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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 17th EUSAAT 2016 3Rs congress in Linz/Austria on August 24-27, 2016 and the 25th Anniversary Congress on Alternatives in Linz.

During the past two decades the "Linz-Congress" has emerged in Europe as the major scientific event in the field of the 3Rs and in the year 2016 the EUSAAT 2016 3Rs Congress Linz will be the largest international 3Rs congress. We are particularly pleased that our international partner societies from Japan, JSAAE, the Japanese Society for Alternatives to Animal Experiments, and from the USA, ASCCT, the American Society for Cellular and Computational Toxicology, are actively participating in our session on "Global Cooperation on Implementing the 3Rs."

EUSAAT 2016 is hosting oral and poster presentations to facilitate discussions and the exchange of ideas for the benefit of alternative methods to animal experiments. The Scientific Committee has identified the most important subjects related to the 3Rs that are currently of interest to scientists in Europe. The EUSAAT 2016 conference focuses on 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."

This year we will for the first time be hosting two round table discussions on topics that are of particular interest to EUSAAT members. The first on "Building a career in the 3Rs area: successful biotech SMEs" and the second on "Implementing the concept of 'Integrated Approaches to Testing and Assessment (IATA)' into international regulatory testing."

The EUSAAT Board is pleased that during the EUSAAT 2016 congress the EU Commission and FELASA will be offering half-day information workshops free of charge on "Severity Classification & Reporting according to Directive 2010/63/EU."

We are very proud that the "Young Scientists Travel Award" (YSTA) program, which we launched last year at the EUSAAT 2015 congress and which was a big success, has attracted several sponsors. We are, therefore, able to continue the YSTA program in 2016 and we want to thank the sponsors for their support. The YSTA program provides young scientists with the opportunity to share their ideas on how to reduce the suffering as well as the number of animals in research, product development and regulation with international colleagues.

The EUSAAT Board is pleased that the number of sponsors of the EUSAAT congresses has increased over the years, since without their continuous support we would not be able to maintain the high scientific standard and to keep the congress fee low. Both elements are equally important in order to attract young scientists from all over Europe and beyond. Therefore, the EUSAAT Board and Scientific Committee thank all of the sponsors of EUSAAT 2016 on behalf of the participants.

Finally, we are also pleased that the abstracts of the EUSAAT 2016 conference on the 3Rs are again published in ALTEX Proceedings and we thank the editor and the CEO of ALTEX for helping us to pave the way for future cooperation for the benefit of implementing the 3Rs in Europe.

I finally want to thank my colleagues on the EUSAAT Board and the Scientific Committee for their continuous support in planning EUSAAT 2016 congress.

Horst Spielmann
Secretary General of EUSAAT

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An automated system for monitoring barrier function in 3D air-liquid interface cultures

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Toxicology studies increasingly rely on more complex cellular cultures to further reduce the usage of animal models and improve the predictive power of *in vitro* screening for skin and lung toxicity in industrial applications. In turn, culturing cells at the air-liquid interface (ALI) is being established as a valuable alternative method for creating *in vivo*-like skin and lung tissue models based on human cells [1,2]. Measurements of the transepithelial electrical resistance (TEER) can be used to evaluate the barrier function of these tissues as a representative metric for the absorption potential in these models, particularly in epidermal models. However, acquiring the TEER values in response to chemical reagents in these tissues still require manual dosing by removing tissues from the thermally regulated culture environment inside the incubator. The resultant temperature change directly affects the measured TEER of the sample, often resulting in large fluctuations in measured values [3]. Additionally, the "chopsticks"-style electrodes often used for obtaining TEER values require manual positioning; causing even further measurement fluctuations. Electrode chambers overcome this limitation, but still must be removed from the incubator for sample inoculation. For this reason, we developed an automated system to expose ALI cellular cultures to test substances dissolved in aqueous solutions while simultaneously monitoring the TEER of the culture.

The presented automated platform is based on the Intelligent Mobile Lab for *in vitro* diagnostics (IMOLA-IVD) [4]. Special BioChip encapsulations that support cell cultures on porous membrane inserts and maintain a stable air-liquid interface were designed and printed with an Ultimaker 2 3D printer. Custom fluidic heads were designed to plug directly into the tops of culture inserts and periodically apply and remove different solutions to membrane-bound cells via a single inlet/outlet. Either phosphate buffered saline (PBS) was applied for recording basal TEER values or test chemicals dissolved in PBS were applied for recording the TEER response upon exposure. An overflow valve incorporated into the fluidic head prevented overfilling and leakages. TEER measurements were recorded via a gold wire electrode placed directly into the fluidic head to create an electrical bridge with planar electrodes on the BioChip. To enable automated perfusion with culture medium and exposure to test substances, a fluidic switching network was developed using IMOLA fluidic modules and a peristaltic pump. Fluidic switching as controlled via a PC loaded with the DALiA Client 2.0 control software. Finally, proof-of-concept studies were performed where ALI cell cultures were exposed to 1% Triton-X solution dissolved in PBS. The resulting system can be used for automated toxicology studies of commercially available ALI cultures.

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A proposal for a catalogue of criteria to objectify the harm-benefit analysis of proposed projects including animal experiments

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Within a project funded by the Austrian Ministry of Science, Research and Economy, a catalogue of criteria to objectify the harm benefit-analysis to support the project evaluation has been developed. Our proposed catalogue will be presented, as well as an earlier version of this catalogue, which has been developed within the project. The catalogues comprise a different degree of differentiation and extend of criteria, which is due to the interpretation of the legal frameset of the Austrian Animal Welfare Act and a stakeholder process that was part of the project. The catalogues consist of different categories that comprise different questions to be answered by the applicant. The answers are assessed and scored. The applicant is also asked to provide the justifications. The catalogue of criteria provides an algorithm that calculates the scores of harms and benefits leading to an overall assessment of the harm-benefit analysis. The different methodological approaches will be presented and discussed.

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A proposal for a table to support the classification of the overall severity of a proposed project including animal experiments

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According to Directive 2010/63/EU Annex VIII Section II the assignment of the severity category shall take into account any intervention or manipulation of an animal within a defined procedure. It shall be based on the most severe effects likely to be experienced by an individual animal after applying all appropriate refinement techniques (Annex VIII, Section II: Assignment criteria). Thereby the type of procedure and a number of other factors that contribute to harm shall be taken into account. All these factors have to be considered on a case-by-case basis (ibid.). Besides the type of manipulation and handling, nature of pain, suffering, distress or lasting harm caused by all elements of the procedure, and its intensity, the duration, frequency and multiplicity of techniques employed, the aspect of cumulative suffering within a procedure is crucial for assigning the procedure to a particular severity category. To provide a better estimation of the overall severity of all procedures, an appropriate overview over the single procedures seems to be crucial, taking into account for instance measures to reduce or eliminate pain, suffering and distress, including refinement of housing, husbandry and care conditions, the application of humane endpoints, as well as the question if the animals are to be reused, whereas the actual severity of the previous procedures has to be taken into account. As the category of severity is a crucial factor for the harm-benefit analysis, we developed a template within a project to objectify the harm-benefit analysis, funded by the Austrian Ministry of Science, Research and Economy. This template to support the classification of the overall severity of a proposed project will be presented.

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Successful evaluation of sensitization by DPRA assay coupled with mass spectrometry detection

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The direct peptide reactivity assay (DPRA) is described in the OECD Guideline 442c and is a validated method for the *in vitro* discrimination between skin sensitizers and non-sensitizers in accordance with the UN GHS.

The DPRA assay assesses the reactivity of a substance with proteins. This reactivity is evaluated by exposing the substance to two generic cysteine and lysine peptides. Both peptides depletion is evaluated by measuring the initial and final concentrations of both peptides, and then, the depletion ratio is used to categorize the test item into four classes of sensitizers, from high sensitizers to non-sensitizers.

The initial and final peptide concentration in the incubation medium is classically measured by LC – UV. This detection method provides a full spectrum of UV absorption on the peptide content of the reactional medium but also on the test item components. This problem occurs especially with complex mixtures like botanical extracts composed of numerous molecules. Unfortunately, these types of compounds are namely responsible for many sensitization effects.

In this work, we describe an evolution of the DPRA assay. The original test was adapted to LC-MS/MS detection, in order to achieve a more specific detection of the peptide. This improvement allows more convenient testing of complex mixtures, thanks to the specificity of Mass Spectrometry.

The method performances were evaluated by incubation of references chemicals and by Quality Controls at low, medium, and high concentrations. Consistent correlation was found with the standard assay (UV detection) results in the literature regarding the classification of the chemicals.

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Implementation of the RHE / IL-18 sensitization assay on the Skinethic™ RHE test system – prediction model optimization

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Currently, the assessment of dermato-cosmetic substances sensitization potential is mainly performed using *in vitro* methods. For that purpose, several assays are available; however most of these assays are not suitable for the evaluation of the sensitization process of complex compounds and or lipophilic substances.

The RHE/IL-18 assay, currently under validation, answer this limitation by using Reconstructed Human Epidermises as test system, allowing the topical testing of lipophilic and/or complex mixtures.

This test is evaluated with different type of Reconstructed Human Epidermises, and we present here preliminary results regarding the implementation of this assay on the specific model SkinEthic™ RHE.

A set of 18 substances and mixtures was tested on this epidermal model, following the RHE/IL-18 protocol. The final results of the assay were obtained following 5 interpretation schemes, in order to determine the optimal prediction model for this assay with this specific test system. The data were analyzed with respect to three different gold standards: LLNA, HRIPT and an integrated reference, constructed from all available results.

No important differences were found in the performance levels depending on the three gold standards. The performances obtained with the SkinEthic™ RHE model support that this model may be considered as an alternative to different reconstructed epidermis models (EpiDERM™, EpiCS™, and VUMC-EE) for the performance of RHE/IL-18 assays. The prediction model to be used was refined, and more substances have to be tested in order to determine the right criteria applicable for this assay using the SkinEthic™ RHE test system.

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Preliminary data on the successful micronucleus testing with the EPISKIN® reconstructed epidermis model

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Currently, the assessment of the genotoxic potential of dermato-cosmetic substances is mainly performed using *in-vitro* methods, and the test most commonly used and well accepted by regulatory agencies is the micronucleus assay. However despite its regular use, this assay has some important limitations.

The reconstituted human epidermis (RHE) model is used more and more in the field of skin research for the safety assessment of topical products, thanks firstly to its physiological properties being reasonably similar to the human skin ones, and secondly because it allows testing of substances of a wide lipophilicity range. Furthermore, the metabolic capacity of the RHE model is close to the one of native human skin thus making the identification of the genotoxic potential of metabolites more predictive.

In this work, we describe our preliminary results on the adaptation of the classic cytokinesis-block micronucleus assay to the EPISKIN® reconstructed epidermis model.

We first investigated whether the EPISKIN® reconstructed epidermis test system is suitable to sustain the assay performance by establishing the correct culture conditions, measuring the basal level of micronuclei in the model, evaluating the effect of classic solvent on this basal level and then by testing positive reference (two direct and one indirect (active after metabolisation)), as well as one negative reference.

Optimal culture conditions were successfully established and several suitable solvents identified, with no impact on the micronucleus basal level. Regarding the evaluation of the references molecules, the expected positives were confirmed with clear dose-related responses and the negative reference was confirmed too.

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Recent developments of disease and human *in vitro* models of the blood-brain barrier

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Many different *in-vitro* models of the BBB have been published based on primary cells, immortalized cell lines or tumor cells derived from several species such as bovine, porcine, mouse, rat or even human. However, no standard *in-vitro* model of the blood-brain barrier (BBB) is established nor broadly accepted, although huge demands from basic research as well as pharmaceutical industry exist. Moreover, there is a great need for validated disease *in-vitro* models of the blood-brain barrier considering that almost in every neurodegenerative illness the BBB is altered which presumably contributes to the disease's etiology and progression. The lack of human BBB models mimicking the *in-vivo* situation close enough is dramatic, especially considering species differences and the need for save data transferability from e.g. rodent preclinical models to the human situation. After the introduction about the BBB and its role in different diseases two novel BBB *in-vitro* models will be explained in more detail. First, a mouse BBB *in-vitro* model for ischemia will be introduced comprising characteristics of barrier breakdown, ABC-transporter functionality, morphology and cerebral ischemia relevant enzyme activities by qPCR, western blotting, immunofluorescence microscopy and functional assays [1]. This model was used to investigate therapeutic strategies. Cross-correlation with a mouse model of traumatic brain injury confirmed the usability and most importantly the predictability of the *in-vitro* model. Second, recent developments of stem cell models highlight the potential to generate human *in-vitro* BBB models with *in-vivo* like phenotype paired with high paracellular tightness. As example for this trend a novel human blood-brain barrier *in-vitro* model will be introduced this is based on cells derived from human induced pluripotent stem cells.

In summary, current works show that the development of predictive disease and human *in-vitro* models of the BBB is feasible and further comprehensive studies may pave the way for these models to reduce the number of animal studies.

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A decision-making framework for the grouping and testing

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The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" proposes Decision-making framework for the grouping and testing of nanomaterials (DF4nano) that 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 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 (including manufacture), release and route of exposure are applied as "qualifiers" within the DF4nano to determine if, e.g. nanomaterials cannot be released from a product matrix, which may justify the waiving of testing. The four main groups encompass (1) soluble nanomaterials, (2) biopersistent high aspect ratio nanomaterials, (3) passive nanomaterials, and (4) active nanomaterials. The DF4nano aims to group nanomaterials by their specific mode-of-action which results in an apical toxic effect. This is eventually directed by a nanomaterial's intrinsic properties. However, since the exact correlation of intrinsic material properties and apical toxic effect is not yet established, the DF4nano uses the "functionality" of nanomaterials for grouping rather than relying on intrinsic material properties alone. Such functionalities include system-dependent material properties (such as dissolution rate in biologically relevant media), bio-physical interactions, *in vitro* effects and release and exposure. The DF4nano is a hazard and risk assessment tool that applies modern toxicology and contributes to the sustainable development of nanotechnological products. It ensures that no studies are performed that do not provide crucial data and therefore saves animals and resources. The grouping decisions of DF4nano for 24 nanomaterials were validated against grouping by results of existing *in vivo* data and demonstrated 23 concordant grouping decisions.

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Metaplastic phenotype in human primary bronchiolar epithelial cells after repeated exposure to mainstream cigarette smoke at the air-liquid interface

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3D constructs composed of primary normal differentiated human bronchiolar epithelial (NHBE) cells were repeatedly exposed at the air-liquid interface with non-lethal concentrations of mainstream cigarette smoke (4 cigarettes a day, 5 days/week, 10 repetitions in total) to build up a permanent burden on the cells. Samples were taken after 4, 7 and 10 times of repeated smoke exposure repetitions and the cultures were investigated by histopathological methods. In comparison with the clean air exposed cultures (process control) and incubator control cells the cigarette smoke exposed cultures showed a reduction of cilia bearing as well as mucus producing cells. At the end of the exposure phase, we found non-hyperplastic areas strongly positive to CK13 antibody, commonly seen in squamous cells as a marker for non-cornified squamous epithelium, thus suggesting a transition of the normal bronchial epithelial cells towards metaplastic cells. The control cultures (clean air exposed and incubator cells) showed no comparable phenotypical changes. In conclusion, our *in vitro* model presents a valuable tool to study the induction of metaplastic alterations after exposure to hazardous airborne material.

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Reducing uncertainty in read-across-based chemical safety assessments

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While there is a growing demand from regulatory authorities worldwide to generate new toxicological information even for chemicals long-existing on the market, societal and ethical considerations necessitate the reduction and subsequent phasing out of animal testing for hazard assessment purposes. This demand is already reflected in the promotion of the 3R principle under the EU REACH or the animal testing ban under the EU Cosmetics Regulation. In this frame, the toxicology of the 21st century has developed in such a way to increasingly use the power of computational chemistry in combination with *in chemico/in vitro* technologies and advanced systems biology to predict the hazard of chemicals. However, even in combination, these tools still have significant weaknesses in predicting systemic toxicity endpoints. In light of these limitations, read-across is currently considered to be the most actionable short/mid-term strategy with the ultimate goal to reduce or replace animal tests in chemical safety assessment [1]. Nevertheless, the reliability of read-across varies significantly and uncertainties are often not sufficiently described or addressed and this is evidenced by a considerable number of ECHA rejections of read-across strategies in the context of REACH submissions. This poster will present two case studies following good read across practices by demonstrating target chemical and analogue similarity with respect to structure and chemical reactivity, structure activity, metabolism and toxicokinetics [2] and by addressing identified uncertainties. The first read-across case revealed uncertainties for the skin sensitisation endpoint and differences in the predicted systemic availability between the analogue and the target substance. Accordingly, an *in vitro* testing program has been proposed to strengthen the read-across at both biological and toxicokinetic levels. In the second case, uncertainties related to the reproductive and developmental toxicity endpoint could be reduced by utilizing AOP-linked biological activity data from the ToxCast database.

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Pre-validation of human small intestinal tissue model system to screen drug induced gastrointestinal toxicity and wound healing

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Development of reliable and reproducible primary human cell based small intestinal (SMI) tissue models that recapitulate *in vivo* SMI tissue phenotype, structure and function are critically needed to study gastrointestinal (GI) permeation, drug toxicity and inflammation. The validity of commonly used Caco-2 cell models is questionable due to a lack of physiological relevance and animal models often fail to predict human responses. This study describes development of 3D SMI models from primary human SMI epithelial cells and fibroblasts. Long term culture of the models and application for drug toxicity and drug permeation studies are demonstrated. The utility of the reconstructed SMI tissue models for GI drug toxicity studies was evaluated using the GI toxicant drug, indomethacin. Outcome measurements include transepithelial electrical resistance (TEER), histology and apical protein washes (sloughed epithelium). Drug permeation studies were performed using 8 drugs that utilize specific transporters (Pgp, BCRP, MRP-2, etc.). Uptake or efflux transport was analyzed by LC-MS/MS. Specific findings include: 1) The SMI tissue models can be cultured for extended time (up to 28 days) with no significant change in TEER or Lucifer yellow leakage (< 2%); 2) Dose dependent toxicity of indomethacin was noted with respect to reduction of TEER, epithelial damage, and epithelial sloughing; and 3) Functionally active drug transport (B-to-A transport with efflux ratios > 2fold) for 7 of 13 test compounds was observed. Furthermore, drug efflux transporter inhibitors increased drug absorption while decreasing the efflux ratio. Efflux ratios for talinolol, digoxin, and loperamide (Pgp substrates) were reduced by 45%, 40%, and 60%, respectively, in the presence of the Pgp inhibitor, verapamil. The efflux ratio of the BCRP substrate, nitrofurantoin, was reduced by 63% in the presence of novobiocin, a known BCRP inhibitor. In terms of wound healing, tissues cultured in human serum completed the wound healing process at day 6 of the culture period compared to > 10 days for control tissues cultured in the absence of serum. In conclusion, the newly developed SMI tissue models appear to be promising new tools for drug safety, permeation, and wound healing studies.

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A new validated *in vitro* skin sensitization test: SENS-IS

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The SENS-IS assay previously developed by Immunosearch [1] is a new approach based on a reconstructed human skin model (Episkin) as test system and on the RT-PCR analysis of the expression of 62 biomarkers relevant to the considered biological processes.

Briefly, the test item is applied on the skin model at 4 different concentrations (50%, 10%; 1% and 0.1%) in the appropriate vehicle. After exposure of the test item, gene expression of three groups of genes is measured: one group (REDOX group) includes a selection of 17 genes that have an antioxidant responsive element (ARE) in their promoter and monitor the redox protective signals induced through the interaction of sensitizers binding to cysteine amino acids of the Keap1-NRF2 complex [2]. The second group (SENS-IS group) includes a selection of 21 genes involved in inflammation and cell migration to address the complex cascade of events leading to activation of DCs by a sensitizing chemical. A third group is involved in irritation signals by measuring the expression level of 23 genes. This combination attempts to reproduce the human skin situation and aims to deliver a detailed analysis of the skin response to the stress induced by the exposure to a test chemical or a mixture of ingredients. To evaluate both hazard and potency, 4 dilutions (50%, 10%; 1% and 0.1%) of the test chemical are applied on the skin model in an appropriate vehicle.

The prediction model is based on the number of over-expressed genes according to the following rules: if at a given test concentration a compound induces at least 7 genes in either the REDOX or SENS-IS group of genes, it is classified as a sensitizer. Potency, using the same classification as in the LLNA, is deduced through the minimal concentration needed to induce the over-expression of ≥7 genes in the REDOX or SENS-IS groups. A compound is classified as an extreme, a strong, a moderate or a weak sensitizer if detected as a sensitizer at a test concentration of 0.1%, 1%, 10% or 50% respectively.

While most of the currently available assays for the sensitization evaluation gives only a qualitative response, sens-is is the only test giving a quantitative response correlated with the murine local lymph node assay (LLNA; OECD TG 429). Its capacity to predict hazard is excellent [3] as demonstrated by Cooper statistics values over 95% on a large panel of chemicals

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Non-human primates in neuroscience research: can it be ethical, if it isn't scientifically necessary?

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Public opposition to non-human primate (NHP) experiments is significant and increasing, yet it continues in spite of this because those who defend them cite minimal harm to NHPs, and substantial human benefit. Both claims are refuted by evidence. Investigations and scientific papers over the years have documented the severe suffering that can be experienced by monkeys used in biomedical experiments, perhaps most notably in neuroscience. Neurophysiology experiments can be some of the most severe, typically involving brain surgery to implant recording devices into monkeys' skulls, harsh water deprivation regimes, immobilisation in primate chairs during experimental procedures which last several hours per day, and which can be conducted for years. We argue that such circumstances cause severe stress and distress, which not only adversely affect welfare, but also the reliability of experimental data. We have also comprehensively reviewed claims of human benefit, specifically in neuroscience, and show that: a) there is a default, speculative assumption of the human relevance and benefit of NHP neuroscience, rather than robust evidence to support it; b) the human relevance and essential contribution and necessity of NHP neuroscience are wholly overstated; c) the contribution and capacity of non-animal investigative methods are greatly understated; and d) confounding issues, such as species differences and the effects of stress and anaesthesia, are usually overlooked. This is the case in NHP research generally, but we have specifically focussed on salient historical and contemporary claims of NHP neuroscience researchers for the necessity of their work, namely: the development and interpretation of functional magnetic resonance imaging (fMRI), deep brain stimulation (DBS), the understanding of neural oscillations and memory, and investigation of the neural control of movement and of vision/binocular rivalry. The increasing power of human-specific methods, including advances in fMRI and invasive techniques such as electrocorticography and single-unit recordings, renders NHP approaches redundant. We conclude that the defence of NHP use is groundless, and that neuroscience would be more relevant and successful for humans if it were conducted with a direct human focus. We have confidence in opposing NHP neuroscience, both on scientific as well as on ethical grounds, and contend that the harm:benefit balance of NHP neuroscience is much more biased against it than is commonly accepted.

