the mouse to the test tube

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Inflammatory bowel diseases (IBD: Crohn's disease and ulcerative colitis) are characterized by chronic destructive, inflammatory alterations of the gastrointestinal tract leading to bloody diarrhea, weight loss, fatigue and abdominal pain. Mucosal inflammation in IBD is driven by activated immune cells in the inflamed bowel wall. In particular, mucosal T lymphocytes have been shown to produce large amounts of pro-inflammatory cytokines as well as induce barrier alterations and tissue damage in patients. Both genetic and environmental factors play a fundamental role in the pathogenesis of ulcerative colitis. These factors appear to initiate acute mucosal inflammation in this disorder that can be followed by progression from acute to chronic intestinal inflammation. Further studies identified differences in the cytokine profile between Crohn's disease and ulcerative colitis, therefore many different experimental IBD animal models are used in research. All these models have specific characteristics that can only mimic more or less parts of the human inflammation, but allow to target specific gene/pathways of the multifactorial disease. Recently, using mouse models in gut microbiota studies has brought insights in defining the more important role of microbiota in the pathogenesis of IBD and obesity. Additionally, organoid systems are generated by using intestinal stem cells intending to study the influence of cytokines or further factors on the mucosa. The first part of the talk will provide a comprehensive overview about the use of mouse models and organoids and discuss their impact as well as limitations. In contrast, the second part of the talk will focus on complex *in vitro* cell culture models of the inflamed intestinal mucosa and their role in safety assessment studies as well as in absorption studies. Beyond simple cell monolayer models of the intestinal mucosa (Caco-2 cells), advanced three-dimensional co-cultivated models additionally involve different cell types such as immune-competent macrophages and dendritic cells. In these models, macrophages and dendritic cells are embedded into collagen layers on the apical side of cultivation inserts such as Transwells. In a next step, intestinal epithelium cells are seeded on top. Physiological ultrastructure and significant barrier properties could successfully be proved. In such a three-dimensional construct, controlled inflammation can be induced by proinflammatory stimuli like cytokines, thus allowing to mimic pathophysiological changes which would occur in the human body. Advanced three-dimensional co-culture models of the inflamed intestine show a high potential to complement and even replace animal models for safety assessment studies as well as for absorption studies on the intestinal epithelium in the state of inflammation.

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EPAA – 10 years of progress and more to do

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Aim: At its 10th anniversary EPAA can demonstrate that it has been a successful pioneer in promoting 3Rs. Still many challenges remain to fulfil our Vision to replace, reduce and replace (3Rs) animal use for meeting regulatory requirements through better and more predictive science. In its next 5 year circle EPAA will focus on closer co-operation with European regulatory agencies and with national regulators striving to promote international harmonization of regulatory safety testing requirements.

Method: EPAA brings together 36 companies from 7 industry sectors and 5 DGs of the European Commission: this unique knowledge-sharing platform launches working groups or studies to define research gaps in the development of 3Rs as well as to improve their implementation in safety regulation. EPAA operates as a dialogue-enabling Public Private Partnership between regulators and regulated and across the sectors.

Results: As of June 2015, EPAA fosters 9 ongoing projects related to Science and Regulation. A total of 6 scientific workshops is planned for this year, covering topics as varied as skin sensitisation, vaccines consistency approach and harmonization of 3Rs in Biologicals. The EPAA project teams involve EPAA members as well as international regulators, civil society representatives and industry representatives from all over the world. Through its extensive stakeholders network EPAA is at the forefront of European and International dialogue on 3Rs initiatives. EPAA also liaises with SEURAT-1, the IIVS, ICCVAM and OECD.

Conclusion: The unique range of partners in EPAA including industry, regulators and welfare groups give it the ability to act as a forum for cross-sector dialogue and help provide a unified EU voice. Through the involvement of the EU Commission and global companies, EPAA has the potential to liaise with the wider international community.

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28-day microphysiological monitoring of human hepatocellular cells