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Modernizing law and policy to support innovation in preclinical pharmaceutical testing in the United States

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Modernizing preclinical testing should mean shifting the testing focus from animals to existing and emerging tests based on human biology that generate predictive data for humans. While preclinical research is essential to gathering safety information before a treatment is tested in humans, the existing process largely depends on animals to predict what will happen in humans.

Today, 95 percent of new drugs fail because they do not work in humans or are unsafe, despite previously appearing safe in preclinical animal tests. According to U.S. FDA, adverse drug reactions cause over 100,000 deaths annually, making adverse drug reactions the 4th leading cause of death in the United States.

Modern, human-relevant test methods have the potential to accelerate the discovery, development and delivery of safer and more effective treatments. The U.S. FDA may accept human biology-based tests for preclinical drug testing. However, in practice, drug developers continue to use animal tests- even when a more human-relevant method may exist – because FDA regulations and reviewers continue to require them.

Laws, regulations and policy should create a path for scientific innovation and at the very least must keep pace with science to avoid stifling innovation. In order to bring written policy in line with stated policy, and to pave the way for further development and use of human-focused preclinical test methods, multiple law and policy modifications should be made. Such changes may include legislation, regulation updates, agency guidance, reforming the way new methods are evaluated and offering training programs to FDA reviewers and industry.

This presentation will describe current issues in drug development, such as cost, time, failure rate and adverse reactions; review acute toxicity data obtained through an analysis of new drug applications; highlight existing human-focused technologies, discuss emerging human-focused tests; and offer suggestions for law and policy change within the United States and through the International Council for Harmonisation that will increase the use of existing technologies and spark innovation of additional modern tests. A comprehensive, collaborative approach will have major impact through improving patient safety and reducing and replacing animal tests for pharmaceutical development.

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A novel method to predict fatty liver drugs using metabolic network based target identification

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Hepatocytes exhibits a wide range of functions extending from removal of toxic substances, homeostatic regulations and synthesis of most plasma membrane constituents as well as production of bile and hormones. Hepatocytes have higher metabolic activity in human and play an important role in human metabolism. Deficiency or alterations in the metabolism of hepatocytes can lead to complicated liver diseases like hepatitis, non alcoholic fatty liver disease (NAFLD), cirrhosis and liver cancer, and can be serious threats to public health. NAFLD is considered as the hepatic manifestation of obesity and metabolic syndrome as a result of series of pathological changes, which ranges from reversible fatty liver (Steotosis) to a non alcoholic steatohepatitis (NASH).

In recent years, the most common cause of chronic liver disease in USA is contributed by NAFLD. A recent study shows the need and cost associated with medication related to liver diseases as well as organ transplantation. Increase rates of obesity diabetes and high cholesterol have results in growing concern for liver diseases. NAFLD and Steatohepatitis are well linked but rare forms of drugs induced liver injury. In addition, fatty liver is often chronic than acute even when drug induced. Even though it is well known that the lipid accumulation in the liver is a hallmark of the NAFLD; the underlying mechanism leading to steotosis and further transition to NASH is still remains elusive. It is therefore difficult to track the onset and progression or to diagnose and design effective therapeutic techniques. The adverse outcome of this pathology may be possibly prevented once the molecular mechanisms involved in the metabolism of Hepatocytes are unraveled. On the other hand, this requires understanding of the coordinated behavior of a very large number of interconnected networks of drugs, molecular target as well as off-target, pathways, metabolic network and metabolites. Recent developments in the field of computational systems biology made it possible to predict the functional effects of systems perturbations using large scale network models. Subsequently, advances in the field of structural bioinformatics and chem-informatics have led to the prediction of protein-drug off-target effects based on their ligand structures and binding site information. Integration of these expertises provides a platform for evaluating metabolic drug response *in silico*. The combination of these approaches was applied to investigate the drugs that can cause fatty liver disease in human. Currently, there is no efficient treatment or explanation involved in the mechanism of NAFLD.

This study represents a novel integration of the trio 'computational systems biology, structural bioinformatics and chem-informatics' approaches to predict drugs involved in metabolic syndrome and the possible underlying mechanism.

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Migrating to the 3rd dimension: the "Neurosphere Assay" to study migration disturbances during neurodevelopment

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Neural progenitor cell migration and neuronal migration are key events during neurodevelopment. The "Neurosphere Assay" based on human neural progenitor cells growing as neurospheres is an *in vitro* tool to study specific disturbances of these neurodevelopmental key events. Using the "Neurosphere Assay", we have characterized different pathways involved in human neural progenitor cell migration, as well as pathways involved in cell migration induced by neurodevelopmental modulating factors present in the embryonic and fetal brain. Besides, to evaluate different possibilities of 3D structural organization of the model, we have established several supporting scaffolds for the short- or long-term culture of human neurospheres and tested the possibilities of compound testing depending on the scaffold provided. The "Neurosphere Assay" is an alternative *in vitro* approach to identify compounds disturbing independent migration, or migration responses to guidance cues during brain development and thus can support neurodevelopmental risk assessment by performing inter-species mode-of-action analyses.

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An *in vitro* system to predict the pro-fibrotic potential of carbon nanotubes in the lung

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With the increasing use of nanomaterials there is a need to investigate their possible adverse effects in humans. Our work has focused on multi-walled carbon nanotubes (MWCNTs) as they are one of the most commonly used nanomaterials in consumer products such as sporting goods and electronics. Humans are exposed to MWCNTs primarily via inhalation when they are released as aerosols into the environment during production [1]; therefore there is a need to predict possible adverse health effects. Pulmonary fibrosis is a key adverse effect that may be linked to MWCNT exposure such as Mitsui-7 [2]. Therefore, a human cell-based *in vitro* assay is required that can predict the (pro-)fibrotic potential of MWCNTs is needed.

The aim of this study was to mimic human inhalation of MWCNTs, using a cell exposure system (VITROCELL© Cloud system) for aerosol exposures to deposit MWCNTs at different concentrations onto cells cultured at the air-liquid interface. A new triple cell co-culture model was established consisting of human cell lines: macrophages (activated THP-1), epithelial cells (A549), and fibroblasts (MRC-5), and exposed to MWCNTs (Mitsui-7) at two concentrations (~1 μg/cm^2 and ~2 μg/cm^2) for 24 or 96 hours. The release of pro-fibrotic markers, such as osteopontin, platelet-derived growth factor, and transforming growth factor-β, was measured in the cell culture medium using ELISA. Our preliminary results showed that prolonged exposures (96 hours) are more suitable to predict pro-fibrotic effects. Further studies are ongoing to compare the effects of MWCNTs in a co-culture composed of cell lines to the one with primary cells.

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Commitment of Italian academic LARF-DIMES for teaching and training in alternative approach to animal testing

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Teaching on alternative methods is important within academia, including educating students on how to find alternative methods as appropriate so that every researcher knows about relevant alternatives, as well about the scientific limitations of an animal approach. Education and training also needs to include lessons by relevant scientists and regulators.

To underline the importance of both the ethical issues of alternative approaches to animal testing and the life sciences in the 21st century, LARF-DIMES, at the University of Genova (Italy), is engaged since 2008 in organization of training courses for dissemination of international resources using the over twenty years of experience of its staff in various fields of experimental pathology and teaching which demonstrates the greater effectiveness of animal-free testing. Moreover LARF team organizes stages for graduated and graduating students of Medicine, Biology, Biotechnology, Pharmacy degrees, PhD courses, and 2nd level Master on Reach regulations.

The courses, focused on practical part and demonstration/lessons of specialist(s) working in the field, provide basic knowledge or improve existing expertise, on alternative methods. In each course, the participants get an update on innovative *in vitro* models with particular emphasis on 3D models. The organization plan foresees a substantial practice to allow everybody to set up cytotoxicity tests, according to OECD guidelines, and other emerging alternative *in vitro* models. These at least 2 days training courses are opened to 24 participants and include already experts of *in vitro* methods as well undergraduating students. The participants came from all over Italy, and the interest has been so high that courses are consistently oversubscribed, with unlucky participants asking to be registered on future courses to secure a place. On 2013 LARF team was one of the winners of international Lush Prize for Training category.

From 2013 LARF, with a contribution by an animal rights society, have just organized 5 editions of a Basic Theoretical and Practical course, with the clause of a very low registration fees to allow even young ungraduated students to participate. The next edition will be planned on September 2016.

Two editions of an Advanced course (in 2014 and 2015) were funded by other international animal-right societies. In this course the new emerging technologies were further highlighted, such as 3D reconstructed human tissues, bioreactors, 3D spheroids and MEA systems.

In April 2016, the close cooperation between LARF and Cell-Tox allowed to organize a 3 days course (From cell to QSAR: predictive models in toxicology) with internationally acclaimed speakers among the prominent experts in *in vitro* models.

All training modules were carried out by LARF staff and specialists from leading companies in *in vitro* research and models, such MatTek, ETT, Lonza, Biopredict, IvTech and others.

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

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Establishment of an education program that meets the needs of researchers and animals

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Education and training of scientist is the basis for high quality research in accordance with animal welfare. This idea is also strongly reflected by the EU-Directive 2010/63/EU, the basis of the latest amendment of the German Animal Welfare Act.

Lately, new recommendations were given which support modular training courses. Not the least because the still existing block courses (f.e. compact courses "A" till "D" after FLEASA recommendations) suffer from similar problems. First there is a lack of species specificity. This is contrary to the EU- Directive and the German Animal Welfare Act, which claim species-specific training. Mostly, researchers also get trained in working with at least two species, whereby one is often not used in later all-day lab work and therefore unnecessarily increases the number of animals for educational purposes. Second, the courses are not flexible and thus often do not meet the needs of the researchers. A person performing subcutaneous injections in an animal experiment does not necessarily want and need to learn how to perform a surgical intervention. Third the courses are not based on one another. To get a higher certification level the participant has to complete a course, which involves subject matter often already educated in a prior course. This again increases the number of animals used.

To overcome these problems we established an individual modular system tailored to the often very specific needs of the scientist working with animals. The basis course imparts the handling and general rules for working with laboratory animals. On this basis people working with animal can gradually obtain further qualification. All the qualification necessary for working with animals, up to being head or deputy on an animal proposal, can be obtained. The provided modules are based on one other, take place regularly and are species-specific. This flexible module system allows the people working with animals, from students to group leaders, to gradually obtain exactly the qualifications they need for their actual work.

References

EU-Directive 2010/63/EU, German Animal Welfare Regulation Governing Experimental Animals, FELASA recommendations

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The "BINACLE" (binding and cleavage) assay allows *in vitro* determination of botulinum and tetanus neurotoxin activity

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The extremely potent neurotoxins produced by Clostridium botulinum and Clostridium tetani show high similarities with regard to their structure and mode of action. They consist of two disulfide-linked subunits: a heavy chain mediating the binding to specific receptors on neurons and a light chain displaying a metalloprotease activity. Following receptor binding and translocation of the toxin into the neuron, these proteases specifically cleave proteins required for the release of neurotransmitters from the synaptic vesicles. Depending on the preferred type of target neuron, the resulting inhibition of neurotransmitter release causes either severe muscle spasms (tetanus) or a flaccid paralysis (botulism).

Methods for the activity determination of these toxins are prescribed by the European Pharmacopoeia for the following applications:

– Tetanus vaccines contain tetanus neurotoxin (TeNT) which has been chemically inactivated. In order to ensure an adequate detoxification, each batch of the inactivated material must be tested for residual toxicity. Up to now, this is exclusively performed by *in vivo* toxicity testing in guinea pigs.
– Increasing amounts of the botulinum neurotoxin (BoNT) serotypes BoNT/A and BoNT/B are produced for pharmaceutical and cosmetic purposes. Due to the high toxicity of these proteins, these preparations have to be thoroughly tested for their potency. Although several animal-free methods have been described, no generally accepted method applicable to all relevant BoNT products is available to date. Accordingly, very high numbers of botulinum potency tests are still performed *in vivo*. In Germany, for example, almost 50000 mice were used in 2015 for this purpose [1].

Reliable alternative methods for the activity determination of these toxins should mimic as closely as possible their natural mode of action. We have developed a binding and cleavage ("BINACLE")-assay, which is based on the receptor-binding preferences and on the proteolytic activities of the respective toxins: In the first step, active toxin molecules are bound to their specific receptor molecules which were immobilized on a microtiter plate. Then the toxin's protease domain is released by chemical reduction and transferred to a second microtiter plate containing a specific substrate protein. Finally, cleaved substrate molecules are detected using antibodies. By taking into account the two most important characteristics of the toxins, this *in vitro* assay format offers a very high specificity combined with a good sensitivity.

The BINACLE assay for the detection of residual TeNT [2-5] is currently being validated in an international collaborative study, which is an important step for promoting both the acceptance of the method by manufacturers and regulators and the implementation of the method in the European Pharmacopoeia.

For BoNT/B, a BINACLE assay has recently been developed, and its applicability for a pharmaceutical product has been demonstrated [6]. A BINACLE assay for the potency determination of BoNT/A has also been developed and is now entering the validation phase.

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Towards lung disease-on-chip models and personalized medicine application

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Organs-on-chip are widely seen as being the next generation of *in-vitro* models. In contrast to standard models based on Petri dish technology, they allow to reproduce the cellular environment found *in-vivo* in an unprecedented way. A further benefit of these systems is the small amount of cells required to run an assay. This is an important condition when scarce patient material is tested. Ultimately, healthy and diseased cells from each patient will be tested in such systems to predict the drug response and tailor the best therapeutic treatment for each patient. Here we report about two organs-on-chip that reproduce key aspects and/or functions of the lung. The first model is a lung-on-chip that reproduces the alveolar barrier including the physiological strain generated by the respiration [1]. The second model mimics a perfusable and functional lung microvasculature [2].

In contrast to the lung-on-chip reported by Ingber and colleagues in 2010 [3], the present lung-on-chip not only reproduces the ultra-thin alveolar barrier, but also the three-dimensional cyclic strain generated by breathing movements. In addition, the open design of the lung-on-chip enables to accurately control the cell density seeded on each side of the 3um thin, porous and flexible membrane. Lung epithelial and endothelial cells were cultured on both side of the membrane to create to an alveolar barrier. Upon confluence of the cells, the alveolar barrier is cyclically stretched in three-dimensions as it is in *in-vivo* at a physiological strain level (corresponds to 10% elongation). The intact lung alveolar epithelium was scratch with a pipette tip to mimic lung epithelial microinjuries believed to be at the origin of idiopathic lung fibrosis. Upon exposure to a potent wound-healing promoter, HGF, the wound healing process was monitored during 24 h either in static or in dynamic mode (10% linear stress). First results show that a physiological mechanical stress significantly inhibits the wound healing process.

The lung microvasculature-on-chip model was created using primary human endothelial cells and pericytes from patients. The cells were embedded in fibrin gel contained in a microfabricated compartment. The cells self-assemble in tight and stables microvessels that can be perfused. The role of the pericytes, which lined on the endothelial vessels, enables the creation of stable (several days) and functional vessels. Upon exposure to phenylephrine, a vasoconstrictor the vessels contract within a few minutes. When the microvasculature was only created with endothelial cells, the vessels were wider and did not contract upon exposure to phenylephrine.

Organs-on-chip enable the reproduction of key aspects of the cellular microenvironment, such as the mechanical stress of the respiration and or the perfusability and contractibility of microcapillaries. Such features may soon be of great benefit for the drug discovery process to efficiently screen drug candidates.

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Replacement of goat xenobiotic metabolism studies beyond OCED 503 by use of rumen simulation technique (RUSITEC)

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Introduction

For registration of plant protection products (PPP) 14C-metabolism studies are required in rat (OECD 417), livestock (OECD 503) and plants (OECD 501, OECD 502). Occasionally there are specific questions occurring on the ruminant xenobiotic metabolism: 1) Are the observed metabolites ruminant specific and formed directly in the rumen? 2) Are ruminants able to cleave plant specific metabolites like glycosides to the respective aglycon? In the past new additional *in vivo* goat metabolism studies beyond OECD 503 goat metabolism study with at least one animal were performed to address these open questions.

Literature research identified the rumen simulation technique (RUSITEC) as a potential appropriate method to simulate the rumen and its metabolic behaviour *in vitro*. The aim of this project was to elucidate if RUSITEC is able to address robustly specific questions on xenobiotic metabolism in ruminants for registration of PPP beyond OECD 503.

Materials and methods

Fresh ovine rumen fluid was incubated *in vitro* 7 days by using RUSITEC. The conservation of the physiological conditions was proven by measurement of pH and Redox potential. The microflora composition of the rumen fluid and its viability was monitored by microscopy, incubation on agar plates and the detection of β Glucosidase.

The metabolic behavior and performance of the rumen fluid was tested by e.g. incubating 14C-triazole derivative metabolites (TDM) like triazole alanine (TA), triazole acetic acid (TAA) and triazole lactic acid (TLA), which are usually formed in plants after application of triazole-containing fungicides. 14C-TA, 14C-TAA and 14C-TLA were applied to the RUSITEC and their metabolic stability was tested by Radio-HPLC over 96 hours. The glucosidase activity was tested directly by β-Glucosidase Activity Assay and indirectly by application of 14C-Octyl-β-D-glucopyranosid and 12C-Polydatin by Radio/UV-HPLC within 96 h.

Results

The pH (mean = 6.70 ± 0.07) and the redox potential (mean = –301 mV ± 30) kept constant within 192 h. The bacteria count kept constant from 120 h (Mean 4.1xE7 ± 0.8) to 192 h (Mean 4.8 xE7 ± 1.4 per mL rumen fluid). Radio-HPLC showed that TA was cleaved within 72 h to 1,2,4-triazole, while TAA and TLA were stable, what is already proven in an *in vivo* situation (OECD 505). β-Glucosidase activity was determined at 4.8 ± 1.0 U/L between 48-192 h. These data were supported by a fast and complete degradation of 14C-Octyl-β-D-glucopyranosid or 12 C-Polydatin to the respective aglycon.

Conclusion

All vitality tests confirmed that the RUSITEC is a successful tool to maintain sheep rumen fluid for at least 7 days *in vitro*. The rumen fluid maintained its main metabolic performance by using RUSITEC. BASF identified the RUSITEC method as an appropriate method to investigate specific questions on xenobiotic metabolism in ruminants. In future BASF will replace *in vivo* animal studies on ruminant metabolism studies beyond OECD 503 by performing RUSITEC studies. The method will be included in the common method portfolio of BASF leading to a significant contribution to the 3R strategy.

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Infection of epithelial cells and fresh human lung tissue with persistent *Pseudomonas aeruginosa*

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Background

Pseudomonas aeruginosa is an opportunistic pathogen known to cause persistent pneumonia, especially in patients suffering from cystic fibrosis. Selection of antimicrobial treatment against *P. aeruginosa* infection is commonly based on microbiological diagnosis using e.g. the determination of antibiotic minimal inhibitory concentrations (MIC). However, the standard assays do not consider the microenvironment of biofilms leading to antibiotic resistance and failure of therapy in clinics. To mimic the *in vivo* situation more closely, we infected respiratory epithelial cells or fresh lung tissue with *P. aeruginosa*.

Methods

Air-liquid-interface cultures of A549 human epithelial cells or PCLS (precision-cut-lung-slices) from rat or human lungs were prepared and infected with *P. aeruginosa* strain PAO1 (105/well). Infected cells and lung tissue were treated with different concentrations of tobramycin. Cell and tissue viability was measured 6 h and 24 h post infection via LIVE/DEAD® staining and confocal microscopy or photometric quantification. Bacterial load was determined by dilution plating and counting of colony-forming units. Furthermore, PAO1 localization within the lung tissue was investigated using fluorescence labeling and confocal laser scanning microscopy.

Results

P. aeruginosa infected epithelial cells A549 and lung tissue *ex vivo*. Cells and tissue remained viable within the first 6 hours of infection. Progression of infection caused loss of tissue viability and cell death most probably due to tissue necrosis. Addition of low doses of tobramycin to *P. aeruginosa*-infected cells or tissue achieved sustained 80% viability after 24 h of cultivation.

Conclusion

Low doses of tobramycin enabled a stable co-cultivation of 10^6 *P. aeruginosa* and respiratory cells or fresh lung tissue for 24 h which is a prerequisite for the development of persistent pneumonia. Further experiments will focus on an expansion of the cultivation period. This setup can be used in the future for efficacy and toxicity testing of novel anti-infectious compounds in the microenvironment of lung tissue

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Use of alternative methods for the prediction of combined effects from chemical mixtures

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Humans and wildlife can be exposed to an infinite number of different combinations of chemicals in mixtures via food, consumer products and the environment. Examples in the literature show that single substances present below their individual threshold of effect, can still be of concern in combination. It is practically unfeasible to test all these possible mixtures experimentally. Therefore smart strategies are needed to assess the potential hazards using new tools that rely less on *in vivo* testing and incorporate instead alternative experimental and computational tools.

In order to explore the use of such tools for the hazard assessment of mixtures, recent literature has been reviewed and a survey on expert experiences in the assessment of combined effects been conducted. The current state of the art for the application of alternative tools was evaluated, focusing on the adverse outcome pathway (AOP) concept, *in vitro* methods, omics techniques, *in silico* approaches, toxicokinetic and dynamic energy budget (DEB) modelling, and on integrated approaches to testing and assessment (IATA).

Expert opinions in the survey regarding the use of novel tools in the risk assessment of mixtures were split between those applying them (often more in a research context) and those that generally think these tools are valuable but their use is currently limited because of lack of guidance, lack of data, or lack of expertise. The literature review showed how these tools allow deriving meaningful information on individual mixture components or whole mixtures, leading to a better understanding of the underlying mechanisms of their individual and combined effects. Using the above-mentioned tools in smart combination and an integrated way, different aspects regarding the hazard from combined exposure to multiple chemicals can be put into context. This will finally allow a better, mechanistically based prediction of mixture effects.

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Analysis of ToxCast data – *in vitro* and physiochemical properties – in the accurate classification of chemicals that induce hepatocarcinogenesis *in vivo*

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Background
In vitro and *in silico* methods continue to be evaluated for their potential to inform chemical toxicology evaluation. The research arm of the Environmental Protection Agency has been one of many research bodies evaluating the potential of such methods as part of their ToxCast initiative. We set out to advance the on-going discussion of improving toxicity testing by exploring whether or not ToxCast physiochemical properties and high throughput assay data could be used as covariates in predictive models to accurately classify chemicals that either do not or do induce hepatocarcinogenesis *in vivo*.