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Hep-G2 human hepatocellular carcinoma cells were seeded onto BioChips and monitored for 28 days by means of extracellular acidification and morphology using the IMOLA-IVD system. 24 h prior to the start of the experiment 100,000 Hep-G2 cells were seeded in 300 μl medium onto the BioChip and incubated in a CO_2-incubator (5%) using standard buffered medium to let the cells adhere to the surface of the BioChip. After pre-incubation, pictures of the transparent BioChip were taken, showing a confluent monolayer of the cells. During the course of the 28-day experiment, the cells were supplied in a stop and go mode (5 min off, 5 min on) with RPMI 1640 medium without sodium bicarbonate, with Gentamicin and 10% fetal bovine serum (FBS). L-Glutamine (day 10) and FBS (day 16) were added to investigate if resupply of those substances is necessary to achieve the long term monitoring. After 28 days, 10% SDS was added as a positive control. Pictures after the experiment show that the cells were lysed by the SDS. Extracellular acidification was monitored using a pH-microsensor and morphology of the cells was monitored with an interdigitated electrode structure. The microsensors were read out about every 10 seconds. A stable extracellular acidification rate established after about 2 h. The real part of the impedance value decreased during the first days until it was stable after day 5. The results show that multiparametric, label-free, long-term monitoring of a living human cell line is possible and paves the way for development of new microphysiological models e.g. mimicking the OECD guideline for repeated dose 28-day oral toxicity study in rodents (OECD TG 407). This work was funded by the German *Bundesministerium für Bildung und Forschung* during the BIOGRAPHY project, BMBF No. 02PN2241. The author want to thank the *Deutscher Tierschutzbund – Akademie für Tierschutz* (German Animal Welfare Federation – Animal Welfare Academy), Neubiberg, Germany and the colleagues at *Heinz Nixdorf-Lehrstuhl für Medizinische Elektronik* of *Technische Universität* München, Munich, Germany.

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Towards the prediction of nanoparticle-induced inhalation toxicity: evaluation of an *in vitro* macrophage assay

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To asses the safety of the increasing number of nanomaterials (NM) and modifications thereof, grouping, ranking and integrated biological testing is highly desirable. However, current *in vitro* models revealed little correlation with rat inhalation studies. In previous projects (NanoCare, NanoGEM) promising correlation was found between studies using primary alveolar macrophages (AM) and/or the NR8383 cell line and short-term inhalation studies (STIS) or intratracheal instillation studies (ITI). This work, therefore, was conducted to continue on this issue. Measuring endpoints of the so-called vector model (cytotoxicity, macrophage activation, TNF release, oxidative burst), and converting these into a sum index, indicates inasmuch NMs interfere with particle clearance and basic immune function of the lung. A total of 28 previously characterized NM (pure and mixed oxides from Al, Ti and/or Zr various CeO_2 unmodified and surface-modified amorphous SiO_2 coated ZrO_2 graphite nanoplatelets different organic and Fe_2O_3 pigments Ag ZnO) were tested against micron-sized quartz DQ12 and corundum. Results were compared to the outcome of 18 ITI and 18 STIS. The ranking of the NM *in vitro* ranged from ion-shedding silver or ZnO NM, to Al-doped CeO_2, over SiO_2 and ZrO_2 modifications, to $SrCo_3$ and $BaSO_4$ as being least active. All particles have been previously characterized and/or used *in vivo* [1-6]. The *in vitro* ranking in principle matched the in vivo results. However, a more detailed comparison of *in vitro* toxicity and STIS data also had to consider particle surface size. Thus, when vector model data were used to distinguish between passive materials (no further testing suggested) and active materials (further testing necessary), the majority of particles were grouped into the right category as determined by STIS. The overall predictivity of that approach was better than 80%. Taken together, the *in vitro* approach with NR8383 cells appears promising and well suited to analyze the hazard potentials of inhaled nanomaterials and, therefore, is suggested as part of a tiered approach. This study was supported by BMBF (03X0021 and 03X0105)

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In vitro assays for the potency determination of botulinum neurotoxin serotypes A and B

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Botulinum neurotoxins (BoNTs) have a paralytic effect, which is broadly applied in the fields of medicine and cosmetics. The toxins produced by the bacterium Clostridium botulinum have to be tested for their specific activity and potency, what is currently mostly done with LD_{50} tests in mice. As these tests cause a high distress for the animals, it is desirable to find alternatives to the LD_{50} test, for which at least 600,000 mice are used annually [1]. The combined assay presented here could be a suitable candidate for this task [2]. BoNTs use an elaborate intoxication process leading to a high toxicity with a human lethal dose of approximately 1 ng per kilogram body weight. During intoxication, BoNTs specifically recognize receptors on efferent nerves preceding neuromuscular junctions. In consequence of the binding, the toxin is taken up via endocytosis. Conformational changes caused by alterations of the pH and redox milieu in the endosome allow the light toxin subunit containing the protease domain to traverse the endosomal membrane and to simultaneously separate from the receptor bound toxin subunit. This leads to an activation of the protease, which then starts to cleave specific proteins involved in the release of the neurotransmitter acetylcholine. The blocked neurotransmitter release from the efferent nerve causes a flaccid paralysis of the respective muscle. The combined assay for potency determination of BoNTs which has been developed in our laboratory is based on the binding capacity as well as the proteolytic activity of the toxins. As the medically relevant BoNT serotypes A and B differ in their receptor proteins as well as in their substrates, two separate combined assays were developed. In these assays, BoNTs are bound to receptor molecules coated on a microtiter plate. As receptor molecules, the ganglioside GT1b as well as a receptor peptide (derived from synaptic vesicle protein 2c for BoNT/A, and synaptotagmin for BoNT/B) are applied. After binding, the protease domain is separated from the residual molecule by reduction and transferred to a second microtiter plate coated with the substrate for the protease (synaptosomal-associated protein 25 for BoNT/A, and synaptobrevin for BoNT/B). Finally the cleaved substrate is detected with antibodies directed against the cleavage site. The development of the assay for the activity determination of BoNT/B has already been completed. In-house validation studies have shown that the assay is highly sensitive, with a detection limit below 1 pg/ml. For BoNT/A, assay optimization is ongoing. In addition to being preferable from an ethical point of view, the combined assay is also easier to perform, cheaper and faster than the LD_{50} test.