Results
ToxCast physiochemical and high throughput assay data were evaluated against known chemicals and *in vivo* endpoints from the ToxRef curated data set. Hepatocarcinogen causing chemicals were found to be larger, more lipophilic and complex in shape than control group chemicals. Adjusted logistic regression models using physiochemical properties as covariates accurately classified 71 percent of the chemicals into the case or control groups, with overall higher specificity than sensitivity. ToxCast *in vitro*, high throughput assays revealed that the activity of two transcription factors exhibited differences across the case and control groups: Nrf2 and e2f. Logistic regression using high throughput assay data as covariates resulted in an adjusted model that correctly classified 71 percent of the chemicals into the case or control groups, also with overall higher specificity than sensitivity. A combined logistic model using physiochemical properties and high throughput assay data as covariates exhibited similar performance compared to the two adjusted models previously discussed.

Discussion
We found that logistic regression models using physiochemical properties and high throughput assay data as covariates perform similarly well, accurately classifying chemicals at similar sensitivity and specificity. This analysis suggests that either form of data can be used in the accurate classification of hepatocarcinogenesis, and possibly other apical endpoints. This finding represents a valuable, incremental step forward in the use of such data in the evaluation of chemicals against apical endpoints of health concern. Further study is needed particularly with regard to sensitivity across models, irrespective of the use of physiochemical properties or high throughput assay data.

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The US EPA's methodology to inform TSCA premanufacturing notification decision-making: a critical analysis based on chemicals regulated by consent order

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Background

Regulatory toxicology has emerged as a necessary discipline to ensure the chemicals introduced into commerce are safe. In the United States, a number of federal laws guide regulatory toxicology, including the Toxic Substances Control Act (TSCA). The New Chemicals program is administered by US Environmental Protection Agency (EPA). This program evaluates premanufacture notifications (PMNs) of new chemicals or significant new uses of existing chemicals. PMN submitters are not required to include human health or environmental effects data, nor are they required to undertake any toxicity testing. These statutory restrictions place constraints on the EPA with respect to the basic data available to inform decisions, and the EPA has only 90 days to make regulatory decisions on PMN substances.

Using publically available information about decisions on new chemicals, we sought to determine the methodology used by the TSCA New Chemicals Program, including data proffered or generated, to inform such regulatory decisions; specifically, consent order decisions.

Results

The consent order regulatory decisions evaluated in this study applied analog analyses, or *in silico* methods to inform two-thirds of the decisions. For these decisions, no new *in vivo* or *in vitro* tests were carried out by EPA or at the direction of EPA. PMN submissions included testing data about one half of the time, which informed one third of the decisions to regulate by consent order. More broadly, test results submitted by PMN submitters were often not aligned to the health concerns identified by the EPA. Multiple human (3 or more) and environmental health concerns were associated with decisions to regulate.

Discussion

Because of the way in which TSCA is written and implemented, the EPA must operate in a data poor information environment. In response, the EPA has developed alternative testing methods, which by definition include non test methods, to inform and assist in PMN regulatory decision making. This demonstrates that TSCA has resulted in the utilization of these alternative methods, demonstrating that laws can provide an opportunity for innovation and efficiencies even if explicit incentives are not designed into the law. TSCA is not the only law to promote such innovation. For instance, the European Commission required the phase out of animal tests in cosmetics. The adoption of alternative testing methods is consistent with the National Academies vision of toxicity testing in the 21st century. This study and findings are important for other regulators in the potential adoption of alternative testing methods to inform regulatory decisions going forward.

Reference

Abstract based on in press paper at: http://www.inderscience.com/info/ingeneral/forthcoming.php?jcode=ijram

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Ethical aspects of the Crispr/Cas9 technology

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Say we completely master Crispr/Cas9 technology. How, then, would we go about judging this technology from an ethical perspective? Is the large scale application of the Crispr/Cas9 in fact ethically permissable?

In my lecture, I will explore various considerations while searching for an answer to this question. The standard way to approach this issue would be to draw up a Harm Benefit Analysis. I will make an attempt here to assess the "Benefits" and the "Harm" within the Crispr/Cas9 technology. In doing so, I will outline the various perceptions from which we can analyse the concepts of "harm" and "benefit". This, however, will only add to the confusion, because different fields of perception offer different definitions of Harms and Benefits. What could help here are the EU guidelines. In these guidelines one can find an unequivocally ethical approach, namely consequentialist and zoocentric. This in turn provides an answer to the ethical permissibility of the technology, but at the same time, the possible objections to this answer also emerge.

My goal is to join forces with the participants in Linz to come up with creative solutions for the stumbling- blocks to large scale use of the Crispr/Cas9 technology.

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Establishment and characterization of a lung/liver organ-on-a-chip model. Phase 1: model characterization

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Over the past few years, we have invested considerable resources into the testing of systems toxicological responses of human bronchial organotypic cultures to acute cigarette smoke and prototype or candidate modified risk tobacco products' (MRTP) aerosol exposure. Recently, we have extended these efforts in an attempt to establish quality parameters for 3D lung cultures, to facilitate future assay development and validation and to establish baseline parameters that could aid in designing repeated exposure experiments.

Increasing public efforts aimed at replacing animals for research purposes and the drive toward employing novel tools for toxicological assessment of new products has led us to consider the integration of additional *in vitro* systems such as those mimicking biological processes in the liver and their combination with some of our existing *in vitro* lung models. We expect this to be feasible by leveraging the recent advent of microphysiological systems such as organs-on-chip models and 3D liver spheroids. Naturally, it is imperative to also fully characterize any additional tissue that will be included in a two-organ microfluidic system. The qualitative and quantitative data obtained will not only help to understand the long-term stability of the liver model, it will also shed light on its responsiveness to various stimuli. Ultimately, once combined, studies are needed to explore the crosstalk between the two tissues at several levels and to gauge whether such a system is amenable to toxicity testing.

For the first phase of this study, we constructed 3D differentiated airway epithelia from primary human bronchial epithelial cells at the air-liquid interface and examined morphology, apical ATP secretion and metabolic capability over a 4-week period. In addition, we produced HepaRG spheroids and evaluated their morphology in addition to metabolic capability and albumin production for 4 consecutive weeks to characterize this liver model in more detail. Furthermore, we started to adapt and test cell culture media to enable the combination of the individual models in one closed system in the next phase. The results presented here provide a summary overview of our efforts to date. We also highlight useful insights into the behaviour of the cultures over time and conclude with an outlook on future steps.

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CAAT Academy: from inception to launching

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Over the last thirty years, dozen of validated alternative test methods exist in the EU and even more thanks to ICATM collaboration. Nevertheless, when one looks at the number of testing proposals submitted to REACH it is clear these methods are not being put to sufficient use. While ad-hoc events, tailor-made training, webinars, and scientific meetings regularly provide training in these new methods, more efforts should be invested into "after-sales" services to disseminate the emerging technologies and reach new audiences. The European Commission and the member states are actively filling the gaps in training via EU research programs such as Framework Program7, Horizon2020, and the innovative medicines initiatives. This presentation will illustrate the mission of CAAT Academy's 3Rs training to increase the use of validated alternative methods and provide feedback and lessons learned from the first two trainings in Romania (in silico tools under REACH) and in France (Hepatoxicity testing and best practices). Last, the sustainability of such initiatives will be described and the objectives of the medium-term announced.

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3Rs education in veterinary science: a course on *in vitro* toxicology

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The University of Milan, offered a new 3 credits course on *in vitro* toxicology, 30 hours, inclusive of 6 hours of lectures and 24 hours of practice, from the AY 2015/2016, first semester, in the second year of the master degree of Veterinary Biotechnology Sciences. The course, entitled "Toxicology and *in vitro* model", is mainly practical, while the theoretical is only of support, in order to provide to students knowledge of fundamental *in vitro* toxicity tests, information on new methodologies and applicability domain. In the lab, students first became familiar with classical cytotoxicity assays i.e. cytokines release (IL-1alfa), LDH release, MTT test, NRU test and subsequently they learn the use of different *in vitro* models from 3D to epithelial barriers, and their applicability in toxicology, with a particular attention on the emerging techniques. Through a meeting of experts, an approach of comparison between *in vitro* and *in silico* data was addressed. At the end of the course a feedback of students was also required, by filling a questionnaire.

Acknowledgement: Emilio Benfenati, Helena Kandarova, Thomas Hartung

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G-protein based ELISA as a potency test for human rabies vaccines

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Human rabies vaccine potency is currently measured using the NIH test and is a key criterion for vaccine release. This test is based on mice immunization followed by intracerebral viral challenge.

While the test is well accepted as a measure of vaccine potency/efficacy, this *in vivo* challenge assay presents several critical issues such as its high variability and the large number of animals required for each assay.

As part of global efforts to reduce animal experimentation and to comply with Sanofi Pasteur internal 3R policy, we developed an ELISA as a promising alternative to the NIH test.

This ELISA is based on the capacity of 2 conformational monoclonal antibodies to specifically recognize the native form of the viral G protein. It has been shown previously that the G protein is the major antigen that induces neutralizing antibody response to rabies virus.

Our data shows that the Sanofi Pasteur rabies G-protein based ELISA:

– is a rapid test and is well adapted to in-process rabies vaccine production monitoring;
– is able to distinguish between potent and different types of artificially generated, sub-potent vaccine lots;
– yields results in good agreement with the *in vivo* NIH test and meets the criteria for a stability-indicating assay

In conclusion, the Sanofi Pasteur rabies G protein ELISA is a scientifically relevant and validated *in vitro* potency assay for release testing of rabies vaccines.

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Cardiotoxicity gene and miRNA biomarkers induced by anticancer drugs

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The safety evaluation of candidate drugs is usually cost-intensive and time-consuming and is often insufficient to predict human relevant cardiotoxicity. The purpose of this study was to develop an *in vitro* repeated exposure toxicity methodology allowing the identification of predictive genomics biomarkers of functional relevance for drug-induced cardiotoxicity in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Anticancer drug therapy is very often accompanied with the development of heart failure (HF). The hiPSC-CMs were incubated with 156 nM doxorubicin, which is a well-characterized cardiotoxicant, for 2 or 6 days followed by washout of the test compound and further incubation in compound-free culture medium until day 14 after the onset of exposure. An xCELLigence Real-Time Cell Analyser was used to monitor doxorubicin-induced cytotoxicity while also monitoring functional alterations of cardiomyocytes by counting of the beating frequency of cardiomyocytes. Unlike single exposure, repeated doxorubicin exposure resulted in long term arrhythmic beating in hiPSC-CMs accompanied by significant cytotoxicity. Global gene and miRNA expression changes were studied using microarrays and bioinformatics tools. Analysis of the transcriptome and miRNA data revealed early expression signatures of genes involved in formation of sarcomeric structures, regulation of ion homeostasis and induction of apoptosis. Eighty-four significantly deregulated genes related to cardiac functions, stress and apoptosis were validated using real-time PCR. The expression of the 84 genes was further studied by real-time PCR in hiPSC-CMs incubated with daunorubicin and mitoxantrone, further anthracycline family members that are also known to induce cardiotoxicity. A panel of 35 genes was deregulated by all three anthracycline family members and can therefore be expected to predict the cardiotoxicity of compounds acting by similar mechanisms as doxorubicin, daunorubicin or mitoxantrone.

Doxorubicin (156 nM) induced early deregulation of 14 miRNAs (10 up-regulated and 4 down-regulated) and persistent up-regulation of 5 miRNAs during drug washout.

Quantitative real-time PCR analyses confirmed the early deregulation of miR-187-3p, miR-182-5p, miR-486-3p, miR-486-5p, miR-34a-3p, miR-4423-3p, miR-34c-3p, miR-34c-5p and miR-1303, and also the prolonged up-regulation of miR-182-5p, miR-4423-3p and miR-34c-5p. Several of the deregulated genes and miRNAs have been identified to be similarly deregulated in cardiac diseases such as HF. The identified genes and miRNAs represent sensitive biomarkers for the safety assessment of potential cardiotoxicity hazards.

References

More recently, we have published this work in the following publications:

Chaudhari, U., Nemade, H., Wagh, V. et al. (2015). *Arch Toxicol*, Epub ahead of print.

Chaudhari, U., Nemade, H., Gaspar, J. A. (2016). *Arch Toxicol*, Epub ahead of print.

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Towards a harmonized approach: role of the German National Committee for the protection of animals used for scientific purposes in the process of implementation of the Directive 2010/63/EU

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Implementation of a directive by the Member States always implies at least two potential sources of inconsistency: on the one hand, Member States are granted a certain margin of appreciation concerning the shape of the national implementing measures; on the other hand, differences in the application of law on the national level emerge between different responsible authorities. The latter occurs often in countries with a federal structure like Germany, where enforcement of the animal welfare law is a sovereign task of each federal state.

In this context, the National Committee, created to advise the competent authorities and animal welfare bodies, is an excellent tool to promote harmonization and dialog between all parties involved in the process of law enforcement and project evaluation. The German National Committee is part of the German Centre for the Protection of Laboratory Animals (Bf3R) at the Federal Institute for Risk Assessment (BfR). The legally guaranteed scientific independence of the BfR is an important asset of the National Committee's way of working. Pursuing a strictly scientific and impartial approach facilitates the identification of problems and paves way for a dialog with all involved parties. The instruments used by the National Committee to develop solutions for a harmonized enforcement of animal welfare law will be presented along with some recent examples from the daily work.

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Development of a reconstructed human scalp with microfollicles using three-dimensional techniques

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The hair care sector represents a promising and competitive market for cosmetic industry, in which development of new products and adoption of safety tests and assertive claims are essential requirements. *In vivo* tests are associated with high costs and ethical implications, while *in vitro* methodologies provide quick and practical alternatives for the screening of new ingredients. The present work focused on the *in vitro* development of a reconstructed human skin containing spheroids of hair cells to simulate the human scalp. First, microfollicle-like spheroids formed by keratinocytes, dermal papilla cells, endothelial cells and melanocytes were obtained. After 1-2 weeks, spheroids were transferred to an equivalent dermis for subsequent obtainment of equivalent epidermis. Differentiated reconstructed tissues were submitted to immunohistology for analysis of specific cell markers in comparison to *ex vivo* tissues. Cytokeratin 15 (CK15) – an epidermal stem cell marker– and vimentin (VIM) – a mesenchymal marker present in hair cells and dermis – showed similar pattern to *ex vivo* follicles as well presented similar form and stratification of the different kind of cells. Hematoxylin-eosin staining evidenced a similar deposition of basic proteins when reconstructed and *in vivo* follicles were compared. The results herein observed represent a promising alternative for development of an *in vitro* scalp, which might expand possibilities for efficient and assertive evaluation of innovative hair care products without necessity of human volunteers and/or animals.

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Self-assembled 3D spheroids of MSC-derived hepatocyte-like cells for *in vitro* toxicity studies

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3D cultures of human stem cell-derived hepatocyte-like cells (HLCs) have emerged as promising models. Primary hepatocytes cultured as spheroids maintain the hepatic phenotype in short and long-term *in vitro* cultures, by better resembling the *in vivo* environment of the liver and consequently increasing the translational value of the resulting data [1]. Additionally, human-relevant data on hepatic drug metabolism toxicity must ideally rely on cell systems that exhibit all liver-specific functions with a highly available source of human cells.

In this study, the first stage of hepatic differentiation of human neonatal mesenchymal stem cells (hnMSCs) was performed in 2D monolayer cultures for 17 days [2]. The second stage was performed by either maintaining cells in 2D cultures for an extra 14 days as control, or alternatively cultured in a 3D system as self-assembled spheroids. hnMSC differentiation into HLCs is shown by positive immune-staining of hepatic markers CK-18, HNF-4α, albumin, hepatic transporters OATP-C and MRP-2 and drug metabolizing enzymes as CYP1A2 and CYP3A4, as well as displaying relevant glucose, phase I (EROD, CYP3A4, CYP2C9 and CYP1A1) and phase II (UGTs) metabolism, and the ability to produce urea and albumin. Particularly, EROD activity and urea and albumin production were increased in 3D culture. At day 27 spheroids revealed higher bupropion conversion whereas 2D culture, upon diclofenac exposure, produced higher amount of 4-OH-Diclofenac. To further exploit the applicability of HLCs for predicting drug induced cytotoxicity, HLCs were exposed to diclofenac, at concentrations ranging from 0.05 to 4 mM, and cytotoxicity profiles were calculated in 2D and 3D systems by MTS and LDH. This resulted in an IC_{50} value of 1.51 ± 0.05 and 0.98 ± 0.03 mM in 2D and spheroid cultures (MTS), respectively. The results herein observed may suggest that a more complete bioconversion of diclofenac and its metabolites may be occurring in the spheroid-based system, resulting in higher diclofenac cytotoxicity in this cell culture model.

In conclusion, 3D spheroid culture improved HLC maturation showing relevant biotransformation capacity, providing a more reliable human model for *in vitro* toxicology applications, namely in mechanistic studies, resulting in more accurate hepatotoxicity assessment *in vitro* and, eventually, reducing animal experimentation, following the 3R principles.

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OncoCilAir™: organotypic human *in vitro* model for lung cancer research

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With more than 1 million deaths worldwide every year, lung cancer remains an area of unmet needs. Realistic human 3D models are required to improve preclinical predictivity. To that end, we have engineered a novel *in vitro* lung cancer model, OncoCilAir™, which combines a functional reconstituted human airway epithelium, human lung fibroblasts and lung adenocarcinoma cell lines. Because of its unique lifespan (> 3 month) and its dual composition (healthy and compromised human tissues), OncoCilAir allows for the concurrent testing of the efficacy of drug candidates against malignant cells and their non-toxicity against healthy tissues, all in a single culture well [1,2]. Accordingly, a first proof of concept study performed on a panel of anti-cancer drugs including the investigational drugs selumetinib and Mekinist® demonstrated that OncoCilAir™ carrying the KRASG12S mutation showed responsiveness in agreement with first clinical reported results, validating this unique tissue model as a predictive tool for anticancer drug efficacy evaluation [3]. To replicate *in vitro* patient's stratification we have now extended the model to EGFR mutations which account for 16% of lung adenocarcinoma. Results showed that as expected, OncoCilAir™ EGFRdel19 is sensitive to Tarceva® and Iressa® treatments and therefore provides a useful model to decipher *in vitro* mechanisms of resistance. In addition, we found that OncoCilAir™ may be used for translational testing of inhalation therapies, like functionalized nanoparticles. The efficacy of aerosol therapies depends on physical properties of the airway, including the secretion of mucus and the strength of cilia beating. OncoCilAir™ recapitulates *in vitro* such pitfalls, with the presence of goblet cells producing mucus and functional ciliated cells. Moreover, repeated airborne delivery of compounds to OncoCilAir™ is achievable by nebulisation, making possible aerosol *in vitro* chronic treatment simulation up to three months repeated dose testing. Lastly, because it retains *in vitro* the specificities of human tropism to viral infection, OncoCilAir™ stand out as an ideal model for testing oncolytic virus therapies [4]. All together, these data highlights the OncoCilAir™ model as a versatile platform which provides a unique opportunity to accelerate the development of optimal lung cancer therapies while sparing the lives of many animals.

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Establishment and characterization of a novel 3D human *in vitro* small airway model

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The small airways are non cartilaginous airways with a diameter < 2 mm, which are extremely venerable to external insults such as tobacco smoke, mineral dust, air-pollutants, allergens, drugs, bacterial and viral infections. They play an important role in many lung diseases including chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), sarcoidosis and obliterative bronchiolitis (OB). However, the small airways constitute one of the least understood areas of the lungs due to the inaccessibility *in vivo*. Therefore, an i*n vitro* human small airway model would be tremendously valuable for toxicity testing of chemical substances and for studying various respiratory diseases.

We report here the establishment and characterization of a novel *in vitro* human small airway model (SmallAir™). The primary epithelial cells were isolated from the distal lungs by enzymatic digestion. After amplification, the cells were seeded on the microporous membrane of Transwell inserts. Once confluent, the cultures were switched into air-liquid interface. After 3 weeks of culture, the epithelium became fully differentiated, with morphology of columnar epithelium, and a thickness of 10-15 µm. The epithelium is electrically tight (TEER ≈ 400 ohm.cm^2). Most significantly, CC-10, a specific marker of Clara cells, was highly expressed in SmallAir™. CC-10 was detected by both immuno-histochemisty and Western Blot. As expected, SmallAir™ contained few Muc5-Ac positive cells (goblet cells). In contrast, CC-10 was not detected in MucilAir™, an *in vitro* model of the human bronchial epithelium. Instead, Muc-5Ac was highly expressed in MucilAir™. SmallAir™ contained also basal cells and ciliated cells, showing cilia beating (8 Hz) and mucociliary clearance (15 mm/s). SmallAir™ represents a unique and powerful tool for studying the physiology and function of small airways and it should provide new insights into this major area of lung diseases.

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The use of *in vitro* human airway epithelia for the development of novel antivirals

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Objective

Respiratory viral infections cause mild to severe diseases in worldwide, such as common cold, bronchiolitis and pneumonia and are associated with huge costs to society. To test new molecules for shortening, alleviating the diseases or to develop new therapies, relevant *in vitro* human models are necessary. We propose here human reconstituted standardized nasal epithelia, MucilAir™ for a proof of concept study of viral drug screening. The MucilAir™ respiratory epithelium possesses defence mechanisms, comparable to *in vivo* situation, such as mucus production, mucociliary clearance, and secretion of defensive molecules. For each virus, several different parameters were assessed: the replication kinetics, the cell tropism, the impact of the virus on tissue integrity and cytokine secretion.

Methods

Clinically relevant Rhinovirus (A16, C15), Enterovirus (EV68) and Influenza A virus (H1N1, H3N2) strains were added to fully differentiated MucilAir™-Pool (mix of 14 donors) cultures at the apical side. Apically released viral genome copy number, overall mucin secretion, cilia beating frequency, velocity of mucociliary clearance and tissue integrity were assessed daily during 4 days. Basal media of the MucilAir™ cultures served for cytokines as well as toxicity measurements. Different concentrations of antiviral treatments were carried out parallel and all endpoints were compared with the control conditions.

Results

MucilAir™ cultures showed an excellent host characteristics and high rate of replication for all tested viruses, including difficult-to-culture Rhinovirus C15. Infections in MucilAir™ cultures permitted us a detailed description of the early viral pathomechanisms in the nasal epithelia for the different viruses. Rupintrivir efficiently inhibited the replication of HRV-A16 and HRV-C15 in a dose and time dependent manner and restored the mucociliary clearance impaired by EV68. Oseltamivir reduced the replication of H1N1 and H3N2 and restored the impaired barrier function monitored by transepithelial electrical resistance.

Conclusion

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

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Evaluation of reconstituted human bronchial epithelium (MucilAir™) suitability for repeated dose testing

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The airway epithelium reconstituted *in vitro* is a powerful tool for studying the respiratory diseases as well as for the assessment of airborne toxicants. However, one of the problems in long term culture is the de-differentiation of the ciliated cells: after certain period of culture, the cells lost cilia and the cilia-beating became weak or absent; the morphology of the epithelia may also change with time; the ratio between the different types of cells could also be altered, etc.

In order to use MucilAir™, a 3D cell model of the human airway epithelium, for long term or repeated dose toxicity testing, it is important to assess its stability in term of morphology and function. Fortunately, most of these changes can be easily monitored on a fully differentiated bronchial model. This study, designed to assess the stability of bronchial MucilAir™, was performed in three different laboratories and by different operators over 80 days of cultures. Most of the endpoints used are non-destructive, for example, TEER, Cilia Beating Frequency, mucin secretion, which allows monitoring the same tissues over a long period of time. In addition, histological analysis and immunostaining for specific biomarkers were also performed to evaluate each type of cells precisely. Namely, (i) H/E-Alcian Blue, to see the pseudo-stratified architecture, as well as the goblet cells (ii) p63 for basal cells (iii) Ki67 for proliferating cells, (iv) Muc-5Ac for Goblet cells and (v) FoxJ1 for ciliated cells.

Despite of some minor differences, the overall results demonstrated that the fully differentiated bronchial epithelium (MucilAir™) is indeed stable at different laboratories and the endpoints used are highly relevant and reliable.

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Development of an innovative moving interface for an *in vitro* model of the epithelial intestinal barrier

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Most physiological barriers are exposed to a dynamic environment, characterized by flow of biological fluids and tissue contraction, exposing epithelial cells to continuous mechanical deformation. *In vitro* models using human cells can represent a valuable alternative to animal experimentation because they enable the investigation of specific phenomena in a controlled environment [1]; however, the traditional cell culture models are static and fail to reproduce the physiological dynamics. Some efforts have been made to develop more sophisticated alternatives capable of providing mechanical stimuli to the cells as is the case of systems composed of a deformable membrane that can be stretched using pneumatic devices [2-5].

Our goal is to recreate the dynamic environment of the intestine with an advanced *in vitro* model that mimics the moving interface between the intestinal lumen and the internal mucosa. The system consists of a cell culture chamber actuated with motion through the use of an electroactive polymer (EAP) as cell substrate, which simulates intestinal peristalsis. EAP's are a class of polymers that undergo mechanical deformation when an electric field is applied [6]. The DEA membrane was made porous in the cell culture region by engineering an array of tungsten microneedles controlled by a micropositioner. To make the membrane electrically tuneable, deformable electrodes consisting of conductive liquid silicon were sprayed on the both sides of the active region of the DEA film.

Results demonstrated that the system is biocompatible, and suitable for Caco-2 cell adhesion and proliferation. After the culture period, the device was actuated for four hours of stimulation, generating strains typical of human intestinal motion (10%) at physiological frequencies (0.15 Hz). Cell imaging confirmed that the integrity of the cell barrier was maintained and epithelial cell markers were expressed.

This work establishes the potentiality of the DEA technology to develop relevant *in-vitro* models of the human physiological barriers, increasing the correlation between the *in vitro* data and the human case, thus reducing the need for animal testing.

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Improving confidence in read-across: a case study for the oral repeated dose toxicity of β-olefinic alcohols

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Read-across is an established technique for data gap filling. Historically it relied on the premise that compounds that share similar chemistry would have similar toxicological properties. Applied to homologues, this can work adequately. A number of other scenarios for read-across have been developed, one being compounds that are metabolised to a common metabolite. Such a read-across argument is more difficult to confirm and justify without supporting information on the presence of the metabolite and rates of metabolism. As such, justification of a read-across argument moves from being based on chemistry to requiring biological evidence. In order to investigate how read-across can be optimised, including supporting chemical similarity with biological evidence from a variety of traditional and non-standard (non-animal, alternative) assays, a series of case studies has been undertaken [1]. The findings of the case study to read across the No Observed (Adverse) Effect Level (NO(A)EL) from the 90 day repeated-dose toxicity of β-olefinic alcohols are pertinent to the issue of common metabolites. Such compounds are assumed to be metabolised by alcohol dehydrogenase (ADH) to polarised α,β-unsaturated aldehydes and ketones. These metabolites are reactive in nature and are assumed to be able to react with thiol groups in cellular proteins (via a Michael addition reaction mechanism). Such reactivity with proteins may result in cellular apoptosis and / or necrosis of hepatocytes and could cause fibrosis of the liver which can be simulated in hepatic co-cultures [2]. A category of short chain unsaturated alcohols was formed, differing in terms of the position of the double bond and branching. The structural variation is considered to have an effect on the relative reactivity of the metabolite, hence affecting potency. Readily available toxicity data were compiled for this category and organised according to a published framework [3]. The data compilation shows that there are few, high quality, *in vivo* 90 day repeated dose toxicity data. However, the category is supported by *ex vivo*, *in chemico* and *in vitro* data from a number of assays as well as *in silico* predictions and profiling. The results indicate that read-across of the NO(A)EL is possible with acceptable uncertainty. The non-standard data support the read-across justification in key areas including confirmation of the metabolite and its relative reactivity. The category has been evaluated according to the European Chemicals Agency's (ECHA) Read-Across Assessment Framework (RAAF). The RAAF assessment structures the evaluation formally and demonstrate how uncertainty in the read-across justification is reduced by the inclusion of non-standard (*in silico, in chemico, ex vivo* and *in vitro*) data.

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If non-animal alternative tests are accepted by regulatory agencies, will industry use them?

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Recently the U.S. Environmental Protection Agency's Office of Pesticide Programs (EPA OPP) announced their intent to replace all the animal testing requirements of the "EPA six-pack" with non-animal methods as soon as possible. This would include tests for ocular irritation, dermal irritation, dermal sensitization, acute oral toxicity, acute dermal toxicity and acute inhalation toxicity. Their replacements could be waivers, e.g. dermal toxicity replaced with results of oral toxicity, or with single *in vitro* tests, or with integrated testing strategies (ITS) or with Integrated Approaches on Testing and Assessment (IATA). Efforts to address skin sensitization and expand the eye irritation policy are now ongoing in conjunction with ICCVAM and NICEATM, and to address skin irritation (for anti-microbial cleaning products [AMCPs] and some other pesticides) with the Institute for *In vitro* Sciences (IIVS). However, will industry use the non-animal methods if these methods are not specifically required? The OPP's experience with an existing non-animal ocular irritation policy for registering AMCPs is that only a fraction of registrations took advantage of the non-animal testing option. Most companies submitted Draize test data for commercial, monetary, reasons. These decisions were primarily due to lack of world-wide harmonization, but other significant reasons will be discussed. Ways forward to increase the use of the non-animal options have been proposed by both animal welfare groups and EPA, and will be discussed in this presentation.

Reference

Clippinger, A., Hill, E., Curren, R. and Bishop, P. (2016). *ALTEX*, Epub ahead of print. http://dx.doi.org/10.14573/altex.1601311

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Role of the Swedish national committee in the harmonization of project authorization and evaluation

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In Sweden, six regional animal ethics committees separately handle the authorization and evaluation of projects involving animal research.

To harmonize their work, a number of actions have been taken and the national committee has a central role here. Actions include an educational programme for the members in the animal ethics committees, visits to the animal ethics committees, as well as creating a strategy for recruiting suitable committee members. Furthermore, the work of the animal ethics committees is a standing point on the meeting agenda for the national committee.

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ATERA: advanced 3D human tissue models and exclusive testing services for cosmetic, consumer product and pharmaceutical industry

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In line with industrial innovation and many EU regulatory encouragements or requirements (EC 1223/2009, 2010/63/EU, EC 1272/2008, EC 1907/2006, 98/8/EC), the need for reconstructed human tissue models is still increasing. Advanced Tissue Engineering for Research Applications (ATERA) is a new tissue engineering company specialized in the development, validation, manufacturing and commercialization of "advanced" human tissue models. These models can be used as a reliable, cost-effective, and predictive alternative to animal experimentation in product evaluation, industrial safety and efficacy testing as well as medical research. ATERA is currently marketing a portfolio of 3D human tissue models including 3 types of reconstructed human epidermis, ocular models, oral mucosal epithelial models, vaginal, bladder and colon mucosa. To show the capacity of the ATERA – RHE skin irritation test method to replace the *in vivo* Draize test for regulatory accepted testing, a catch up validation study was performed. Results with an overall accuracy of 85% and reproducibility of 93% are currently under assessment by EURL-ECVAM's Scientific Advisory Committee. All human tissue models are produced in a ISO 9001/V2008 certified state-of-the-art production facility. In addition to these models several advanced models are available or will become available. These including full thickness skin models, a human intestine model, a model to mimic the Blood Brain Barrier (BBB) and corneal epithelial models. A variety of highly specialised *in vitro* testing services are offered by ATERA and its partnering laboratories such as standardized *in vitro* test protocols for efficacy and safety testing but also sophisticated assays based on impedance spectroscopy to assess compounds and formulations for sub-clinical irritation, epidermal barrier regeneration and wound-healing. Testing services are also performed on tissue models that are currently not mass produced. A perfused vascularised skin model allows to evaluate both systemic efficacy and toxicity of test compounds, in the presence or absence of circulation of other cell types (e.g. different immune cells) thus providing a new *in vitro* tool helpful to develop candidate methods assessing potential systemic toxicity. Advanced Tissue Engineering for Research Applications now provides new opportunities to promote alternative methods and to develop predictive tools more relevant to human health.

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Studying liver toxicity and disease modelling using bioprinted 3D human liver tissue

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Human tissue biology is strongly influenced by the unique interplay and extensive cross talk that exists between different resident cell populations. These cell types are often spatially arranged in a specific architecture which defines their biological function and mechanistic response to drug treatment over time. Three-dimensional bioprinted tissues that model this cellular complexity and form offer major advantages over conventional *in vitro* systems with respect to predictive modeling. Our tissues incorporate key architectural features and primary cell types and can be maintained in culture on a timescale of several days to weeks. We will describe various tissue models under development, including liver and kidney. More specifically, we will discuss case studies using exVive3D™ Bioprinted Human Liver Tissues to assess drug toxicity and metabolism, and their use in the generation of complex disease phenotypes such as fibrosis.

Collectively, these results suggest bioprinted tissues respond selectively to known toxicants following biologically relevant dosing regimens, and that the combination of biochemical, metabolic, and histologic endpoints provide a comprehensive means of examining the progression of tissue injury on a mechanistic basis.

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Good Cell Culture Practice for stem cells, stem-cell-derived models and organ-on-chip models

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The advances in cell culture techniques and the establishment of *in vitro* test systems in research need to be accompanied with approaches to standardize processes and documentation. The "Bologna Statement on Good Cell Culture Practice" in 1999 prompted the ECVAM Task Force on Good Cell Culture Practice (GCCP) to produce two seminal guidance documents [1,2]. Additional refinements were introduced in 2011 [3]. With growing availability of cultured organoids, and the increasing generation and use of pluripotent stem cells and their differentiation progeny, there is a need for revision and update of the GCCP guidelines for these new technologies. This includes quality assurance of supplied biological materials as well as consideration of ethical and legal aspects. This Presentation will give an insight in the preliminary outcomes of two think tanks, which were held in 2015 in Baltimore (USA) and Konstanz (Germany) on Good Cell Culture Practice for pluripotent stem cells, iPSC and organoids. These expert groups intend to enhance the GCCP principle regarding human cells and tissues and the quality assurance of cells obtained by reprogramming.

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Ex vivo infection with rhinovirus in mouse precision-cut lung slices

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Respiratory viral infection may trigger exacerbation of several lung diseases such as asthma. Hereby human rhinovirus (HRV) is mainly linked to cause adverse effects in patients with preconditions. HRV replicates mainly in epithelial cells of upper and lower airways. This leads to the activation of host innate immunity by triggering several Toll-like receptor (TLR) pathways such as TLR-3 and TLR 7/8. During viral replication production of double-stranded RNA (dsRNA) activates mainly the TLR3 receptor, therefore the role of this pathway in anti-viral response was studied *ex vivo* in mouse precision-cut lung slices (PCLS). HRV1B infection was compared to stimulation with the synthetic analog of dsRNA, poly(I:C). Pathways that are induced during viral infection may give new insights in the pathoprogression of asthma exacerbation.

PCLS were prepared from healthy Balb/c mice. The tissue was incubated with HRV1B (105 TCID50/mL) or 100 µg/mL poly(I:C) for 24 h. Inactivated UV-HRV was used as a replication independent control. The induction of cytokines, such as IL-28, MCP-1, IL-6 and IP-10 were assessed by ELISA.

Host immune response during viral infection was induced in the mouse *ex vivo* model by upregulation of anti-viral and pro-inflammatory cytokines. HRV1B infection induced significant expression IL-6 (2.2-fold), MCP-1 (2.1-fold), IP-10 (8-fold) and IL-28 (2.3-fold). No induction of these cytokines was detected in the UV-inactivated HRV1B control, showing replication dependency. Poly(I:C) induced significant secretion of the IL-6 (1.9-fold), MCP-1 (1.9-fold), IP-10 (14-fold) but not of IL-28, revealing differences in virus and poly(I:C) induced pathways.

This study shows that PCLS can be used to reflect host response to viral infection. Moreover, poly(I:C) only partly mimics virus infection and leads to slightly different immune response in lung tissue. Future studies will focus on viral infection in asthmatic environment to study exacerbation and impaired immune response.

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Creating organotypic oxygen tension in cell culture chambers using perfused flow and flexible chamber geometry

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There is currently great interest in applying microfluidics technology to the construction of complex *in vitro* models with the eventual goal of simulating the behaviour of an organ or even a whole organism. It is widely recognised that 3D cell cultures are more representative of human physiology than 2D monolayers of cells growing on a flat surface. Correct organ function also requires gradients of oxygen and metabolites and removal of waste material, as well as co-culture of multiple cell types-hence advanced and accurate models also require flow of media.

Using the proprietary Quasi Vivo® technology, cells cultured under optimum flow conditions are more metabolically competent than those cultured using traditional static techniques, and cells in interconnected chambers can create homeostatic conditions. We present here modelling data which shows that modifying the geometry of the QV900 chamber gives us the ability to connect chambers in series with different oxygen concentrations in each chamber, allowing the creation of organ and multi-organ models. Therefore, the currently available meso-scale Quasi Vivo® technology can lead the way towards relevant and easy-to-use *in vitro* techniques for animal replacement.

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Transient receptor potential (TRP) ion channels activation induce increased bronchoconstriction in passive sensitized vital human *ex vivo* Precision-Cut Lung Slices

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Introduction

Transient receptor potential (TRP) proteins function as ion channels in sensory nerve endings of human airways. The transient receptor potential ankyrin 1 (TRPA1) and the transient receptor potential vanilloid type 1 receptor (TRPV1) contribute to chemical hypersensitivity, chronic cough and airway inflammation in asthma and COPD. However, the underlying molecular pathways are not completely understood. Here we show in *ex vivo* human organotypic lung tissue slices the intensive interplay between peripheral sensory C-fibers and mast cells, involving neuronal mediators and specific IgE antibodies, which lead to increased bronchoconstriction to TRPV1 agonists in passively sensitized tissue.

Methods

Human Precision-Cut Lung Slices (PCLS) were generated using resection material from patients. PCLS with cross-sectioned airways were stimulated with tear gas (TRPA1 agonist), capsaicin (TRPV1 agonist) or a synthetic substance P analog (NK1 agonist). Furthermore passively sensitized PCLS were preincubated with the NK1 receptor antagonist, a therapeutic IgE antibody or an H1 receptor inhibitor before challenging the airways with capsaicin. The reduction of the airway area (RAA) was calculated from videomicroscopic images of bronchoconstriction as percentage of the initial airway area. Simultaneously, peripheral nerve fibers and SP were immunostained. Three-dimensional reconstructions of the sensory nerve fibers and mast cells were generated using confocal microscopy. Subsequently, mast cell-nerve interactions and neuropeptide SP content was volumetric analyzed in PCLS +/- TRPA1 agonist treatment.

Results

Airway stimulation with tear gas, capsaicin and the SP analog reduce airway area to 20%, 25% and 70% (RAA), respectively. Passive sensitized airways show increased bronchoconstriction to capsaicin up to 47% RAA. The application of NK1 receptor antagonist, H1 receptor inhibitor and a therapeutic IgE antibody blocked bronchoconstriction after capsaicin agonist stimulation in passive sensitized PCLS. Immunostainings revealed the presence of sensory nerves containing SP in the airways of PCLS. Volumetric analysis showed 20% reduced SP content in peripheral nerves after tear gas treatment in PCLS. Furthermore, close sensory nerve-mast cell interactions are detected. Capsaicin treatment of the lung slices resulted in mast cell degranulation.

Conclusions

The activation of peripheral sensory nerves in *ex vivo* PCLS via TRP ion channels provokes release of the neuropeptide SP and mast cell degranulation and thus lead to bronchoconstriction. TRP-induced increased bronchoconstriction in passive sensitized PCLS is NK1 receptor, H1 receptor and IgE-dependent. Thereby, a close interplay between sensory nerve fibers and mast cells was detected. Here we show the applicability of human PCLS as a suitable *ex vivo* model to study TRP ion channel-induced bronchoconstriction, including the functional reactivity of nerve terminals, mast cells and airway cells to reflect this complex processes under asthmatic conditions without the need of *in vivo* animal experiments.

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LUCS (Light-Up Cell System), a novel live cell assay for acute and other regulatory toxicity applications

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Light-Up Cell System (LUCS) is a novel live cell toxicity assay based on light-induced Reactive Oxygen Species (ROS) production and efflux (ABC/SLC) transport regulation. Application of light (480 nm, 0.3 J/cm^2) on normal growing cells treated with thiazole orange (TO, 15 min, 4 µM) induces an increase (up to 15 times) in fluorescence emission. This property is lost when cells are pre-treated with toxic compounds. Therefore, measure of fluorescence before and after light application reflects cell homeostasis status. Dose-response experiments allowed for sigmoid curve plotting and toxicity IC_{50} determination. The LUCS method was optimized for live cells cultured in 96- or 384-well plates and for phylogenetically distant cell models (from E. Coli to plant, yeast, fish or human cells) [1].

LUCS mechanism of action (MoA) was deciphered. Electron paramagnetic resonance experiments showed that singlet oxygen and hydroxyl radical are involved downstream of light effect, presumably leading to deleterious oxidative stress and a massive intracellular dye entry. Cell efflux/influx experiments showed that cell TO entry is regulated by a multi-drug resistance-like process.

In order to evaluate LUCS assay for acute toxicity testing, a dose-response study was performed using 58 substances described in the ACuteTOX EU database [2]. HepG2 IC_{50}s could be calculated for 53 substances and were correlated with human IC_{50}s with a R2 = 0.69. A prediction model (PM) was calculated as: Log(human LC_{50}) = 0.97 Log(HepG2 LUCS IC_{50}) - 0,82. Effect size was evaluated using negative (48 wells) and positive (48 wells) controls giving a Z factor of 0.8. In another study, HepG2 LUCS IC_{50}s were correlated with 3T3-NRU IC_{50}s giving an R2 = 0.8 and a linear regression curve in the form of Log(HepG2 LUCS IC_{50}) = 0.94 Log(3T3 NRU IC_{50}) - 0.23. LUCS improvement over NRU cytotoxicity assay relies in a better standardization and easier laboratory implementation with readouts obtained on any commercially available microplate fluorescence readers.

A last study was done in the context of the EU Regulation No 1272/2008 (CLP, Classification, Labelling and Packaging of substances and mixtures) to investigate whether LUCS can allow classification according to animal regulatory assay data. A second PM in the form of Log(mouse LD_{50} in µmol/kg) = 0.52 Log(LUCS EC_{50} in M) + 4,6 was calculated from 34 different substances. Assuming an uncertainty of 3% (0.12 Log), the PM allowed to classify 29/34 substances (85.29%) in the right class while 4/34 (11.76%, potassium cyanide, thallium sulfate, isoniazide, caffeine) were underclassified and 1/36 overclassified (2.94%, chloroquine) by one class.

LUCS is an evidence-based cell assay standardized on 96- and 384-well plates with applications in toxicity assessment. As it was optimized for integrated testing strategies in acute toxicity, a positive impact in terms of the 3Rs principle is expected. Other applications are envisaged in phototoxicity, skin/ocular corrosion and irritation, repeated-dose toxicity and multidrug resistance.

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Effects of implementation of EU Directive 63/2010 on education at University of Debrecen

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The University of Debrecen Committee of Animal Welfare (UDCAW) has been operating since 1999 (formerly, under the name of University of Debrecen Committee of Animal Research). The main goals of Committee are to improve the animal health and welfare, to promote the principles of replacement, reduction and refinement (3R) in research and education. In our presentation we would like to show, with examples and statistical data, how the implementation of Ordinance 40/2013. (II. 14.) of the Hungarian Government (on behalf of EU Directive 2010/63/EU of the European Parliament and of the Council) has changed the everyday teaching and experimental methods at University of Debrecen; for examples a significant part of animals used for education and trainings has been replaced by different type of models, also the number of experimental animals was decreased, thanks for careful planning of scientific projects, furthermore the number of undergraduate and postgraduate staff members, who are attending the lectures and trainings in laboratory animal science, has been increased significantly.

Reference

Ordinance 40/2013 (II. 14.) of the Hungarian Government EU Directive 2010/63/EU of the European Parliament and of the Council

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Screening of endocrine disruptors and their impact on human health

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Endocrine disruptors are hormonally active substances, of natural or synthetic origin, affecting the endocrine (hormonal) systems of humans. They have been associated with clinically observed adverse developmental, reproductive, neurological and immune effects. It is a specific group of compounds, which may be the ligands of human estrogen or androgen receptors due to their molecular weight and structure, and thus, depending on the degree and frequency of exposure, have the ability to affect endocrine system either by blocking or activating the receptor. Growing evidence suggests that low concentrations under chronic exposure during developmental periods of the organism may result in adverse effects in later periods of life. Certain compounds with endocrine disrupting effects can be found in a number of chemical groups, e.g. steroids, cyclic hydrocarbons, phenols, flavonoids, phtalates, parabens, biocides, plasticizers, surfactants, fire retardants, distinct antimicrobials, UV filters, toxic metals and other substances. They may be released from products intended for consumers, e.g. cosmetics or toys, everyday items, household products, medical devices and other products coming from industry or agriculture. Packaging materials, from which the hazardous substances may migrate into finished products, may represent health risk as well. Estrogenic endocrine-disrupting chemicals are found in environmental and biological samples, commercial and consumer products, food, and numerous other sources.