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InvertTox presents its new *in vivo* alternative testing strategy for predictive toxicology and efficacy screenings: the NeoMac assays

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InvertTox performs contract research based on its in house developed irritation and carcinogenicity test portfolio using Invertebrate models: The Slug Mucosal Irritation assay (SMI) and the newly developed Flatworm Stem Cell Proliferation assay (NeoMac). We here present the NeoMac assay. The NeoMac assay predicts carcinogenicity (safety assessment) and the effect (efficacy assessment) of compounds. For this, the flatworm Macrostomum lignano is used as a test organism. Flatworms possess pluripotent adult stem cells which have been shown to functionally correspond to human stem cells and whose proliferation dynamics can easily be experimentally assessed *in vivo* and used as an endpoint. Six compounds were subjected to the NeoMac-Carcinogenicity assay. Our assay correctly predicted the carcinogenicity of the compounds in both carcinogenic categories (genotox and non-genotox) and did not predict false positives. Moreover we developed a chemically induced *in vivo* cancer model with a tumor phenotype and present a proof of concept of its applicability in anti-cancer drugs efficacy screenings. We characterized the tumor phenotype by monitoring adult pluripotent stem cell dynamics during tumor formation and we treated flatworms bearing tumors with an anti-neoplastic agent which resulted in the recovery of normal stem cell proliferation pattern demonstrating the potential future applications of this model in pre-clinical efficacy screenings. Altogether, these assays contribute to the improvement of the toolbox for predictive toxicology and alternative efficacy screenings.

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Decision algorithm for 3R (refine, reduce, replace) programs – a case study in vaccines

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Since cutting-edge technology is not yet sufficiently able to mimic live organisms in their entirety, or the interplay of organs and organ systems, animal testing is still indispensable in many areas of R&D for new vaccines and adjuvants. However, in many cases the use of animals in batch release testing is historically driven from previous experiences with the same or related substances. The 3R principles described by Russell and Burch have outlined core principles for responsible animal usage guided by scientific advances in animal welfare and technologies available for safety and effectiveness testing. The replacement of animal resources, the ultimate target of 3Rs, in product development and/or release processes is not linear process but more often a series of smaller investments and a continuum of changes involving refinements, reductions and ultimately replacement(s). The presentation will introduce an algorithm for documenting influencing factors and measuring impact of 3R principles to product development. The model is broadly applicable and can be used in private and/or public organizations and across product sectors. The intended output of the algorithm is to provide a modular process for guiding resource investments, tracking progress and identifying risks and benefits for scientists and stakeholders (executive leadership, commercial partners, and non-scientific partners). The proactive and consecutive implementation of step-by-step 3Rs approaches within projects and programs will facilitate the process of building new experience, practices, and routines, resulting in "animal-free" batch release testing in the not-too-distant future.

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In vitro models of skin wounds – potential, limitations and perspectives