We have used *in silico* approach and two *in vitro* accessible tools suitable for detection of estrogenic and anti-estrogenic chemicals – ER TA assay (OECD TG 455) and the yeast based Xenoscreen YES/YAS assay (Xenometrix®). Bisphenol A and its analogues showed estrogenic activity, which may be similar to 17β-estradiol and already legislatively regulated Bisphenol A. All tested bisphenols showed agonistic and antagonistic activity to the human estrogen receptor. Only BPZ showed weak agonistic activity to the androgen receptor. Analogous chemicals, which are produced and available on the market, are suspected to be replacing those already evaluated and regulated (e.g. Bisphenol A) and thus should be of considerable interest for testing and evaluation. Chemicals with positive results in the *in vitro* systems are suspected to cause adverse effects *in vivo*. At the international level, the improvement of testing strategies and methods alternative to animal testing is highly required and has been discussed in the context of EU and global legislation. Increasing pressure on testing of endocrine disruption potential in the near future is very anticipated, along with efforts to reduce the potential exposure of humans, particularly of sensitive populations.

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Label-free monitoring of HepG2 spheroids – a platform for automated liver toxicity screening

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In the human body, the liver plays a central role in metabolizing and detoxifying foreign substances like drugs or chemicals. Therefore, hepatotoxicity is the most common reason for drug-induced liver injury, causing maladies that range from biochemical disorders to acute liver failures. Current testing strategies fail as effective screening tools, indicated by failures in clinical testing as well as high attrition rates [1]. Hence, more physiologically significant 3D cultures, such as Liver-on-a-Chip systems, are poised to replace existing strategies by identifying the adverse effects to micro-organ models induced by chemical entities in highly controlled environments. However, emerging Liver-on-a-Chip systems still rely heavily on end-point assays which lack the ability to survey multiple time-points during testing. Furthermore, the chemical labels used in these tests can alter metabolic function, producing false toxicity results. For this reason, liver toxicity assessments can benefit greatly by integrating sensor-based, real-time, label-free monitoring of 3D tissues. The inclusion of these automated testing schemes is capable of reducing the use of animal models significantly.

Herein, a new tissue encapsulation was designed and evaluated for long-term spheroid monitoring on the Intelligent Mobile Lab for In Vitro Diagnostics (IMOLA-IVD), capable of recording extracellular acidification rate (ECAR) and oxygen consumption rate [2]. A microwell array disc was fabricated using a commercial 3D-printer, and incorporated onto BioChips with the addition of a porous layer to hold the spheroids in their microwells and prevent wash out due to medium flow. Flow velocity and pump cycle were set for optimal cellular viability and experimental exposure, and were then uploaded into the control software. HepG2 spheroids were seeded with 1000 cells initially and cultured into spheroids measuring 622 microns in diameter. Spheroids were then transferred at day four onto the encapsulation within the chips. A stop and go mode (1 hour off, 1 hour on) was applied to enable the monitoring during the pump off phase, and to wash out the metabolites during the pump on phase. Spheroids were perfused for 36 hours prior to lysis with 1% SDS as a positive control within the proof-of-concepts study. Static culture tests exhibited spheroidal morphologies on assembled 3D-printed microwell arrays for up to 21 days, demonstrating the ability to perform long-term monitoring on the designed platform. Microphysiometrical monitoring revealed a repeatable pattern of extracellular acidification and oxygen consumption throughout the experiment, indicating metabolic activity of spheroids embedded in microwells. After perfusion for 36 hours with medium, with the addition of SDS resulted in a drop-off in ECAR signals from 36.6 mV/h (±1.8) to 9.2 mV/h (±7.6) for nine HepG2 spheroids embedded on a single BioChip.

In conclusion, the developed microphysiometer platform represents the next generation Liver-on-a-Chip assay platform for label-free monitoring of real-time dynamics during toxic response. To our knowledge, with the designed platform it was possible for the first time to achieve high reproducibility through automation with 3D spheroids on a microphysiometer. The new system provides a "ready-to-use" platform for further toxicology testing, paving the way towards more accurate predictions in drug safety and efficacy in the future.

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Arxula Yeast Estrogen Screen (A-YES®) – a non-animal method to evaluate the estrogenic potential of chemical compounds

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Endocrine-disrupting compounds pose a global threat to human health and the environment [6]. There is a strong need for standardized and reliable methods of evaluating the endocrine-disrupting potential of chemicals with a special emphasis on estrogen-active substances [7,1]. Currently, the Endocrine Disruptor Screening Program and OECD Conceptual Framework propose several *in vitro* and *in vivo* assays to assess the risk of estrogen-active substances and some of the *in vitro* assays even include the use of animals [2,5]. As a fast and cost-effective alternative to animal testing, we have developed a microorganism-based, effect-related reporter gene assay to measure estrogenic potentials of chemical compounds [3]. The lyophilized salt- and temperature-tolerant yeast, *Arxula adeninivorans*, is used as a biological component in this test kit. The Arxula Yeast Estrogen Screen (A-YES®) assay is applicable for pure chemicals suspected to have estrogenic potential as well as extracts and native water samples [4].

Within the framework of the ISO standardization process (ISO/CD 19040-2), an interlaboratory trial was performed by thirteen independent laboratories to demonstrate the reproducibility of the A-YES® assay. Part of the interlaboratory trial was the quantification of the estrogenic activity in a mix of bisphenols in different dilutions. Our analysis demonstrates a high reproducibility of the assay with a repeatability standard deviation of 7.5%. The limit of detection was determined to be in the range of 0.7 - 3.7 ng/L 17ß-estradiol equivalent (EEQ) with a calibration range between 0 and 80 ng/L EEQ. Additionally, the A-YES® is able to measure the estrogenic potency of various substances, including parabens, phthalates and drugs. Our results show that the A-YES® is a precise and robust method for detecting estrogenic potentials of chemical compounds and could provide a non-animal alternative to current *in vitro* assays.

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PBTK modeling of potential endocrine modulators: *in vitro-in vivo* extrapolation (IVIVE) and *in silico-in vitro* based risk assessments

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Physiologically Based Toxicokinetic Modeling (PBTK) is an *in silico* tool to predict compound kinetics based on test substance related properties and physiological parameters of the organism. PBTK is a key element for inverse dosimetry to relate effect concentrations *in vitro* to external, e.g. oral doses. In our investigations, we use 8 compartment models for the rat including adrenals and testes or ovaries. Test substance specific properties taken for PBTK modeling are molecular weight and logPO/W as well as IVIS based tissue specific partition coefficients, hepatic clearance, intestinal permeability and plasma protein binding. Berkeley Madonna Software was applied to solve consequent differential equations.

Here we present the above described model for the 3 test substances bisphenol A (BPA), fenarimol (FEN) and genistein (KETO). Using the lowest effect concentrations (LOECs) of BPA, FEN and GEN from 1) an *in vitro* yeast based assays with human estrogen and androgen receptor combined with a reporter gene and 2) the interaction of steroidogenesis model calculations were made to relate *in vitro* concentrations to oral doses in the rat. Model calculations, based on *in vitro* LOECs of 10 µM (BPA), 3 µM (FEN) and 1 µM (GEN), for concentrations in target organs resulted in estimated oral LOELs of 5, 2 and 2 mg/kg. When calculations were made for plasma levels oral LOELs were estimated to be 477, 129 and 141 mg/kg for BPA, FEN and KETO, respectively. When compared to existing *in vivo* data with endocrine related LOELs of 375 mg/kg bw day for BPA (1), 50 mg/kg day for FEN [2] and 50 mg/kg day for GEN [1,3], it can be concluded that for the exemplary test substances addressed, IVIS related risk assessment approaches based on target tissues seems overpredictive whereas plasma related LOELs were closer to the *in vivo* situation,

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Towards the replacement of fetal bovine serum in cell culture applications

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Foetal bovine serum (FBS) is used as a supplement for cell culture media that provides an undefined mixture of macromolecules to maintain cell viability and function. Serum is obtained from more than 1 million calf foetuses per year during the slaughter of pregnant cows by the meat and dairy industries (Jochems et al 2002). Supplementation of cell culture media with FBS presents significant scientific and animal welfare concerns. Considerable batch-to-batch variation exists because of the variable composition of FBS. There is also the risk of contaminating cells with problematic animal proteins and pathogens such as viruses. Since variability and biosafety risks can lead to unexpected and undesired outcomes, more defined media supplements for cell cultures are needed (Gstrauntaler et al 2013, van der Valk et al 2010). Furthermore, there are animal welfare concerns because blood is collected during the last two-thirds of gestation. At this stage, there is evidence that foetal calves are aware (Jochems et al 2002). As foetuses are not anesthetised for blood collection, it is very likely that they experience pain (Jochems et al 2002, van der Valk et al 2004).

However, there are alternatives to the use of FBS in cell culture media that overcome these concerns. Advances in biotechnological protein production, mostly in mammalian cells, allow for the commercial production of large amounts of recombinant proteins, which have the benefit of being analogous to the proteins from specific species (Grillberger et al 2009, Keenan et al 2006). Use of application-specific cell culture media supplements eliminates the variability and biosafety issues, eases product purification and reduces costs (Sung et al 2004).

Many laboratories are using supplements that are not animal-derived for *in vitro* testing for basic research or regulatory purposes. In basic research and R&D testing in industry laboratories, FBS can be replaced relatively easily; media recipes have been optimised for many cell types, although the concentration of supplements for some cell types will need further optimisation. In regulatory testing, there is official support for the use of serum-free media (ECVAM 2008), and their use is prescribed in some guidelines using reconstructed human tissues. The use of serum-free media should be extended to more research areas. A concerted effort by multiple stakeholders will be necessary to make the complete transition to non-animal media supplements. For example, information on concentrations of media supplements and educational resources need to be made available. Alternatives to FBS should be used in development and validation of new *in vitro* methods. When using existing test guidelines prescribing the use of FBS, alternatives can be used in parallel to evaluate their range of applicability.

This poster includes recommendations for the use of FBS alternatives in both regulatory and non-regulatory testing as well as information about companies that sell serum-free medium or cell culture supplements.

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Emedia skills lab for Laboratory Animal Science – alternatives to animal experiments in teaching and learning

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The BMBF-funded project emedia skills lab for Laboratory Animal Science (emsl VTK) focuses on the establishment of a sustainable, 3R-conform media-supported training concept for Laboratory Animal Science (LAS), which also includes the use of training models. The goal of the project is to qualify personnel planning, performing and evaluating animal experiments in order to improve the performance in animal experiments (Refinement), which will subsequently result in long-term Reduction and Replacement of animal experiments. An important prerequisite for the successful use of media and models in teaching is the acceptance of them by the user. Therefore, we evaluated the concept in four evaluations of different media types generated for the training of specific skills and treatments in LAS.

Media containing texts, illustrations, photographs, animations, quizzes and videos were combined to interactive, interdisciplinary and inter-professional e-learning modules. The content suits for several target groups (e.g. students/participants of FELASA courses/lecturers) and were made available via the e-learning platform emedia VTK. Sustainability is ensured by the integration into the curriculum of the master program for laboratory animal science (MLAS) and the LAS-interactive platform.

For the implementation of the 3R's the transfer from theoretical fact-based knowledge to practical application of the 3R's has to be ensured. Therefore, scripts/handouts for self-study followed by media-supported lectures/webinars and e-learning-modules as well as homework were provided. Depending on the course concept practical skills are first demonstrated via video and/or via tutor, before the application is trained under supervision on training models and/or animals.

To confirm the acceptance and effect of this training concept 4 evaluations were conducted, regarding the acceptance of teaching media/use of videos, interactive video-formats, combination of instructional teaching-video and use of training model (silicone-ear SIKO) for learning venous blood sampling and its effect on conducting the practical skill on the rabbit.

Results of the evaluations showed that media like scripts (87%) and videos (58%) were preferred media formats. Videos were to 79% favored when included in lectures/webinars and to 67% when included in self-learning media to acquire new practical skills.

The interactive video format of catheterization techniques in rats was highly accepted (> 80%) and positively assessed (> 90%). Based on this result the RWTH Aachen completely replaced the live-rat-catheterization demonstration during FELASA B course. For learning a practical skill, the combination of teaching-video and practical demonstration showed highest acceptance (100%). The users rate the training model SIKO as "good preparation" and "ideal addition" to training on the live animal, but they "rather disagree" that the model can replace the animal. They estimated their knowledge on blood collection as improved ("good") and the stress load on the animal as reduced. However, the results of the tutor's evaluation showed no significant difference of the training effect of the combination video/model on venous blood sampling.

With the here presented study we successfully implemented the 3R's in our training concept, especially in the areas of reduction and refinement. The high acceptance of the here evaluated media is encouraging to develop further media for teaching in the field of LAS.

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Implementation of 3Rs for livestock animals in bioscientific research-rationale and objectives of the newly established expert working group – LaNiV*

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Livestock animals are of high importance in bioscientific research and frequently used for agricultural, veterinary, basic, safety or translational studies [1,2]. In contrast to small laboratory animals, livestock animals are usually obtained directly from livestock production, resulting in lower homogeneity, variating animal health and hygienic status. Further challenges are the generally high requirement of personnel expertise and housing conditions, including the amount of space and pathogen infection control. Currently, comparable widely used standardized health and hygienic guidelines as well as frequently offered certificated education trainings for laboratory rodents are lacking for livestock animals used in scientific procedures. Hence, these factors may have adverse impact on animal welfare and the experimental outcome of scientific procedures.

In accordance to the legal requirement to implement the 3R principle (European Union directive 2010/63) the use of livestock animals for bioscientific research needs to define standardized guidelines and skilled educated staff members, who have the knowledge of species-specific needs, which includes among others the ability to recognize and assess pain.

To overcome this dilemma we established a working group in 2015 aiming for both the refinement of farm animals in research and the reduction of the animal numbers used in experimental trials. This working group, named LaNiV*, is officially accepted as a part of the German society for laboratory animal science (GV-SOLAS) and consists of a broad scientific network including experts for livestock animals from Germany, Switzerland and Austria. Currently, five work packages were developed: animal acquisition/health (WP1), housing/hygienic strategies (WP2), experimental design/procedures (WP3), disposition/tissue allocation (WP4), and education (WP5). Objectives of the WP include: WP1 and WP2 focused on identification of relevant pathogens to implement a standardized health certification scheme for animal acquisition and housing. WP3 designed definitions for assessments and recommendations of standardized anaesthetic and analgesic strategies. WP4 evaluate possibilities for animal disposition, organ- and tissue-sharing at the end of an experiment. In WP5 basic courses (according to FELASA) are in preparation and the contents of further education programmes for staff member on livestock animal trials will be defined. Additionally, international specified interdisciplinary workshops of the WP topics will be developed. Furthermore, different refinement targets, e.g. environmental enrichment strategies, will be included within prospective research projects.

Until now the implementation of the 3R principle in experimental set-ups using livestock animals, is limited due to the lack of standardized protocols for animal housing, health monitoring, personnel training strategies, anaesthesia and pain relief as important refinement strategies. This may result not only in higher suffering and/or mortality rates but also affect the validity and reporting of experimental and might increase the number of animals necessary. Therefore, our expert group aims to develop recommendations, standardized guidelines and training courses for researchers working with livestock animals to improve animal welfare. Continued international networking among bioscientific researchers will ensure that these improvements will spread across the European Union.

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Integration of conducting polymer devices with 3D human tissue models for *in vitro* toxicology testing

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The Blood Brain Barrier (BBB) provides a physical separation between blood and fluid compartments of the central nervous system. It is composed of polarized endothelial cells maintained together by adherent and tight-junctions whose quantity and localization are responsible for barrier selectivity modulation. The BBB is more generally part of the NeuroVascular Unit including glial cells (Astrocytes and Pericytes), which modulate the barrier effect through a variety of cellular and molecular mechanisms, which are not fully understood. In order to increase our basic understanding in this field, but with a view of future application in *in vitro* toxicology assessment, we are developing a biologically relevant BBB model with integrated real time monitoring of the barrier integrity. Electrical impedance sensing (EIS) has emerged as a dynamic method, with demonstrated potential for use in monitoring barrier function, cellular adhesion, proliferation, micro motion, and wound healing. We have previously developed EIS systems based on Organic ElectroChemical Transistors (OECT) [1,2]. We have shown that these devices can be used as a non-invasive technique for *in vitro* toxicology [3]. Recently, using this technology we have developed a three-dimensional model of the BBB based on co-culture of human astrocytes and brain capillary endothelial cells (hBMEC) on collagen gel scaffolds that allows us a sensitive and continuous monitoring of barrier resistance. To validate the functionality of our model we correlated an increase in barrier resistance with expression and localization of tight-junction proteins. Integration of the model with a microfluidic system to mimic physiological flow is ongoing.

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Enhancing effect of 100 kHz alternating electric field on the ChIFN-γ-like molecule inducing capacity of LCL (lens culinaris lectine) and 10% PBS washout of holocene minerals

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Macrophages are heterogeneous cells that play key roles in host defence and tissue repair following challenges from injury, infection, or malignancy [1,2]. Macrophage functions are determined by the integration of signals from the microenvironment that to date, have been predominantly characterized as biologic and chemical stimuli, such as microbial products, cytokines, and metabolic factors. One potentially important physical cue that has been overlooked in the regulation of macrophage functions is the endogenous, direct current EF. Situations where EFs arise include wounded tissues, where epithelial barriers have been broken. For example, directional ion transport leads to a trans-epithelial potential difference of 50-100 mV across intact skin. This collapses locally to 0 at the breached epithelium, giving rise to a steady voltage gradient of 40-200 mV/mm directed toward the wound edge and parallel to the epithelial layer, with the wound negative with respect to distal tissue [3-5]. It was reported [6] that one or a few strong electric impulses can induce an HuIFN-α like protein in human leukocytes, that is antigenic ally different from any known IFN molecule. In addition, it was found by Kovacs et al. [7] that in Porcine leukocytes after the addition of Sendai virus and electric impulses induce the PoIFN-α like molecule. The pulsed electromagnetic fields of 50-Hz enhance the induction of different cytokines: Interleukin 1β, Interleukin-2 and Tumour Necrosis Factor (TNF-α) by PBMC challenged with PHA [8]. The presented experiments were aimed to find in the chicken macrophage system, the appearance of ChIFN-γ like molecule after the challenge with LCL and 10% PBS washout of different Holocene minerals and the alternating electric fields of 100 kHz. The ChIFN-γ molecule activity was assayed by Antiproliferative assay on CoMA (Chicken macrophage cell line) and ChIFN-γ (40.000 I.U./ml) as standard. All samples were analysed by RP-HPLC measured at 280/214 nm. The following results were obtained: LCL alone give 6372.917 I.U./ml, (2) LCL + 100 kHz/2 min give 22 191.187 I. U (3) 100 kHz/ 2 min give 1550.123 I.U./ml, (3) 10% PBS give 10316.914 I.U./ml (4) 10% PBS + 100 kHz/2 min give 28077.774 I.U./ml, (5) 10% PBS + LCL give 48028.774 I.U./ml, (6) 10% PBS + LCL + 100kHz/2 min give 105476.626 I.U./ml. In addition, we have tested different samples of Holocene minerals and theirs 10% PBS washout. So we tested Sample 3 (Sand from Koprivnica) and in combination: 10% PBS (3) + LCL + 100 kHz/2 minutes we got 162122.40 I.U./ml. So it can be concluded that 10% PBS washout of different Holocene minerals enhance in a different way the LCL challenge of ChIFN-γ-like molecules in addition of 100 kHz electric field. Definitely it depends from the composition of the Holocene minerals.

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Hepatic differentiation of hiPSC in co-culture with endothelial cells using different culture media

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The derivation of somatic cells from human induced pluripotent stem cells (hiPSC) holds great potential for *in vitro* toxicity testing as an alternative to animal studies. Since the liver represents the central organ for drug metabolism and a main target organ for drug-associated toxicity, hepatocytes are of particular interest for toxicological investigations. However, when compared to primary human hepatocytes, full maturation of hiPSC-derived hepatocytes has not yet been achieved [1,2]. It has been reported that *in vivo* liver organogenesis is promoted by endothelial cells prior to vascular function [3]. *In vitro* small liver buds can successfully be created by co-culturing hiPSC-derived definitive endodermal (DE) cells with endothelial cells and mesenchymal stem cells [4]. Based on these findings a co-culture model of hiPSC-derived cells with human umbilical vein endothelial cells (HUVEC) was investigated in this study, including evaluation of different co-culture media.

In an initial experiment, the medium composition was optimized in a way that hiPSC-derived DE cells are supported in differentiation and HUVEC in growth. For the co-culture experiments the hiPSC line DF6-9-9T was pre-differentiated into DE-cells by adding activin A and Wnt3a to the culture medium. Afterwards 1 million HUVEC per 2 million hiPSC were added to the cultures and the optimized co-culture medium was applied. This approach was performed in conventional 2D cultures and perfused 3D bioreactors based on a hollow-fibre membrane technology for high-density cell culture.

The results showed a distinct positive effect of the optimized co-culture medium on hepatic differentiation of hiPSC with respect to mRNA expression and secretion of alpha-fetoprotein and albumin. Activities of cytochrome P450 (CYP) enzymes CYP2B6 and CYP3A4 were 5- to 30-fold increased compared to the control medium. Furthermore, it was shown that co-cultivation of HUVEC with hiPSC-derived DE cells was feasible in both 2D cultures and 3D bioreactors showing expression of hepatic markers such as albumin and cytokeratin 18 and activity of different CYP enzymes. In addition, this study emphasizes the value of dynamic 3D culture systems in stem cell differentiation especially regarding the formation of tissue like structures. This dynamic 3D culture system also provides the possibility of a constant perfusion with test compounds and therefore enables chronic toxicity studies as already shown for primary human hepatocytes [5].

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The "Neurosphere Assay" as a valuable part of a DNT testing strategy

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Neurospheres gained from time-matched fetal human and postnatal rodent brains are three dimensional (3D) cell culture models consisting of neural progenitor cells (NPCs) which proliferate in culture and – under differentiating conditions – migrate and differentiate into neurons and glia cells. We have set up SOP-based protocols to reproducibly assess neurodevelopmental endpoints – NPC proliferation, migration, differentiation into neurons and astrocytes – with the purpose of developmental neurotoxicity (DNT) testing *in vitro* in the "Neurosphere Assay".

For understanding the biological application domain of our assay we performed "omics" analyses at timepoints of "Neurosphere Assay" test endpoint, 0, 3 and 5 days of differentiation. Moreover, we analyzed different pathways known to be implicated in brain development. Testing of a variety of positive and negative compounds adds information on the "Neurosphere Assay's" application domain.

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Human placental explants as an alternative for animal testing

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Objectives

The placenta is a highly specialized organ supporting normal growth and development of the fetus [1]. Since the anatomy and function of human placenta differs more than any other organ between species, human placental tissue is a promising approach for the evaluation of reproductive toxicity [2]. Therefore, this study aims to analyze the effects of chemotherapy on placental cells and to further characterize exposed placental tissue by different methods.

Methods

After preparation and 24 hours recovery time, placental villous tissue explants (PVTEs) were incubated with doxorubicin, docetaxel, 5-fluorouracil or vincristine for at least 48 hours (n = 3). Supernatant analyses of glucose, lactate, lactate dehydrogenase (LDH), human chorionic gonadotropin (hCG), estrogen and progesterone were performed. Metabolic activity was evaluated via MTS assay. PVTEs were embedded in paraffin; sections were stained with HE- and IHC (Ki-67, cPARP). Evaluation was performed for trophoblast nuclei and membrane, fibrin deposits, syncytial knots and subepithelial vacuolization. Additionally, a scoring system for histological evaluation of placenta tissue was generated.

Results

Glucose levels in supernatants of treated PVTEs were increased compared to controls, whereas lactate levels were decreased. After 24 h incubation, estrogen levels were elevated compared to the control. HCG and progesterone remained unchanged. The metabolic activity was only reduced by doxorubicin or docetaxel. Histology (HE, IHC) showed tissue damage in control cultures over progress of time. The most conspicuous additional impairment by drug treatment was induced by vincristine and 5-fluorouracil, as reflected by intense subepithelial vacuolization of PVTEs.

Conclusion

PVTE cultures allow the detection of harmful effects of chemotherapy. Highly concentrated cytostatic drugs lead to significant damage. HE scoring improved sensitivity of IHC analyses, because it includes several different evaluation criteria. Nonetheless, there is a need for further optimization of culture conditions to reduce decreased vitality of control PVTEs.

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Three dimensional cell culture model for toxicity and efficacy tests

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Objectives

Three dimensional cell cultures in terms of spheroids are well established models, which resemble avascular tumors within pH, oxygen, metabolic and proliferative gradients [1]. Moreover, compared to conventional monolayers spheroids with no stratification better simulate intact tissue [2]. Therefore, in this study two types of spheroids were used to analyze wanted (effective) and unwanted (toxic) effects of chemotherapy. We generated organoid spheroids simulating placenta tissue and tumor spheroids mimicking breast cancer in order to identify optimal treatment during pregnancy.

Methods

Trophoblast (HTR-8/SVneo, JEG-3) spheroids and MCF-7 breast cancer spheroids were generated via hanging drops with 25% methyl cellulose. MDA-MB-231 breast cancer spheroids were formed using liquid overlay technique with 3.5% Matrigel. After generation, trophoblast spheroids were incubated with doxorubicin, docetaxel, 5-fluorouracil and vincristine for 24 or 48 hours. Before treatment, breast cancer spheroids were cultivated 2 days to ensure their structural subdivision in proliferative and necrotic areas. The effects were evaluated via light microscopy, MTS assay, histological and immunohistochemical (Ki-67, cPARP, p27 Kip1) analyses. Lactate, human chorionic gonadotropin, estrogen and progesterone were measured.

Results

After treatment with doxorubicin, docetaxel or vincristine morphology of trophoblast spheroids was harmed and hormonal secretion was reduced. Metabolic activity was significantly reduced by doxorubicin and docetaxel. In contrast, morphology of breast cancer spheroids was only affected by doxorubicin and docetaxel. Nonetheless, vincristine treated MCF-7 spheroids presented a three layer structure suggesting impairment of morphology. Moreover, after incubation with doxorubicin or 5-fluorouracil cPARP positive cells were detected in the outer spheroidal layer of MCF-7 spheroids.

Conclusion

Toxicological analyses demonstrate wanted effects on breast cancer spheroids but also unwanted effects on trophoblast spheroids. Doxorubicin and docetaxel revealed strongest toxicity in both types of spheroids. Therefore, fluorouracil and vincristine leading to no reduction of metabolic activity in trophoblast spheroids might be interesting candidates for further investigations.

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The toxicity of sunscreen formulations containing zinc oxide on zebrafish embryos

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With the rapid expansion of zinc oxide (ZnO) nanoparticles in our life, they will ultimately be released into the environment and it is very important to understand the biological effects on aquatic organism to reveal their potential safety on human and environment. In this study, the effects of ZnO nanoparticles in sunscreen were investigated, using zebrafish (*Danio rerio*) embryos as the model. Toxicological endpoints, such as hatching rate, heart rate, abnormal rate, mortality and body length were evaluated.

In order to study the toxicity of ZnO nanoparticles in sunscreen on zebrafish embryos, five different groups were set up: (1) sunscreen containing ZnO nanoparticles in (SN-group); (2) pure ZnO nanoparticles (PN-group); (3) sunscreen containing ZnCl (SI-group); (4) sunscreen containing all accessories but no ZnO (SA-group); and (5) Zebrafish culture medium (Control). The Zn concentrations in SN-, PN-, SI-groups were 0.1, 1, 10, 20, 50 mg/L. During the exposure period, these toxicological endpoints were recorded, such as hatching rate, heart rate, abnormal rate, mortality and body length. The results have shown that the solution in each group did not significantly affect the toxicological endpoints at low concentrations (0.1, 1, 10 mg/L), but the hatching rate was lower and mortality rate was higher in SN-group and PN-group than those in Ion-group at high concentrations (20, 50 mg/L). Meanwhile, the mortality rate is higher in PN-group than that in SN-group. With the rising of Zn-concentration, mortality and abnormal rate increased, while body length and hatching rate reduced.

In conclusion, pure ZnO nanoparticles have more severe toxicity on zebrafish embryos than zinc ions and ZnO in sunscreen. The sunscreen accessories could alleviate the ZnO nanoparticles toxicity on zebrafish embryos. To further investigate the toxicological mechanism of ZnO nanoparticles, I plan to characterize the physical and chemical properties of ZnO nanoparticles in the exposure solution.

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Assessment of Hen's Egg Test Chorioallantoic Membrane (HET-CAM) for screening of anticancer activities of drugs

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Introduction
The hen's egg test chorioallantoic membrane (HET-CAM) assay is considered an experimental alternative for animal model. It serves as an ideal model for studying the pro- and anti-angiogenic properties of compounds. CAM has been also considered as an ideal model for grafting of tumors and assess the anticancer activities of drugs. A wide range of methodologies depends on using CAM with different experimental protocols. The methods currently used, however, depend on semi-quantitative or complicated quantitative procedures for assessment of results. The main drawback of using the CAM assay was the difficulty to quantify angiogenic response with reasonable speed, ease and accuracy.

Aim
The study was designed to fulfill the following aspects:
1. Development and establishment of a simplified, standardized method for qualitative and quantitative investigation of angiogenesis using HET-CAM model.
2. Evaluation of pro- and anti-angiogenic materials on the new established model.
3. Development of a new simplified live imaging method for angiography in HET-CAM model suitable for both qualitative and quantitative assessment of angiogenesis.
4. Investigation of tumor growth inoculated on CAM.

Materials and methods
A special designed incubator was built up to study the suitable environment for chick embryo growth. Fertilized eggs were incubated at 37.0°C. The proper factors for CAM development, CAM visualization, blood vessels formation and angiogenesis were assessed. Copper sulphate (CuSO4) and dexamethasone were applied on filter paper and placed on CAM. Their proper concentrations were tested for chick embryo viability and CAM development as well as the ideal concentrations for angiogenic activity were determined. Ehrlich ascites carcinoma (EAC) was cultured on CAM. Its growth and angiogenic activity were assessed. A new live imaging angiograph methodology was carried out and both qualitative and quantitative parameters of angiogenesis were studied through computerized analysis.

Results
The proper factors for studying angiogenesis and tumor growth using CAM model were determined. The proper concentrations of $CuSO_4$ and dexamethasone as ideal positive controls for pro- and anti-angiogenesis were found to be 50 µg/0.02 ml and 0.02 µg/0.02 ml, respectively. Both qualitative and quantitative angiogenesis parameters were assessed. EAC colony was developed on CAM and was identified under microscope. EAC possessed high angiogenic activity showing high dense blood supply to the colony. A new live imaging method for CAM was developed providing a suitable platform for computerized image analysis of blood vessels and tumor cells transplanted on the CAM.

Conclusion
This present study revealed the proper factors for CAM to be used as an alternative to animal model for studying angiogenesis, assessment of pro- and anti-angiogenesis drugs as well as studying the tumor growth. In the present study, the newly developed procedure for angiography provided:
1. A new live imaging method for CAM providing a new accurate angiography procedure with less variability.
2. An established methodology for computational digital image analysis of blood vessels and tumor transplanted on the CAM providing new methods for assessment and an accurate source for statistical data.

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

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Introduction
For diagnostics of discogenic low back pain, local anaesthetics are frequently used. They are injected into intervertebral disc under the fluoroscopic control. Lidocaine and bupivacaine are most commonly used for controlling and relieving the pain in interventional spine procedures. However, they may be cytotoxic to intervertebral disc cells and may initiate or accelerate the intervertebral disc degeneration. The potential effects of these agents still remain unclear, as many studies so far have been accomplished on animal cells. The study aim was to evaluate the effect of local anaesthetics on isolated human intervertebral disc cells *in vitro*.

Materials and methods
Annulus fibrosus and nucleus pulposus cells were isolated from human lumbar intervertebral disc fragments following discectomies. The cell culture was established on microtitre plates and exposed to various concentrations of lidocaine, bupivacaine and their mixture. Saline solution was used as a control. Three different dilutions of local anaesthetics were tested: undiluted, 1:2 and 1:4 dilutions. The cells were treated for 6, 24 and 48 hours and then examined with for viability.

Results
Human intervertebral disc cells demonstrated a time and dose depended response to lidocaine and bupivacaine. Nulceus pulposus cells were more susceptible than annulus fibrosus cells to the toxic effects of both anaesthetics. Lidocaine was more toxic than bupivacaine. In lidocaine, the final cell survival fraction was 0%, 10% and 20%. Bupivacaine presented less cytotoxicity with the final cell survival of 10%, 60% and 80%. Lidocaine-bupivacaine mixture showed an intermediate toxicological effect on the nulceus pulposus and annulus fibrosus cell culture. The cell death was mainly caused by necrosis rather than apoptosis.

Conclusions
When incubating the human intervertebral disc cells *in vitro* with a combination of anaesthetic agents commonly used for discography, the cytotoxic effects were observed in a dose- and time-dependent manner. According to our study, lidocaine and its mixtures should be avoided due to their high toxicity to the intervertebral disc cells. Bupivacaine was the least toxic, especially when used in 1:4 dilutions and may be thus recommended for the intradiscal diagnostics. It is assumed that the genesis of disc degeneration might be contributed also by the toxic effects of the anaesthetics used, culminating to progressive tissue damage after the diagnostic measures.

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The process of isolation and characterisation of HUIEC, Human Intestinal Epithelial Cell Line

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Introduction

This study describes the development and characterization of human intestinal epithelial cells (HUIEC), a spontaneously arising cell line established by selective trypsinization and cloning of the intestinal epithelium, resulting in a uniform population of highly epithelial cells with a strong growth potential. The line can be used as a replacement for animal cell lines.

Materials and methods

The HUIEC cell line was derived from the human small intestine. Mechanical and enzymatic decomposition was followed by sequential centrifugation, selective trypisnization and cloning of the intestinal epithelium. Immunocytochemistry was used in order to characterize the cell culture for the presence of specific structural and enzymatic epithelial markers.

Results

HUIEC is a rapidly growing, spontaneously evolved intestinal epithelial cell. A transepithelial resistance greater than 120 Ω/cm was measured. The ability of the epithelium to polarise and to form transepithelial resistance confirmed the *in vivo*-like functionality of the culture. The presence of cell markers suggested the epithelial nature of the isolated culture.

Conclusions

HUIEC is a stable and fast-growing untransformed, adult human epithelial intestinal cell line, which can be used for further experiments with functional cell models as an alternative to animals and animal cell models.

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Animal Friendly Affinity Reagents (AFAs): making animal immunisation obsolete

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Valued at a staggering 80 billion dollars, the global antibody industry produces an indispensable resource that is relied upon by scientists, healthcare professionals and consumers, in all areas of research, safety testing, health and the environment. However, it is an industry that uses millions of unaccounted-for animals with important animal welfare implications [1]. With the emergence of the news regarding mistreatment of animals at a US antibody production facility [2] and the availability of numerous replacement methods, it is timely to reflect upon a golden opportunity for replacement. Animal Friendly Affinity reagents (AFAs) encapsulate all binding molecules that are generated recombinantly using naïve (non-immunized) B- lymphocyte or synthetic gene repertoires, selected *in vitro* by phage, ribosome or yeast display. These are typically antibodies, but also include non-antibody affinity reagents, such as DARPins, affibodies, monobodies, anticalins, and others [3,4]. AFAs do not necessitate animal immunisation at any stage of production, making the use of animals obsolete [5]. Commercially available or developed in-house, they have wide ranging applicability, equal or greater specificity and affinity to a huge repertoire of antigens and offer greater control over their properties, generation time and cost. Directive 2010/63/EU legislates for the replacement of animals used in scientific procedures where alternatives exist. Yet despite the irrefutable maturation of the growing number of techniques to produce AFAs and an abundance of literature to support, animal derived antibody production continues to be authorised. Non-technical summaries do not reflect the status quo. Twenty years ago, ECVAM workshop reports [6,7] advised EU Member States that "in the near future", phage display "without prior immunisation of B-cell donors (would) avoid the need to use living animals" so it is incomprehensible that such an enormous area of needless animal use continues to be overlooked and that the opportunity for replacement has not been seized. To stimulate an EU led replacement program, ensuring Directive 2010/63/EU is implemented, we have engaged in discussions with EURL ECVAM and Directorate-General for the Environment. We recommend that the following actions be prioritized: the replacement of animal immunization methods for antibody production within EU Member States, including the import of antibodies and antibody containing products; adherence to European standards by manufacturers from outside the EU; the establishment of an expert working group to set up a roadmap for replacement; implementation programs to ensure that antibody producers are fully supported and that European statistics on the number of animals used for experimental and other scientific purposes should include data on the use of animals for antibody production as an independent category. These actions must be reinforced through international co-operation and nationally, by agencies who execute government regulation at operational level for commercialised products or who safeguard our health and the environment and should include their import, to avoid that production is outsourced to regions where animal welfare is less well regulated. For the full length article, see Gray et al., 2016 [8].

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A three-dimensional bioprinted liver equivalent for human-on-a-chip toxicity screenings

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The liver is the most important organ in metabolism and toxin conversion. Therefore, many *in vitro* systems have been engineered for liver toxicity screenings. While conventional methods lack cell viability for long-term cultivation and show a decrease in liver specific gene expression over time, more physiological environments are described in three-dimensional tissue constructs with co-cultivation of various cell-types in microfluidic bioreactors. The incorporation of cells in three-dimensional, highly organized hydrogel architectures via bioprinting provides a new method that can also be used for hepatic tissue fabrication.

Here we present a 3D-printing strategy for the creation of a high-resolution, multiple-cell-type liver equivalent, which aims in providing an *in vitro* platform for human-on-a-chip toxicity screenings.

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Complex skin models and impedance spectroscopy as new tools for hazard identification and efficacy testing

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Despite advances in the development of *in-vitro*-tissue-models such as reconstructed human epidermis (RHE), the number of endpoints in toxicity-testing, which can be addressed with these models, is limited. This is due to a lack of key cellular components and a restricted live time of the models. In addition, the analysis of the models is still dependent on invasive methods such as histological processing or 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining.

To overcome these pitfalls, we achieved advanced culture systems and biomaterials which allow long term culture of complex tissue-equivalents. Using these technologies, we have developed the first full thickness skin-model with a perfused vascular network.

Furthermore, as an alternative for destructive methods, we have established a non-destructive technology to analyze the integrity of the epidermal barrier based on impedance spectroscopy. RHE typically exhibits characteristic impedance spectra in a frequency ranging between 1 Hz and 100 kHz, which is comparable to the spectra of freshly isolated human epidermal biopsies. From these spectra, we extracted electrical parameters of the RHE such as the capacitance and the ohmic resistance. These parameters change significantly during epidermal differentiation and were used to quantify the effects of mechanical and chemical disruption of the epidermal integrity. Most relevant, impedance spectroscopy shows a sufficient sensitivity to detect a transient decreased ohmic resistance caused by 2-propanol, which is classified as a non-irritant by MTT assays. This result indicates that impedance spectroscopy can be employed as an additional method to assess mild irritative effects.

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

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In vitro Hollow Fiber System for Tuberculosis (HFS-TB): regulatory qualification and impact in TB drug development

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The Critical Path Institute (C-Path), an independent, non-profit organization working to accelerate the process of drug and medical product development, announced in February 2015 that the European Medicines Agency (EMA) rendered a positive qualification opinion on the *in vitro* Hollow Fiber System for Tuberculosis (HFS-TB) drug development tool. The effort to attain EMA qualification for this innovative *in vitro* pharmacokinetic / pharmacodynamic drug development tool was led by the Critical Path to TB Drug Regimens (CPTR) initiative through its Regulatory Science Consortium. The Critical Path to Drug TB Regimens Initiative, launched in 2010, is a broad collaboration of industry, civil society, government, and regulatory officials working together to develop regulatory science that can be used to identify, develop, and put through formal regulatory review new methods and tools in the development of promising tuberculosis (TB) drug candidate combinations. To support the implementation of the HFS-TB, CPTR is currently developing a laboratory manual for the HFS-TB to allow greater usability by researchers.

C-Path is one of three founding partners with the Bill and Melinda Gates Foundation and the Global Alliance, aids researchers in determining which drugs to combine and at what doses in order to effectively fight multi-drug resistant Mycobacterium tuberculosis (Mtb). EMA's qualification will speed the model's adoption by drug developers, who can now be assured that European regulatory bodies will accept supportive data from research using this method. The HFS-TB provides a deep understanding of how drugs move through the body and exert their pharmacokinetic and pharmacodynamic effect on Mtb. This represents a significant advancement in the development of effective treatments for Mtb, as current testing models do not always predict appropriate drug dosages for human testing.

Worldwide, TB is a public health epidemic, particularly in developing countries, and kills more than one million people a year. Drug resistant tuberculosis is on the rise, with about 450,000 people in 2012 developing a version that does not respond to existing treatments. Developers of new treatments for Mtb face many challenges, including the fact that Mtb manifests very slowly, progresses through distinct stages that are difficult to detect, and affects many patients who live far from medical research and treatment facilities – all issues that the use of the HFS-TB could help address.

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The details of this program work as well as descriptions of both regulatory approaches by EMA and FDA were summarized in a supplemental publication in Clinical Infectious Diseases (CID) journal. This special supplement, "An Evidence-Based Drug Development Tool for Tuberculosis Regimens: The Hollow Fiber System Model", was published in August 2015: Clin Infect Dis. (2015) 61 (Suppl 1). It is comprised of 4 manuscripts, featuring the evidence-based assessment of the predictive accuracy and regulatory review of the HFS-TB, along with editorials provided by EMA and FDA. Hollow Fiber System Model for Tuberculosis: The European Medicines Agency Experience. 2015 Aug 15; 61 Suppl 1:S1-S4 http://dx.doi.org/10.1093/cid/civ484. PubMed PMID: 26224766. Cavaleri M, Manolis E. Strategic Regulatory Evaluation and Endorsement of the Hollow Fiber Tuberculosis System as a Novel Drug Development Tool. Clin Infect Dis. 2015 Aug 15; 61 Suppl 1:S5-9. http://dx.doi.org/10.1093/cid/civ424. PubMed PMID: 26224771. Romero, K., Clay, R., Hanna, D. Systematic Analysis of Hollow Fiber Model of Tuberculosis Experiments. Clin Infect Dis. 2015 Aug 15;61 Suppl 1:S10-7. http://dx.doi.org/10.1093/cid/civ425. PubMed PMID: 26224767. Pasipanodya JG, Nuermberger E, Romero K, Hanna D, Gumbo T. Correlations Between the Hollow Fiber Model of Tuberculosis and Therapeutic Events in Tuberculosis Patients: Learn and Confirm. Clin Infect Dis. 2015 Aug 15;61 Suppl 1:S18-24. http://dx.doi.org/10.1093/cid/civ426. PubMed PMID: 26224768. Gumbo T, Pasipanodya JG, Nuermberger E, Romero K, Hanna D. Forecasting Accuracy of the Hollow Fiber Model of Tuberculosis for Clinical Therapeutic Outcomes. Clin Infect Dis. 2015 Aug 15;61 Suppl 1:S25-31. http://dx.doi.org/10.1093/cid/civ427. PubMed PMID: 26224769. Gumbo T, Pasipanodya JG, Romero K, Hanna D, Nuermberger E. The Hollow Fiber System Model in the Nonclinical Evaluation of Antituberculosis Drug Regimens Clin Infect Dis. 2015 Aug 15;61 Suppl 1:S32-3. http://dx.doi.org/10.1093/cid/civ460. PubMed PMID: 26224770. Chilukuri D, McMaster O, Bergman K, Colangelo P, Snow K, Toerner JG.

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Pro-Test Deutschland, a voice for science

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Scientists often fail to communicate their moral decisions and ethical reasoning for their work to the broader public. It is of no surprise then that some scientists are accused of a supposed lack of ethics, and those scientists conducting research with animals are attacked for alleged inhumane and immoral treatment of animals. Pro-Test Deutschland (PTD) does not think animal research lacks ethical standards or moral fiber. Rather, PTD believes what is missing is an open line of communication. Pro-Test Deutschland understands that it is often hard for people outside of the scientific community to obtain reliable information about the appropriate applications for animal research and why it is needed. Thus, to facilitate an informed and fair debate for the entire society, PTD supplies information through its website and social media platforms. By offering clarification on many scientific, ethical, legal, social, and psychological aspects of animal research, PTD provides a common ground to all those who wish to stand up for science.

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Vascularization of multi-organ-chips for tissue engineering and regenerative medicine

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Organ-on-a-chips provide an adequate and reliable system for drug testing and engineering of small tissues. Yet, a big drawback is the lack of an intrinsic vascularization of tissue-engineered constructs. Here, we demonstrate the incorporation of fibrin scaffolds with endothelial and adipose-derived stem cells embedded into a perfused closed multi-organ-chip (MOC) system. We show that under static conditions vascular network formation is influenced by both fibrinogen and thrombin concentrations. 3D-rendered images using two-photon microscopy analysis show organized microvascular structures in samples cultured under static or flow conditions for two weeks. We furthermore report that the use of serum- and growth factor-free basal media did not impair the maintenance of formed vascular structures when the media switch occurs on day 4 of incubation. Moreover, using fluorescence-labeled fibrinogen we monitored fibrin degradation under static conditions and address the use of the fibrinolysis inhibitor aprotinin for optimal microvessel formation. Finally, we conclude that a vascular network can be established by co-culturing endothelial and adipose-derived stem cells in a perfused organ-on-a-chip model. Further experiments will address whether additional layers of microtissues can be vascularized by this system.