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Due to the increasing incidence of chronic wounds often accompanied with severe wound infections, fundamental insight into molecular processes governing the healing procedure as well as infection is urgently needed. For a rational development of effective therapeutics to eradicate wound pathogens and facilitate wound healing, suitable models are required closely mimicking the *in vivo* situation of a skin wound in the human body. Different animal models with wounded skin exist, however transferability of animal-derived data to the *in vivo* situation in the human body is impaired due to differences in anatomy, immune reactions and wound healing processes. In addition, animal skin is significantly more permeable than human skin based on differences in the composition of the stratum corneum lipids, thickness of individual skin layers as well as the density of hair follicles. Besides the use of excised human skin as *in vitro* model, significant progress in tissue engineering led to advanced three-dimensional skin cultivates which are either used as replacement grafts in case of burn victims or as test specimens for basic research as well as for testing of novel therapeutics and cosmetics. Simple *in vitro* wound healing assays involve keratinocytes cultivated in a monolayer in which a controlled defect area is generated by a blunt specimen. The extent of repopulation of the defected area allows for evaluation of "wound healing". However, this test is not reflecting influences of interactions of different cell types and three dimensional effects. Three-dimensional skin cultivates existing of different cell types as well as excised human skin samples can also be applied as wound models. Different wounding procedures are utilized ranging from mechanical incision and burning up to chemical tissue damage. Such models allow for analysis of different phases of wound healing as well as evaluation of novel therapeutic approaches for wound treatment. As wound infections are a severe clinical problem, attempts for adequate simulation of infected wounds gain more and more interest. However, the controlled and reproducible infection of such *in vitro* models still poses significant challenges. The talk will give an overview about existing wound models highlighting their individual potential and discussing the limitations as well as give a future perspective.

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Adverse outcome pathway knowledge base: a crowdsourcing tool to add value to existing information on alternative methods results

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An Adverse Outcome Pathway (AOP) is a framework portraying existing knowledge – from many scientific sources – of the linkage between a Molecular Initiating Event (MIE), and an Adverse Outcome (AO), connected by a chain of Key Events (KE) and the relationships between them (KER). A sufficiently detailed AOP description can support chemical risk assessment by indicating relationships between perturbations at molecular and cellular level with perturbations and adverse effects at tissue, organ and organism level. To enable the scientific community, in one central location, to share and discuss AOP related knowledge, the OECD has launched the AOP-KB project, allowing interested parties to build AOPs by entering and linking information about MIEs, KEs, AOs and Chemical Initiators. Knowing that pathway elements are not necessarily unique to a single AOP, value is added to existing knowledge by facilitating the re-use of MIE, KE and AO information in multiple AOPs, which prevents redundancy and makes the collective knowledge about those entities available in all AOPs in which they appear. AOP-KB is a combination of individually developed platforms, and the first AOP-KB module is already available to the public: AOP-Wiki, a system that organises, via crowd-sourcing, the available knowledge and published research into a verbal description of individual pathways, using a user friendly Wiki interface. Controlled-vocabulary drop-down lists from which to select Methods, Actions, Biological Objects, Life stages, Species etc. related to the AOP simplify the entry of ontology-based information. AOP-KB collects information derived from alternative methods and displays the knowledge in a way that simplifies the development of "Integrated Approaches to Testing and Assessment" (IATA) by offering biological context. With IATA gaining momentum in the regulatory environment, this puts alternative methods results at the centre stage for regulatory acceptance, which adds value to already existing information. AOP-KB is created jointly by the European Commission's Joint Research Centre (JRC), the United States Environmental Protection Agency (US-EPA) and the Organization for Economic Co-operation and Development (OECD). The presentation will showcase the main elements of AOP-Wiki as well as real-life AOPs already in the review phase.

https://aopkb.org/
http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm#AOP_wiki

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Impaired skin barrier function of cutaneous squamous cell carcinoma

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Non-melanoma skin cancer affects by far the highest number of men with continuously increasing incidences for the last decades. Most important riskfactors are UV radiation, immunosuppression, and deficient DNA repair [1]. Metastasized cutaneous squamous cell carcinoma (cSCC) presents the lethal form of non-melanoma skin cancer. After tumor initiation within the epidermis, transformed keratinocytes may penetrate the dermis and metastasize to distant organs. Immunocompromised patients like graft recipients bear an increased risk of disease progression and lethal outcome [2]. Since current treatment options fail to accumulate efficiently within the cSCC lesion, an improved understanding of the altered skin barrier is needed. Having significantly altered the pioneering cSCC model [3] and after performing a proof-of-concept study, we investigated the impact of transformed keratinocytes on the skin barrier function of disease models for the carcinoma in-situ (usually referred to as actinic keratosis, AK) and the invasive cSCC. cSCC models exhibited a lower to absent occludin- and zonula occludens protein 1 staining compared to the normal construct. The junctional adhesion molecule A expression was not altered. This is well in accordance to the *in-vivo* situation [4] and likely contributes to the impaired barrier function of the cSCC model. The surface pH of the AK model (pH 6.1) remained close to the surface pH of the normal constructs (pH 6.0), but the invasive cSCC model exhibited a drastically increased surface pH (pH 6.6). Thus, even an acidic tumor microenvironment results in impaired formation of the normal pH gradient through epidermal layers. In accordance to OECD test guideline 428, caffeine (logP -0.08) was chosen as test compound. cSCC models exhibited significantly increased permeation coefficients AK: 1.49 cm/s, invasive cSCC: 2.58 cm/s compared to the normal construct(Papp = 0.95 cm/s). Moreover, lag-times decreased from 1.1 h(normal) over 0.9 h (AK) to 0.5 h (invasive cSCC model). In conclusion, our reconstructed normal skin resembles the human *in-vivo* skin barrier. Introducing cSCC lesions into the organotypic skin model caused severe loss of barrier function. The altered surface pH and tight junction proteins question the common hypothesis of low-permeable hyperkeratotic cSCC lesions. Further studies will focus on the stratum corneum lipid composition.

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Geometrically confined cell differentiation and migration model for human teratogen detection

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Spatially and temporally organized cell differentiation and tissue morphogenesis characterize the whole embryo development process, and unintended exposure to teratogenic compounds can lead to various birth defects. However, current animal-based models for teratogen testing is limited by time, cost and high inter-species variability, while human pluripotent stem cell (hPSC) models are only focusing on recapitulating cell differentiation with neither spatial control nor morphogenic movements. Here, we developed a human-relevant *in vitro* model, which recapitulated both spatially confined cell differentiation and migration, to identify potentially teratogenic compounds. Firstly mesoendoderm differentiation was only induced to the periphery of micropatterned hPSC (μP-hPSC) colonies, where there were higher integrin-mediated adhesions compared with colony interior. When further inducing the mesoendoderm differentiation from 1 day to 3 days, tissue morphogenesis could be recapitulated, which was mainly collective cell migration *in vitro*. Cells at the colony periphery actually underwent epithelial-mesenchymal transition (EMT) and directed collective cell migration to form an annular mesoendoderm pattern which was similar as *in vivo*. When treated with known teratogens, the two cellular processes (cell differentiation and collective cell migration) were disrupted and the morphology of the mesoendoderm pattern was altered. Image processing and statistical algorithms were developed to quantify and classify the compounds' teratogenic potential. The μP-hPSC model not only could capture the dose-dependent effects of teratogenicity but also could correctly classify species-specific drug (Thalidomide) and false negative drug (D-penicillamine) in the conventional mouse embryonic stem cell test. This model offers a scalable screening platform to mitigate the risks of teratogen exposures in human.

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Dermal sensitizers – identification and potency ranking using IVSA and epiCS®

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Human 3D reconstructed skin epidermal equivalents have been shown to release IL-18 in response to a wide range of dermal sensitizing chemicals. The concentration of these chemicals that produce greater than a threshold positive response (Stimulation Index, SI ≥ 2.0) is correlated to their potency or strength in an In Vitro Sensitization Assay (IVSA). In our experiments, 4-Nitrobenzyl bromide (NBB) and 2,4-Dinitrochlorobenzene (DNCB) were strong inducers of IL-18 secretion into the culture medium (SI-2 = 0.02% and 0.03%, respectively). The strong sensitizer p-Phenylenediamine (PPD) had an SI-2 of 0.13%. Cinnamaldehyde (CA) (SI-2 = 0.33%) and Isoeugenol (IE) (SI-2 = 0.56%) were moderate sensitizers, while Eugenol (EU) (SI-2 = 0.75), Resorcinol (RES) (SI-2 = 2.9%) and Hexylcinnamaldehyde (HCA) (SI-2 = 8.08%) were weak sensitizers. Sensitizer potency ranked using an SI-2 as follows: NBB > DNCB > PPD > CA > IE > EU > RES > HCA, with NBB, DNCB and PPD classified as strong, CA, IE and EU as moderate, and RES and HCA classified as weak sensitizers. Of the total of 18 chemicals tested, seven were irritants and two were non-sensitizers (Glycerol and Isopropanol) of these, only Chlorobenzene (50%) was incorrectly predicted as a sensitizer. epiCS® gave an Accuracy of 89% and Sensitivity of 89%, and all other Cooper Statistics (Specificity, Negative and Positive Predictivity) values were 89%. In summary, measuring IL-18 release from 3D tissues allows for highly accurate and sensitive identification of dermal sensitizers. Also, the ability to rank-order potency of these chemicals based on SI-2.0 values of IL-18 secretion is a powerful tool for further classification into potency categories.

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NanoSafety research and its challenges!