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Chronologically-aged fibroblasts significantly alter the morphology of reconstructed human skin

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Preclinical drug development relies on animal models and early clinical trials on young men, but demographic changes in western societies ask for the development of medicinal products for elderly patients. Testing might disregard age and sex in addition to the general lack of predictive power due to species-specific differences when considering data generated in animals [1]. Various approaches tried to emulate skin aging, but faced distinct limitations [2]. Herein, we investigated the influence of fibroblasts from donors with varying age and sex on the morphology of reconstructed human skin (RHS). RHS built from fibroblasts isolated from medically indicated circumcised boys (< 9 years; juvenile fibroblasts), was compared to RHS built from fibroblasts isolated from mamma reduction surgeries of 20 to 30 years (adult) and 60 to 70 years (aged) old female donors. Juvenile keratinocytes from the juvenile donors (< 9 years) were used for the epidermal compartment of all RHS to investigate the influence of fibroblast age on epidermal development.

The dermal thickness decreased significantly by 21% in RHS composed of fibroblasts from aged donors which is in accordance with healthy human [3]. The collagen content in RHS with aged fibroblasts also declined compared to the constructs containing juvenile fibroblasts, which is in line with previously published data [4], too. Concordantly, the matrixmetalloproteinase-1 gene expression and the level of promatrixmetalloproteinase-1 protein increased more than 2-fold in RHS containing aged fibroblasts. Moreover, the fibroblast count in RHS with aged fibroblasts was reduced to 54% of the fibroblast count in RHS built with juvenile fibroblasts, which mimics the situation *in vivo* [5]. A thinner viable epidermis at the expense of a thickened stratum corneum was observed in RHS with adult and aged fibroblasts. Finally, the surface pH of 5.1 in the RHS with adult and aged fibroblasts is well in accordance with skin physiology in women between 20 and 70 years.

Taken together, replacing juvenile by chronologically-aged fibroblasts induces hallmarks of skin aging and might also better reflect the diversity of patients in RHS. Thus, our approach processes not only the potential to advance preclinical drug development, but also reduces animal testing upon formal validation.

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Inflammatory skin disease models

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Reconstructed human tissues, including *in vitro* skin models, gained continuously interest during the past 20 years aiming for either the establishment of alternatives for animal testing or for human-based and, thus, presumably more reliable test systems for preclinical studies [1]. The establishment of disease models, however, is still in its infancy although it is of particular relevance to mimic pathological conditions *in vitro* as realistic as possible [2]. This talk gives an overview about new advances and approaches in the development of skin disease models with focus on inflammatory diseases and will demonstrate their versatility and applicability for studying disease-related pathological mechanism *in vitro* and for the assessment of tolerability and efficiency of drugs or drug delivery systems.

Pathologic conditions are induced either by modulating disease-associated genes [3], supplementation of disease-related cytokines [4], implementation of immune cells or the application of patient-derived cells.

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Placental models to analyze the effects of chemotherapeutics on trophoblastic cells during pregnancy

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Purpose

Despite the emergence of vaccines cervical cancer is still one of the most frequent malignancies during pregnancy caused by the lack of vaccination as well as screening examinations in the third world. Besides surgery, chemotherapy in the second and third trimester is indicated to maintain pregnancy and enhance outcome for mother and fetus. Common chemotherapeutics seem to be well tolerated and malformations occur as from normal pregnancies. Nonetheless, data are rare and studies examining the toxic impact of chemotherapeutics on reproduction are usually based on animal models [1]. Therefore, we aim to analyze the toxicity of common chemotherapeutics on trophoblastic cell lines in 2D and 3D as well as on placental villous tissue explants (PVTEs).

Methods

The two immortalized trophoblast cell lines HTR-8/SVneo and JEG-3 were cultured as monolayer or formed to spheroids via hanging drops. Placentas from healthy women were obtained from the University Hospital Jena after spontaneous delivery or caesarian section for preparation of PVTEs (n = 3). Cells and PVTEs were incubated with cisplatin, paclitaxel, topotecan or bleomycin each in three different concentrations for up to 48 hours. Cell viability was analyzed via MTS assay and supernatant analyzes of glucose, lactate, LDH, human chorionic gonadotropin as well as estrogen and progesterone were performed. For histological examinations spheroids and PVTEs were embedded in paraffin.

Results

In monolayer, MTS assay revealed a decrease of cell viability in almost all tested conditions. Further, dramatic effects on JEG-3 spheroid formation have been detected microscopically after incubation with cisplatin and paclitaxel. Levels of glucose, lactate and estrogen in PVTE supernatants were significant modified by cisplatin, paclitaxel and topotecan. Morphologic changes were observed in spheroids and PVTEs including the untreated PVTEs and became more remarkable with ongoing cultivation time.

Conclusions

Spheroids react more differentiated to chemotherapeutics than monolayer culture owed to their stratified composition resembling solid tumors *in vivo*. Consisting of numerous cell types embedded in their natural extracellular matrix PVTEs represent a promising *ex vivo* model to study effects of xenobiotics on human placenta and overcome animal models with limited informative value. Supernatant analyses indicate glucose, lactate and estrogen as potential toxicity marker. In future studies we plan to evaluate placental characteristics such as invasion, migration or secretion of cytokines to broaden the application range of PVTEs.

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Improving cell culture experiments by replacing fetal calf serum with human serum

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Purpose
Most experimental setups include fetal calf serum (FCS) as growth supplement for cell culture media, although its use is associated with a range of ethical concerns [1]. Further, growth factors and receptors differ between species with regard to amino acid sequences, splicing variants or glycosylation patterns in particular [2]. Therefore, cultivation of human cells with FCS might lead to ligand-receptor-interactions which do not mimic the human biology correctly and lead to imprecise experimental outcomes. Hence, we aim to evaluate the impact of human serum (HS) and FCS on proliferation, migration, invasion and spheroid formation of cervical cancer cells and granulosa cells.

Methods
A side-to-side comparison between both sera was performed to assess cell viability, migration and invasion of the two cervical cancer cell lines SiHa and SW756. Further, cell viability of the granulosa cell lines COV434 and KGN as well as of the primary granulosa cells isolated from IVF-ICSI patients were analyzed via MTS-assay. The effects of HS or FCS on spheroid formation of SiHa as well as SW756 cells were analyzed microscopically after forming via hanging drops for up to 72 hours. Subsequently, after transfer to poly-HEMA coated 96 well plates the influence of HS and FCS on pre-formed spheroids was analyzed.

Results
Cell proliferation was stimulated by FCS and HS in the cervical cancer cells similarly. HS enhanced invasion, whereas a slight improvement of migration was detected by wound-healing assay when culture in media supplemented with FCS. Concerning the granulosa cells, the cell viability increased in media with HS. Further, HS significantly improved spheroid formation, particularly in SiHa cells. While SiHa cells in medium with FCS form loose aggregates in the presence of 25% methocel, regularly shaped spheroids could be observed in medium supplemented with HS even in the absence of methocel. Culturing in medium containing FCS led to a degradation of pre-formed spheroids. The effect of HS on spheroid formation was confirmed by histological examinations.

Conclusion
HS affects cell viability, migration and invasion capacity on cervical cancer cell lines similarly and therefore displays a good alternative for FCS in cell culture. Moreover, an increased cell metabolic activity was detected in case of all granulosa cell lines. Culturing cells in HS led to regularly formed spheroids, even when cells grown in FCS did not. We see this as evidence, that xenogeneic sera do not mimic the human situation properly. The exact molecular background of serum-related species differences and its concrete effects on experimental outcomes require further investigations.

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Safety assessment in EU-substance law: an open door for alternative methods

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The Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH, ECNo 1907/2006), the Biocidal Products Regulation (EUNo 528/2012) and the Plant Protection Products Regulation (ECNo 1107/2009) require certain safety assessments if a particular substance or product is to be registered or authorized within the EU. Therefore, the regulatory frameworks – either the regulations themselves or an implementing regulation – determine various tests which ought to be performed by the manufacturer, producer or importer. Several of those methods include the use of animals. However, REACH, the Biocidal Products Regulation as well as the Plant Protection Products Regulation promote alternative testing methods for the safety assessment of substances, thus enabling to reduce and replace animal testing. Against this background, all three regulations enshrine the principles of data sharing and avoidance of unnecessary testing, meaning that no studies involving vertebrate animals shall be repeated, but existing data shall be shared between the registrants resp. applicants. In general, studies affecting vertebrate animals shall be used at most as ultima ratio, that is to say no alternative testing method is available. Moreover, REACH and the Biocidal Products Regulation open possibilities to waive the standard tests (involving animals) in case their practical application is not scientifically necessary, technically not possible or the tests can be "waived" on substance-tailored exposure.

Aside from these general options concerning the reduction of regulatory animal testing, the EU-legislator endeavours the implementation of alternative methods as standard tests for safety evaluations of certain substances. However, to incorporate an alternative method it needs – as a rule – to be validated. The EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) performs the validation process and, where appropriate, validates a method which results (theoretically) in its scientific – and eventually regulatory – acceptance. The process is time-consuming and cost-intensive. It is thus vital to simplify the access to the validation as well as the process itself. Additionally, measures need to be taken to allow immediate regulatory acceptance as currently – even though the alternative is scientifically accepted – the regulatory acceptance may remain pending for years after a successful validation.

This presentation will discuss two approaches on how to minimize and replace regulatory animal testing within the existing legal and regulatory framework. For this purpose, the basic elements concerning the reduction and replacement of animal use for safety assessments within the three regulations will be described before their weaknesses will be analysed. Additionally, the role of the validation process with regard to the replacement of standard tests (involving the use of animals) will be assessed. Finally, the presentation will focus on the enhancement of regulatory acceptance and data sharing by facilitating cooperation between all affected actors. It will be demonstrated that the metaphorical door to not only reducing, but replacing animal testing for safety evaluations of dangerous substances within the scope of REACH, the Biocidal Products Regulation and the Plant Protection Products Regulation is open, we just have to walk through it.

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Human or mouse fibroblasts for assessing acute toxicity?

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BALB/c 3T3 Neutral Red Uptake (NRU) assay is a standardized test method for estimating starting doses for acute oral systemic toxicity tests in rodents (OECD GD 129: Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests. In: Series on Testing and Assessment, ENV/JM/MONO(2010)). The method uses mouse BALB/c 3T3 fibroblasts or normal human epidermal keratinocyte NHK cells. Mouse BALB/c 3T3 fibroblasts is mostly used. We have previously transferred and validated BALB/c 3T3 NRU assay in our GLP- laboratory. Subsequently, in order to obtain more human relevant cytotoxicity data, we have performed an intralaboratory validation using human BJ fibroblasts in the NRU assay instead of mouse BALB/c 3T3 fibroblasts. Human BJ NRU assay passed the criteria for relevance, reproducibility and repeatability, and was implemented as a routine test method in our laboratory. Here we present comparative cytotoxicity data of 23 different test chemicals (pharmaceuticals, industrial chemicals, pesticides and food additives) produced with both BALB/c 3T3 NRU and BJ NRU assays.

Mouse BALB/c 3T3 fibroblasts (CCL-163) and human BJ fibroblasts (CRL-2522) were obtained from ATCC. The assays were performed according to OECD GD 129. The results were given as percentage of viable cells as compared to the untreated controls. Sodium dodecyl sulfate was used as positive control. Solvent was the negative control.

Most of the 23 test chemicals studied were more toxic to mouse BALB/c 3T3 than to human BJ fibroblasts (e.g. Acetaminophen, Acetone, Mercury II Chloride, Octyl gallate and Sorbic acid), or were equally toxic to mouse 3T3 and human BJ fibroblasts (e.g. Nicotine, Bisphenol A, Sodium dichromate dehydrate and Sodium selenite). Only a few of the test chemicals, i.e., Digoxin, Oxytetracycline and Potassium cyanide were more toxic to human BJ than to mouse 3T3 fibroblasts. The most remarkable difference between human and mouse fibroblasts was detected with Acetaminophen and Potassium cyanide; Acetaminophen was toxic to mouse fibroblasts with an IC_{50} of 49.4 μg/ml, but had no effect in human fibroblasts. Potassium cyanide, in turn, was not toxic to mouse fibroblasts but decreased human fibroblast viability with an IC_{50} of 226.9 μg/ml. When comparing the IC_{50} of tested chemicals in human fibroblast assay to human acute dose (mg/kg bw) the correlation coefficient of 0.869 was obtained. The respective correlations of IC_{50} with mouse cell test to human acute dose (mg/kg bw) and rodent acute dose (mg/kg bw) were 0.591 and 0.604, respectively.

A species-specific differences in the toxicity of tested chemicals between mouse and human fibroblasts were noticed. Further, human fibroblast test gave better correlation with the respective human acute dose than mouse fibroblast test proposing that human cell test should be used to assess acute cytotoxicity in man.

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The novel ToxTracker reporter system provides mechanistic insight into the genotoxic properties of compounds and materials

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With the increasing production of new chemicals for a wide range of applications in health care, food and as cosmetics, the demand for rapid and reliable toxicity assessment is growing. Novel innovative *in vitro* systems should ideally not only identify genotoxic properties of chemicals, but also provide insight into the type of cellular damage inflicted in order to more reliably predict human health hazard of novel compounds.

The ToxTracker assay is a novel mammalian stem cell-based assay that detects activation of specific cellular signalling pathways upon exposure to unknown compounds1. ToxTracker consists of a panel of six different GFP-tagged reporters that allows discrimination between induction of DNA damage, oxidative stress and protein damage. These reporters were constructed by first performing whole-genome transcription analysis of mES cells exposed to different classes of chemicals in order to identify specific biomarker genes. Next, GFP reporters were generated by bacterial artificial chromosome (BAC) recombineering. The ToxTracker reporter cell lines are combined in 96-well plates, exposed to the test samples, typically for 24h but longer exposures are possible, and fluorescence in living cells are examined by flow cytometry. ToxTracker provides insight into the mechanisms of toxicity by visualising induction of DNA replication stress, NF-kB associated DNA damage signalling, various cellular anti-oxidant responses and activation of the unfolded protein response. In addition, ToxTracker can discriminate between clastogenic genotoxins and eugenic compounds based on the differential induction and kinetics to the different genotoxicity reporters.

For data analysis, we have developed the software tool ToxPlot. Automated data analysis and graphical representation of the test results allow clear and rapid assessment of the reactive properties of compounds. Furthermore, Toxplot allows comparison of the ToxTracker profile from unknown compounds with a large collection of reference compounds to assess the relevance of the test results.

The ToxTracker assay has been extensively validated using the compound library suggested by ECVAM and has an outstanding sensitivity and specificity compared to the conventional *in vitro* genotoxicity tests. The integrative approach of the ToxTracker assay provides a powerful tool for *in vitro* carcinogenic hazard identification of chemicals by unveiling activation of specific cellular signalling pathways upon exposure and deliver insight into the underlying mechanism of toxicity.

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A novel 3D *in vitro* model of the deep lung in both healthy and diseased state for screening of safety and efficacy of pulmonary (nano)medicines

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Pulmonary drug delivery has gained noteworthy attention by offering the possibility to administer drugs in a non-invasive way, as well as avoiding digestive enzymes and first-pass metabolism. In the last couple of years our lab has worked on novel in vitro approaches to mimic the human air-blood barrier, where gas-exchange occurs, in order to study the interaction of novel pulmonary drug carriers, as well as the biocompatibility of novel anti-infective drugs [1,2,3].

In addition to this scientific output on drug efficacy and effects on the air-blood barrier, using *in vitro* models that mimic the *in vivo* situation as close as possible also conforms to the "3Rs" principles of refinement, replacement, and reduction of animal experimentations in research.

Our *in vitro* model of the alveolar region features the two main players of the air-blood barrier: epithelial type I cells and macrophages. In order to make the model better reproducible and long-lasting human primary cells were replaced by cell lines, i.e. an in-house immortalized Type I epithelial cell line (hAELVi) [4] and differentiated human macrophage-like cells (THP-1). The hAELVi cell line has high morphological and physiological resemblance to alveolar Type-1 cells. Most importantly, the cells express functional tight junctions indicated by a transepithelial electrical resistance (TEER) of > 1000 Ohm x cm^2, a necessary characteristic for a diffusional barrier [5]. Differentiated THP-1 cells on the other hand, represent a key element of the immunological barrier of the deep lung, responsible for uptake of both inhaled particles and pathogens.

Elaborating this co-culture model that represents the deep lung in the healthy state, we also want to focus on *in vitro* systems mimicking a diseased state. Our hypothesis is that similar particles can induce an aberrant cellular response in patients whose cells are in a more vulnerable diseased state compared to healthy persons, something that is challenging to test locally *in vivo*. *In vitro*, however, a diseased state can be simulated with inflammatory stimuli, e.g. Lipopolysaccharide (LPS), since lung inflammation plays a central pathological role in many respiratory diseases, such as cystic fibrosis (CF), pneumonia, chronic obstructive pulmonary disease (COPD), asthma, tuberculosis (TBC) or lung fibrosis.

The first results obtained with LPS of *Escherichia Coli* as an inflammation activator showed no toxicity for both cell types, but a loss of barrier function indicated by a decrease in TEER values. In addition also increased levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 were observed, indicating a direct pro-inflammatory response.

The optimized inflamed model will be used for future validation as an alternative to animal testing in order to evaluate the interaction of nanoparticles as well as the effect of inhaled (nano)medicines in a diseased state compared to healthy state.

This work is part of the IMI Research Project COMPACT – Collaboration on the Optimization of Macromolecular Pharmaceutical Access to Cellular Targets.

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Opportunities for Organ-on-a-Chip models in toxicological risk assessment

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There is a strong desire in the society to develop and apply new and improved alternatives to animal testing in hazard assessments, for both ethical considerations and better prediction of the risks for adverse effects in humans. Use of alternatives are of special importance for the testing of nanomaterials, as information on the toxicity of a wider range of sizes, shapes and surface properties of the same chemical substance have to and will be produced. For the risk assessment of nanomaterials especially toxicokinetics, including possible accumulation, and toxicity after chronic exposure (repeated dose), are considered as the most important information. For these endpoints, unfortunately, validated and standardized non-animal methods are currently not available.

A recent development in *in vitro* systems is the Organ-on-a-Chip (OC) model using microfluidics. The main advantage of OCs over the current *in vitro* models (including static 3D models) appears to be the possibility to apply fluid (or gas) flows and mechanical stress, which create a more relevant physiological environment for cell culture [1]. This in turn leads to cultures that are physiologically better alike *in vivo* tissues and show longer viability (e.g. up to 4 weeks), while the exposure also better resembles real life conditions.

We have studied the current needs for (improved) *in vitro* alternatives for animal tests in toxicological risk assessment and the opportunities OC models provide therein. OC models appear to be useful for assessing the toxicokinetics, acute toxicity, subacute toxicity and possibly reproduction toxicity of chemicals. However, the added value of OC models in comparison to static *in vitro* models, and their value as replacement of *in vivo* models will need to be proven with comparative studies. We designed a roadmap for research and actions to incorporate (or not) OC models in toxicological risk assessment. It is envisaged that high priority should be given to models for toxicokinetics. Data from these models are necessary to be able to evaluate and eventually validate the performance of OC models for hazard endpoints such as repeated dose toxicity. As the *in vivo* studies used in the validation of *in vitro* hazard models inherently include toxicokinetics, toxicokinetic information needs to be added to the *in vitro* hazard data in such a validation for fair comparison.

This abstract does not necessarily reflect the view of the National Institute of Public Health and the Environment.

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Identification of pro-fibrotic biomarkers in precision-cut lung slices (PCLS)

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Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic interstitial pneumonias with one of the worst prognoses among respiratory diseases. The disease is characterized by an abnormal repair process, including the excessive proliferation of fibroblasts and destruction of cellular architecture of the lung. However, the exact pathomechanism of IPF is not yet understood. Current animal models for fibrosis do not entirely reflect all features of the disease as observed in humans which further hampers the development of new therapies. Yet, a general shift from *in vivo* to *ex vivo* models (and from animals to humans) seems to be necessary in order to support the development of translational models. The aim was to establish a novel experimental method to identify biomarkers of pulmonary fibrosis.

PCLS were prepared from lungs of bleomycin-treated rats, NaCl-treated controls or tumor-free lung tissue from cancer patients. Human PCLS were cultured in the presence of TGF-β and TNF-α to induce a pro-fibrotic profile. Rat PCLS were cultivated in medium only. Cytokine responses were measured by ELISA or MSD and genetic profiles were analyzed by RTqPCR.

Analysis of the mRNA profile revealed an up-regulation of important pro-fibrotic genes in lungs from bleomycin-treated rats as compared to the controls. PCLS prepared from these rats showed elevated expression of the pro-inflammatory cytokine IL-1β, which is involved in the initial wound repair mechanism and early fibrotic response. Increased IL-1β levels were also detected in human PCLS stimulated with TGF-β and TNF-α as compared to medium controls. Additionally, distinct pro-fibrotic genes, matrix metalloproteinases as well as important pro-fibrotic growth factors, amongst others, were found to be elevated in TGF-β and TNF-α treated PCLS as compared to unstimulated controls.

Overall, this novel *ex vivo* method shows great potential to investigate important signaling pathways and mechanisms of early onset fibrosis for the identification of pro-fibrotic biomarkers.

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Collaborative industry efforts to optimize and standardize *in vitro* methods suitable for tobacco regulatory science

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Currently U.S. regulatory authorities and industry are actively looking for ways to incorporate more predictive, mechanistic, non-animal methods to replace the traditional use of animals in testing. While some industries have been successful in working cooperatively with regulators to identify non-animal testing approaches, direct collaboration proves difficult when discussing the regulation of traditional and next generation tobacco products. As a way forward, a series of independently organized workshops have been convened with the aim to help optimize and standardize *in vitro* approaches currently utilized both by the tobacco industry for internal assessment of tobacco products, and by non-industry scientists to study mechanisms of toxicity, in acute, repeat, and chronic exposure formats. The collaboration has resulted in the initiation of a multi-laboratory testing program to assess key adverse outcomes associated with the use of tobacco products. The process for the establishment of collaboration, interaction with the regulatory agency and considerations in the development of the testing protocol will be discussed.