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Engineered nanomaterials (ENMs), due to their unique properties, contribute considerably to new and exciting commercial products of daily use. However, these same properties may also be responsible for having unintended and potentially harmful effects on the human health and on the environment. In the last 15 years a new field of research has been established: NanoSafety research is aiming to identify potential adverse effects as soon as possible to and so avoiding any social or economic drawbacks. The gained knowledge in NanoSafety is rather limited [1] despite the immense number of published data to a variety of ENMs. The reasons are manifold and addressed in many commentaries and reviews [2]. An important consideration in developing standards and regulations governing the production and usage of commercial ENMs is the development of robust and reliable measurements that are able to accurately support risk-benefit models. Standardization and assay quality controls should be implemented to increase the comparability of the obtained toxicological results. Such measures are common in the areas of analytical and clinical chemistry [2-3]. A systematic approach is cause-and-effect analysis that has been used successfully to identify sources of variability in analytical assays. Here we present in a prove of concept, the direct application of such an analysis to a frequently used cell based viability assay in nanotoxicology, the MTS assay, together with some of the outcome elaborated in an interlaboratory comparison study. Compared with soluble substances, ENMs exhibit additional dimensions of complexity such as size, shape, chemical composition and different degrees of agglomeration. ENMs agglomeration has a sig-nificant influence on exposure, uptake, biodistribution and fate of ENMs in human beings [4]. There-fore a detailed investigation of particles size and of agglomeration is needed. Of special interest are factors that trigger agglomeration or dissociation in a complex biological environment. The interaction of ENMs with biological barrier is of particular interest, since the ENMs have to cross highly protective biological barriers to cause damage *in vivo*. Beside the intestine, skin or air-blood barrier the placenta barrier may play an important role too. The placenta is a multifunctional organ supporting the fetus with nutrients, removal of waste products and protecting against harmful sub-stances. The disturbance or damage of the placental barrier is directly linked with teratological impact and reproductive toxicology. Previous studies mainly addressed whether nanoparticles are able to cross the placental barrier or not. Though, the transport mechanisms underlying nanoparticle translo-cation across the placenta are still sparsely understood. With the use of the ex vivo human placenta perfusion model we could show that the transport of polystyrene beads is not based on passive diffu-sion, but is likely to involve an active, energy-dependent transport pathway [5].

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Analysis of effects of the adoption of alternative methods on the number of animals used for eye irritation in the European Union

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In regulatory toxicology, progress is made in the areas of development and validation of alternative methods. However, the implementation of these methods is in many cases hardly visible once they are adopted. This can be documented by the number of animals used for a specific endpoint. In our study, we singled out the endpoint eye irritation to investigate if the adoption of alternative methods had an effect on the number of animals used. Traditionally, since John H. Draize and Jacob M. Spines devised the so-called Draize-Test, rabbits were used in testing for this particular endpoint. However, the Draize-Test has been extensively criticized since the 1970s for scientific reasons. Main concerns were differences between the rabbit and the human eye, the subjectivity of the scoring apparatus and the variability of results [1]. The Organisation for Economic Co-Operation and Development (OECD) adopted two alternative methods in 2009: the Bovine Corneal Opacity and Permeability Test Method (BCOP, TG 437) and the Isolated Chicken Eye Test Method (ICE, TG 438) in 2009. These two Test Guidelines (TGs) were then accepted by the OECD for the identification of ocular corrosives and severe irritants and were also included into the Test Methods Regulation (TMR) that lays down the test methods for the EU chemicals regulation, REACH and the EU biocidal products regulation (BPR) in 2010. In 2013, these TGs were updated to now also enable the identification of chemicals inducing serious eye damage and chemicals not requiring classification for eye irritation or serious eye damage [2]. Assuming a well functioning implementation would lead to a decrease or even collapse of the number of rabbits used for eye irritation testing we looked into these numbers in the European Union (EU) starting from 1999 until 2011. In the EU there is a considerable decrease in rabbits used between 2005 and 2008 which may be correlated to the entering into force of the first stage of the cosmetics testing ban (finished products) in 2004. However the acceptance of BCOP and ICE by the OECD in 2009 and the inclusion of these two test methods in the TMR did not cause any significant effect in the European numbers (only a decrease from 2105 in 2008 to 2080 rabbits in 2011). When analysing the available official European statistical data for rabbits used in the eye irritation test, no positive effect of the acceptance of alternative methods by the OECD can be concluded until now. A possible reason may be due to slow implementation of the BCOP and the ICE. However, the EU Directive 2010/63/EU lays down rules that require Member States to ensure that an alternative method or testing strategy shall be used instead of a procedure involving animals and to ensure that the number of animals used is reduced to a minimum. Therefore, we would have expected the European Commission to take action on the ongoing use of rabbits for eye irritation testing.

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Evaluation of an *in vitro* human dermal Sensitization test for use with medical device extracts

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In accordance with ISO 10993-10, the guinea pig maximization test, Buehler test, or murine local lymph node assay are used to assess the dermal sensitization potential of medical devices. Though there are a number of *in vitro* dermal sensitization assays available, only the SenCeeTox® assay is capable of assessing both polar (aqueously soluble) and non-polar (aqueously insoluble) extracts. The SenCeeTox® assay has previously been shown to accurately predict sensitization by monitoring viability, reactivity, and specific genes known to be crucial in dermal sensitization. Here, ten known sensitizers were evaluated. Six were incorporated into medical device silicone (10% final concentration) and extracted in polar (physiological saline) and non-polar (sesame oil) solvents in accordance with ISO 10993-12:2012. The four remaining compounds were added to extracts of plain silicone. This resulted in 20 test solutions (10 compounds in both saline and sesame oil). The assay controls were prepared directly in saline and sesame oil prior to exposure. EpiDerm™ tissues were exposed for 24 hours and tissue viability was assessed by LDH release. Expression of multiple genes controlled by Nrf2/ARE was assessed by qRT-PCR. The test solutions were also assessed for reactivity with glutathione. Eight of the 10 test samples (80%) were correctly identified as negative (non- or weak sensitizers) or positive (moderate/strong/extreme sensitizers) in at least one of the two extracts, and 40% of the potencies were correctly identified. Our results indicate that the SenCeeTox® assay combined with EpiDerm™ tissues can detect the presence of sensitizers in medical device extracts. Although there is more validation that needs to be performed, this model may eventually be a suitable replacement for the animal methods currently used to evaluate medical device biocompatibility.

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Hydra offers potential as a simple alternative model organism for ecotoxicology and environmental genomics assessment

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The development of novel approaches and amenable experimental systems to risk assess the environmental and other chemicals are the need of the hour. REACH Legislation in respect of Chemical Safety Assessment requires that, in view of the large number of chemicals for which safety assessment is needed to be carried out which is not feasible using the conventional *in vivo* test systems, novel and simple model organisms are needed to be brought up into the scenario. Small model organisms are emphasized in Tox21 and ToxCast programs also. In as much as Zebrafish embryo, Drosophila and Caenorhabditis offer substantial scope in this context, there is still need for simpler organisms that are amenable for high-throughput screening. Hydra, a cnidarian, proves to be a potent experimental system in environmental toxicity and risk monitoring due to its many desirable features most important of which is the availability of conserved biochemical and pharmacological targets. We aimed to understand the organismal, cellular and genome level responses in Hydra to environmental heavy metals such as divalent copper, Cu (II), by adopting a systematic approach to unravel the mechanism of chemical action. At the organismal level lethality was observed at environmental relevant concentrations followed by developmental deformity and finally affecting the population fitness. Gene expression profiles of several putative or already established stress markers (Superoxide dismutase, Catalase, Glutathione S transferase, Glutathione peroxidase, HSP70, Bcl-2 and FoxO) were analyzed using real-time PCR. Induction of apoptosis was investigated by finding the expression of Bcl-2, Bax and capspase-3 proteins. The results showed that on exposure to the toxicant the polyp's defense mechanism was hampered and the apoptotic machinery was induced. We conclude that Hydra has the potential to be developed into an efficient experimental system for figuring out the precise mechanism of chemical-induced environmental toxicity in a quick, simple, and inexpensive manner which will aid in understanding the adverse outcome of entities for environmental surveillance.

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Late abstract

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3D cultivation and differentiation of human neural progenitor cells in a biomimetic functionalized hydrogel matrix as a model for developmental neurotoxicity testing

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Developmental neurotoxicity (DNT) poses a threat to society as exposure towards developmental neurotoxicants can lead to neurodevelopmental deficits in children. DNT testing is currently performed by animal experiments, but due to the resource-intensity of the rat DNT bioassay there is an urgent need for the development of alternative methods. This need is strongly supported by the observation that extrapolation of animal-derived findings to humans often hampers predictivity due to species differences. Hence, alternative models, which are based on human material and mimic the physiological situation as well as possible, are desired. Lately, it has become evident that cell responses *in vitro* largely depend on the cell's context, i.e. that cells grown in three dimensions (3D) respond to triggers or stressors in a more physiological way than cells grown in a 2D monolayer. On this background, we established a differentiation model of human neural progenitor cells (NPC) in a hydrogel matrix to mimic the processes of NPC migration and differentiation as crucial neurodevelopmental endpoints in 3D. We used primary human NPCs growing as neurospheres and cultivated them in a defined biomimetic hydrogel, which was functionalized with different laminin peptide sequences. We measured viability and migration distance after 6 days as well as the differentiation into neurons after 25 days of differentiation. We compared the performance of neurospheres, which were cultivated in the hydrogel matrix, to neurospheres cultivated in the biological matrix protein mixture matrigel. As a proof-of-principle for DNT testing applicability, we exposed neurospheres cultivated in the functionalized hydrogel to methyl mercury chloride. Human neurospheres cultivated in a 3D hydrogel matrix in the presence of the laminin adhesion motif IKVAV mimicked basic processes of brain development: NPCs migrated and differentiated into neurons and glia cells. Comparative studies with neurospheres cultivated in matrigel showed better migration behavior in the matrigel than in the hydrogel. Besides, after long-term cultivation of 25 days neuronal networks were formed within the hydrogel matrix. Exposure to methyl mercury chloride affected migration as the most sensitive endpoint without significantly reducing viability in hydrogels. In conclusion, we established a biomimetic hydrogel model for the 3D cultivation of human NPCs, which enables (i) measurements of cell survival, migration and differentiation, (ii) formation of neuronal networks after long-term cultivation, and (iii) the fundamental possibility of chemical DNT testing.

Late abstract

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A human *in vitro* model for studying cholestasis

Yeda Kaminski, Sebastian Klein, Elmar Heinzle, Fozia Noor

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Cholestasis results from an impaired bile acid secretion and flow leading to intrahepatic accumulation of bile acids. Drugs can disorder bile acid homeostasis by interfering with their metabolism and transport. Due to differences in the transporters in human and rodents, a human liver model for *in vitro* toxicity studies of pro-cholestatic compounds is highly desirable. We investigated human HepaRG cell line, comprising of hepatocytes and biliary cells, as an *in vitro* model for cholestasis. In order to investigate whether HepaRG cells are a good model for cholestasis, we studied the effect of inhibitors of bile acid transporters in intracellular bile acid concentrations. HepaRG cells were seeded in 7.2x104 cells/well in 96-well plate (n=3). Cells were treated for 24 h with bosentan or hydrocortisone with or without bile acid supplementation, and with bosentan and hydrocortisone with and without bile acid supplementation. The bile acid concentration used corresponds to that in serum of healthy [1] and cholestatic persons [2]. Cell viability was assessed by ATP assay. For cells exposed to normal or cholestatic human serum concentration of bile acids we observed viability above 80%. Caspase 3/7 assay was used to identify apoptosis in cells exposed to bosentan and/or hydrocortisone with or without bile acid supplementation in the range of cholestatic concentrations reported for human serum. The observed caspase activity increased with increase bile acid concentration irrespective of the presence of bosentan and hydrocortisone. Intracellular bile acid concentrations were quantified by liquid chromatography-mass spectrometry (LC-MS). Cells exposed to bosentan or hydrocortisone and supplemented with bile acids had higher intracellular concentrations of bile acids. In cells supplemented with bile acids corresponding to serum concentrations, bile acid concentrations were either similar to healthy or cholestatic concentrations. Those and treated with hydrocortisone, glycochenodeoxycholic acid, a hydrophobic bile acid considered to be toxic, showed the highest intracellular bile acid concentrations. When supplemented with serum of cholestatic concentration of bile acids and treated with hydrocortisone we observed higher intracellular concentration of bile acids than that in the cells subjected to the other mentioned conditions. We can conclude that HepaRG cells are responsive to the pro-cholestatic compounds bosentan and hydrocortisone with concomitant effect on intracellular concentration of bile acids without compromising cell viability. Therefore, HepaRG cells seem suitable for further *in vitro* toxicity studies addressing the effect of concentration and interaction of pro-cholestatic compounds and to inspect unknown cholestatic potential of other compounds.

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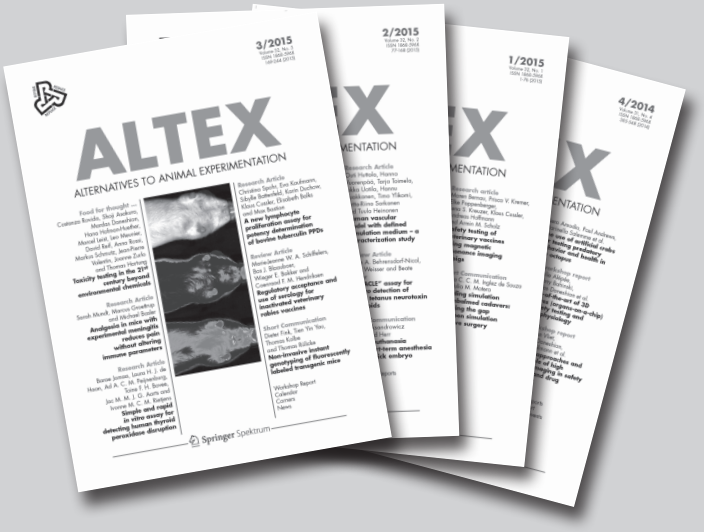

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