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Examination of the gap between validation and (real) regulatory acceptance

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Since the 2007 publication of the National Academy of Sciences report, Toxicity testing in the 21st Century Century: A Vision and A Strategy, efforts to identify, validate and accept non-animal test methods have increased exponentially worldwide. The availability of OECD Test Guidelines for a variety of acute endpoints, as well as the stated acceptance of such tests by several regulatory authorities, should logically result in a similar increase in use by industry. Case studies show, however, that industry – for various reasons – does not utilize identified non-animal approaches to their fullest extent. Through case studies from the US and China, this presentation will explore some identified reasons for lack of use by industry and pose considerations for identifying solutions.

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Combination of endpoints is valuable method for estimating hepatobiliary toxicity in 3D spheroid cultures

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Drug-induced hepatobiliary impairment is one of the serious adverse events in clinical study. The use of animals in toxicology studies is highly controversial due to ethical and interspecies differences. Recently, various three-dimensional hepatocyte spheroid models using HepG2, HepaRG and human primary hepatocyte have been developed. The structural and functional similarity to the *in vivo* situation and long-term survivability of these spheroids are valuable features for an *in vitro* model simulating *in vivo* conditions. To estimate the utility and the possibility for combination of evaluation items for hepatobiliary toxicity, we confirmed the variation of endpoints using *in vitro* HepG2, HepaRG and human primary hepatocyte spheroid treated with hepatotoxicity-induced drugs.

In HepG2, HepaRG and human primary hepatocyte spheroids treated with acetaminophen (APAP), α-naphthylisothiocyanate (ANIT) and tetrachloromethane (CCl_4), their irregular morphology (diameter, volume and sphericity) increased in a dose-dependent manner. Especially, sphericity of spheroids indicated remarkable sensitivity. Furthermore, in living spheroid staining using SYTOX, TMRM, BODIPY and CellRox, intracellular responses including cellular membrane or mitochondrial permeability, lipid droplet accumulation and ROS production, varied between the drugs. In HE and immunohistochemical staining, spheroids demonstrated distinct findings, such as cellular degeneration and/or necrosis and increased vacuolization. miR-122 expressions in supernatant were significantly elevated after treatment of drugs especially in human primary hepatocyte spheroid.

We revealed that chronological drug-induced hepatobiliary toxicity could be detected by measuring morphology, intracellular responses (cellular membrane and mitochondrial permeability, lipid droplet accumulation and ROS production) and supernatant miR-122 level of spheroids. Furthermore, combination of evaluation items was valuable method for detecting hepatobiliary toxicity *in vitro*.

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An improved aerosol deposition device to assess safety and efficacy of dry powder formulations at the air/liquid interface

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In order to replace animal testing in pulmonary drug delivery, the Pharmaceutical Aerosol Deposition Device on Cell Culture (PADDOCC), was originally designed by Hein et al. [1,2] to enable deposition of dry powder formulations from commercial metered dose in halers (DPI's) on pulmonary cell cultures under physiological conditions. However, this prototype was still limited in several aspects which necessitated a complete redesign of such platform for aerosol testing. The newly developed instrument can be used with commercial dry powder inhaler devices and offers flexible adjustment of the flow rate and other process parameters. We investigated different dry powders and their performance in the system. Scanning electron microscopy and HPLC-analysis emphasized the impact of specific instrument settings on a commercial pharmaceutical salbutamol dry powder formulation. Approximately 0.5 µg/cm² salbutamol was deposited for one deposition cycle (10 minutes sedimentation). Innovative formulations based on spray dried mannitol, trehalose and starch-derivatives were also investigated.

After proper adjustment of the instrument's settings, different aerosol formulations can be distinguished. The new instrument offers the opportunity to study the effects of particles as well as of aerosolized drugs, excipients and final formulations on respiratory cell cultures at the air-liquid interface, using the same dose and device as later by the patient. As dry powder inhalers cannot be tested on animals, this new instrument may provide important information on the safety and efficacy of new dry powder aerosol medicines and thus facilitate their translation in to the clinic.

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Repeated isoflurane anesthesia in female mice – assessing the severity of stress

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Introduction

The ultimate goal of the EU Directive 2010/63 is to phase out all animal testing. Wherever animal experimentation is necessary, the 3-R-principle of Russel and Burch is meant to be applied. The 3-R-principle stands for replacing animal testing with alternative methods. If no alternative method can be applied, the total number of animals is supposed be reduced. Consequently, some animals are used multiple times in the course of an experiment. For example, in imaging studies, rodents are repeatedly anesthetized in order to control the progress of a disease. However, the Directive claims that "the benefit of reusing animals should be balanced against any adverse effects on their welfare" [1].

The aim of this study is to investigate if repeated anesthesia causes more stress in female mice than a single anesthesia.

Materials and methods

Female C57/BL6 J and the most common inhalation anesthetic isoflurane were used. All parameters observed were compared between controls and animals receiving a single or repeated anesthesia. Over a period of 3 weeks, the animals were anesthetized 6 times for 45 minutes, respectively [2]. Vital parameters and reflexes were monitored when the animals were under anesthesia. The Mouse Grimace Scale was applied 30 and 150 minutes after anesthesia. Besides pain, the Grimace Scale can also assess stress and discomfort. As the display of so-called luxury behaviors serves as an indicator of wellbeing, nest building and burrowing behavior were observed. Furthermore, activity, food and water intake were monitored for 24 hours. A behavioral test battery including the free exploratory paradigm and rotarod test was performed 2 and 9 days after the last anesthesia. Motor coordination and balance were assessed by the rotarod. The free exploratory paradigm estimated anxiety and exploratory behavior. Moreover, fecal corticosterone metabolites and steroid hormones in fur were measured in order to prove evidence of cumulative stress.

Results

Mice being repeatedly anesthetized lost the righting reflex within a shorter time and showed more excitations during induction than mice which were anesthetized only once. The vital parameters did not differ between animals receiving a single or repeated anesthesia. After repeated anesthesia, the latency to first food intake decreased and the animals ingested less food over 24 hours than after a single anesthesia. This deficit was compensated for 8 days later. Both single and repeated anesthesia caused higher scores on the Mouse Grimace Scale versus control 30 minutes after anesthesia. Although repeated anesthesia reduced burrowing behavior, the nests of all mice were assigned equal scores. No effects were seen in the rotarod test and nocturnal activity. In the free exploratory paradigm, repeated anesthesia increased the latency to explore and decreased the total duration of exploration one day after anesthesia. During the test period neither the body weight nor the preliminary analysis of fecal corticosterone metabolites differed between the groups.

Conclusion

Repeated anesthesia caused more stress in female mice during the immediate postanesthetic period only. Accordingly, we assume that the severity level of a single as well as of repeated anesthesia is mild.

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Progress in preparation of electrically conductive biomimetic scaffolds for cell cultivation

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Remarkable progress in biological science as physiology, molecular biology and medicine in last decades together with advanced technologies are opening the door for preparation of biomimetic scaffolds. In this context the holistic approach must be employed. The human body must be considered as a complex biological system which is employing the bioelectricity on the level of organs, tissues and also individual cells. The alteration on any of these levels can have remarkable impact on many aspects of cell physiology. In context of preparation of biomimetic scaffolds, the conducting polymers are considered as an attractive material due to its intrinsic conductivity, easy inexpensive synthesis, and versatile surface properties. Recently a number of studies about the interaction between the conducting polymers, mainly polyaniline, and cells were performed by our team. The pilot studies focused on the cytotoxicity, skin sensitization and irritation [3], revealing the purification procedures [5] or determination of content of low molecular impurities [4]. Thanks to those works, the crucial factors influencing the biocompatibility were detected. Subsequently, the characterization and modification of surface properties were performed and studied in context of stem cell adhesion, growth, cardiomyogenesis and neurogenesis e.g. in [1,2]. Finally, the 3D structures mimicking the electrically excitable tissues using conducting polymers were prepared. The recapitulation of progress in research of biological properties of conducting polymers as well as preparation of biomimetic materials are the main aim of present work.

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MAD world? Promoting the greater mutual acceptance of data

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Mutual Acceptance of Data (MAD) makes perfect sense in a globalised marketplace.

MAD is a multilateral agreement that allows participating countries to share the results of hazard and risk assessments on chemicals generated using OECD methods and principles. This provides confidence that test data generated under the OECD MAD system, and for the same chemicals, can be universally applied.

Not only does this reduce duplicate testing, it enhances the relevance of scientific datasets for chemical substances by providing consistent, transparent data for risk assessment.

However, the concept is not universally or evenly applied, meaning that there remain overlaps which leads to avoidable duplicate testing. With novel AOP-based methods now emerging, there is also uncertainty as to how the results of these new methodologies will be viewed, and what additional criteria might be imposed before they are accepted under various regulatory regimes.

The example of the OECD IATA for Skin Sensitisation will be used. In particular, the way in which AOP-based assays for skin sensitisation have been adopted into the EU REACH regulation, and the questions and uncertainties that surfaced during and after this process.

Reasons behind hesitance to adopt new OECD validated methods will be explored, as will questions over their application and confident interpretation in a regulatory context, with a focus on reducing uncertainties for assessors and policy makers.

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An inflammatory, filaggrin-deficient skin model demonstrates hallmarks of atopic dermatitis *in vitro*

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Atopic dermatitis (AD) is a chronic inflammatory skin disease which is characterized by an impaired epidermal barrier. In 2006, mutations in the filaggrin gene (*FLG*) were identified as a major predisposing factor for the manifestation of AD [1]. Aside from barrier deficiencies, AD is characterized by over-shooting Th2-mediated inflammatory processes and impaired innate immunity [2]. The Th2-derived cytokines IL-4 and IL-13 significantly contribute to the pathogenesis of AD, but their effects on the skin homeostasis and particularly the interdependencies with *FLG* deficiency are not yet fully understood. In this study, an inflammatory skin model was developed to investigate the influence of *FLG* knockdown on the expression of skin barrier and tight junction proteins, the skin barrier, skin surface pH and expression of human β-defensin 1-3 under normal and inflammatory conditions. Supplementation with IL-4 and IL-13 resulted in distinct epidermal thickening (*FLG*+ 89.5 ± 9.9 µm vs. *FLG*-/IL-4/13 160.7 ± 30.8 µm), spongiosis and parakeratosis and shifted the skin surface pH towards unphysiologically high values (pH 6.37 ± 0.03), well-known features of inflammatory skin diseases like atopic dermatitis. Furthermore, we observed a compensatory 3-fold upregulation of involucrin and occludin in *FLG*- models, which was considerably disturbed by IL-4/13 exposure. Concordantly, these cytokines significantly reduced the expression of the skin barrier proteins *FLG* and involucrin in normal skin models. Most interestingly, for the first time we detected significantly (~ 5-fold) higher expression of β-defensin 2 in *FLG*-models. This was particularly noteworthy because β-defensin 2 is known to be upregulated through bacteria or inflammation but not by a genetic defect [2]. Interestingly, this up-regulation was again markedly reduced under inflammatory conditions.

In conclusion, by the means of an inflammatory *in vitro* skin model exhibiting clinical characteristics of atopic dermatitis we were able to further study basic aspects of AD pathogenesis. The results indicate that defects in the epidermal barrier and cutaneous innate immune response are not primarily linked to filaggrin deficiency but are rather secondarily induced by Th2-related inflammation [3].

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A proteomics approach reveals characteristic changes in THP-1 cells after treatment with contact sensitizers

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Allergic contact dermatitis (ACD) is a widespread skin disease with a high prevalence among the general population. Various organic chemicals such as fragrances or preservatives as well as several metals that may be present in cosmetics, jewellery or other commodities have the potential to induce a ACD. It is required that these low molecular weight electrophils bind to proteins in the skin and thereafter induce a delayed-type hypersensitivity immune response. Due to European legislation *in vivo* testing of cosmetics is prohibited. Despite the fact that two *in vitro* tests, the direct peptide reactivity assay (DPRA) and the KeratinoSens™ test, have already been validated by EURL ECVAM, more research is needed to supplement the existing alternative assays as they, for instance, do not allow the categorization of sensitizers. Here we use a proteomics approach to identify possible biomarkers for contact allergy. This will allow retrieving detailed information on pathways that are involved during activation and maturation of the cells.

Monocytic THP-1 cells were cultured and treated with contact allergens or irritants for 4, 8 and 24 hours. Activation of THP-1 cells was monitored by measuring the activation markers CD54 and CD86 after a 24 h treatment following the criteria of the human cell line activation test (hCLAT) [1] and by measuring the increased release of the cytokine IL-8 using an ELISA [2]. A stable isotope labeling by amino acids in cell culture (SILAC) experiment was performed to identify regulated proteins and possible biomarkers. Subsequently, the cell membrane fraction of the THP-1 cells was enriched using the established FASP (filter aided sample preparation) protocol [3]. Proteins were then analyzed by nano-LC-MALDI-MS/MS.

A proof of principle study using the strong allergen 2,4-dinitrochlorobenzene (DNCB; 5, 10 and 20 µM) was conducted. Our approach allowed us to quantify up to 300 proteins out of which a significant number was regulated. Regulation of proteins was concentration-dependent in response to allergens and much lower for cells treated with irritants. Regulated proteins included proteins like catalase, which are already known to be induced in ACD, as well as others currently not described in the context of ACD.

Overall, the proteomics approach established allows the identification of regulated proteins after treatment of THP-1 cells with contact allergens. Currently, we are still improving the protocol for enrichment of the cell membrane proteome. Eventually, we will perform detailed studies using a set of different allergens.

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Designer gels for cell culture

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Biogelx Limited is a biomaterials company that designs tuneable peptide hydrogels, offering artificial tissue environments to cell biologists for a range of cell culture applications.

The hydrogels are highly tuneable, cell-matched biomaterials, capable of revolutionising the way cell biologists control and manipulate cell behaviour in the laboratory. This is of direct relevance to fundamental cell research, including the study of stem cells and in the design of improved disease models for *in vitro* cell-based assays.

Biogelx technology uses self-assembling, short-chain peptide amphiphiles to design nanofibrous hydrogel scaffolds. This assembly can be controlled in order to design hydrogel platforms that are three dimensional (3D), 99% water and have the same nanoscale matrix structure as human tissue (see figure below). This gives control back to the cell biologist, as the gels can be tuned to meet the needs of any given cell type, through mechanical and chemical modification.

This presentation will showcase the underlying chemistry of Biogelx's peptide hydrogels, highlighting the range of chemical and mechanical modifications that can be implemented within the gels, in order to address a wide range of cell-based applications. Some examples of academic and industrial collaborative work shall also be presented, include how the gel tuneable properties, can be use to influence the differentiation pathway of stem cells.

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In vitro study of melatonin effect on HepG2 cells treated with IL-6 in newly developed liver on chip device

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The liver has diverse functions such as the acute phase reaction, energy metabolism, and bile acid transportation. Interleukin 6 (IL-6) is a cytokines and considered a central inflammatory mediator as well as a major regulator of the acute phase response. Melatonin is a lipophilic hormone, mainly produced during sleeping at night by the pineal gland. This hormone has potent antioxidant and anti-inflammatory activities. The outstanding role of the liver to maintain homeostasis and metabolic regulation prompted us to evaluate the direct modification of IL-6-induced alterations on HepG2 cells by melatonin in a newly developed liver on a chip.

Earlier, we have presented a new human liver cell culture system with three dimensional structures in a microfluidic platform to study liver specific physiology and toxicology study [1]. We generated a culture space for liver cells without any physical barrier between medium flow and cell culture area, using a solidified extracellular matrix layered along the phaseguide to protect from shear stress and to mimic the *in vivo* environment, i.e. indirect contact of the hepatocytes with the blood flow.

Using this newly developed *in vitro* liver platform, we evaluate the interplay of IL-6 induced hepatocellular stress response and the protective action of melatonin. Particularly, administration of IL-6 causes damage of mitochondria, reduced activity of cytochrome p450 1A enzyme, MRP2, and an increase of acute phase response. Furthermore, melatonin alleviates hepatocellular stress by reduction of overwhelming acute phase response and reduces mitochondrial dysfunction.

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Analysis of a novel *ex-vivo* organ model to simulate neurodegeneration in retina

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Purpose

In order to understand the pathological processes of retinal diseases, like ischemia or glaucoma, appropriate models are necessary. Either hypoxia that is a key player in both diseases can be simulated by reducing the amount of oxygen or, even more controllable, it can be induced by administrating cobalt chloride ($CoCl_2$). In this project, the degenerative effect of $CoCl_2$ on neurons and glia cells in a novel retina organ culture model were investigated. We established a reliable and reproducible retina degeneration model which is an alternative to *in-vivo* testing. This will lead to reduced numbers of animal experiments in ophthalmic research.

Methods

Organotypic cultures of porcine retina were cultivated and treated with different concentrations of $CoCl_2$ (0, 100, 300 and 500 µM) for 48 h from day 1 onwards. At day 8, retinas were processed for histological (n = 8/group), Western blot (n = 4-5/group), and qRT-PCR (n = 5/group) analysis. Retinal ganglion cells (RGCs, Brn-3a), amacrine cells (calretinin), bipolar cells (PKCα), microglia (Iba1), and activated microglia (CD16/32) were analyzed. In addition, macroglia response (GFAP, vimentin) was evaluated.

Results

Cultivation with 300 and 500 µM $CoCl_2$ led to a decreased number of Brn-3a+ ganglion cells (300 µM: $p = 0.002$; 500 µM: $p < 0.001$), calretinin + amacrine cells (300 µM: $p = 0.002$; 500 µM $p = 0.001$), and PKCα + bipolar cells (300 µM: $p = 0.007$; 500 µM: $p = 0.001$). In contrast, 100 µM $CoCl_2$ had no effect on the neurons of the porcine retina. All three $CoCl_2$ concentrations reduced the microglia population (100 µM: $p = 0.07$; 300 µM: $p<0.001$; 500 µM: $p < 0.001$) and decreased the number of activated microglia (100 µM: $p = 0.001$; 300 µM: $p<0.001$; 500 µM: $p = 0.02$). Macroglia were not affected by $CoCl_2$.

Conclusions

$CoCl_2$ induced a strong degeneration of the porcine retina starting at a concentration of 300 µM. Especially the neurons of the inner retina layers were affected. However, the degenerative effect of $CoCl_2$ was not restricted to neurons, also microglia cells underwent a death mechanism. The decreased number of microglia was surprising, since $CoCl_2$ is known to induce hypoxia and a pro-inflammatory environment. Yet, high concentrations of $CoCl_2$ seem to be toxic for these cells. Compared to a retinal ischemia animal model, similar degenerative mechanisms, in regard to neuronal cell loss, were observed in this novel organ culture model. In summary, an effective and reproducible model for retinal degeneration could be established, which is easy to handle and ready to use for drug screening studies.

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GARD - the future of sensitization testing using a genomics-based platform

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Chemicals able to induce sensitization in exposed individuals are commonly referred to as chemical sensitizers. Depending on the route of exposure and chemical properties, sensitizers give rise to clinical symptoms known as Allergic contact dermatitis (ACD) or Occupational asthma (OA).

ACD is an inflammatory skin disease caused by immunological responses towards chemical skin sensitizers, affecting close to 20% of western population. While OA is not as prevalent, the socioeconomic effects are considered substantial, as health implications are severe.

In order to prevent adverse effects of consumer products and in occupational environments, chemicals are tested for sensitizing properties. Historically, such testing has been performed using animal-based *in vivo* assays, primarily the murine local lymph node assay (LLNA). Due to new legislations on the registration and use of chemicals, a ban on the use of animals within cosmetics industry, as well as efforts stimulated by public opinion and economic interests, recent years have seen a surge of development of alternative methods for assessment of chemical sensitizers. To date, three *in vitro* methods for assessment of skin sensitizers have been validated. However, while relevant in the perspective of an Adverse Outcome Pathway (AOP), the predictive performance is inadequate for accurate safety assessment as stand-alone tests, and an integrated testing strategy based on these methods is still to be finalized.

We have developed a novel *in vitro* testing platform, called Genomic Allergen Rapid Detection – GARD [1], for the prediction of sensitizing chemicals, based on differential expression of disease-associated genomic biomarkers in human myeloid dendritic cell-like cells. By using panels of reference chemicals with known properties of the biological endpoint of interest, whole genome data sets are created with microarray technology for biomarker discovery. Multivariate statistics and computational methods are then employed to find the most powerful genomic predictors for the endpoint of interest, using data-driven approaches.

To date, applications of the GARD platform have generated GARDskin, a novel, state of the art assay for the assessment of chemical skin sensitizers, exhibiting superior predictive performance compared to both *in vivo* and *in vitro* counterparts. The assay has been transferred from microarrays to a resource effective and easy to use technological platform [2], well suited for industrial screening, and is currently being validated for regulatory use. Similarly, GARDair is an adaptation of the GARD platform for assessment of chemical respiratory sensitizers [3]. The method is unique and highly required, as predictive assays for assessment of respiratory sensitizers are greatly underdeveloped, with no validated, or even widely accepted, *in vivo* or *in vitro* method currently in use.

Here, we describe the development, scientific validation, applications and the current state of the GARD platform. The scientific rationale behind the use of genomic biomarker signatures are detailed, and linked to the AOP in a biological context, and to applications made possible through multivariate computational prediction models in a technological context. In conclusion, we argue that GARD is a next generation *in vitro* assay ready for industrial implementation.

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Novel cystic fibrosis *in vitro* model for safety and efficacy testing of new drug delivery systems against chronic *Pseudomonas aeruginosa* infections

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Thick and sticky mucus, how it occurs in the lungs of cystic fibrosis (CF) patients offers good growth conditions for bacteria. Particularly *Pseudomonas aeruginosa*, known as the major pathogen in CF [1], is almost impossible to eradicate once a biofilm has formed. To overcome drug delivery problems, arising from the thick mucus layer and the biofilm, the development of nano-scaled drug delivery systems (DDS) might be a promising approach.

Evaluating the efficacy and safety of new DDS on suitable *in vitro* models is of course mandatory before going into clinical trials. In the literature a lot of murine CF models can be found. To better mimic disease relevant conditions *in vitro*, our aim was to establish a human based co-culture model of bronchial epithelial cells with *P. aeruginosa* on top, allowing the formation of a biofilm. Two bronchial epithelial cell lines Calu-3 and CFBE41o- were used, representing the healthy and CF-diseased airways, respectively. To resemble the thick and sticky mucus, as can be found in CF patients, an extra layer of human mucus was applied to the human epithelial cells. Once the cells have formed an epithelial barrier they are infected with a preformed biofilm of *P. aeruginosa*.

In order to treat infected human cells, ciprofloxacin-complex-loaded PLGA nanoparticles (NP) were prepared under controlled conditions using the novel MicroJet Reactor technology. The NPs should be able to penetrate the mucus and biofilm and release the drug in a controlled manner at the site of action accordingly. This new DDS was therefore applied after different time points onto the *in vitro* model as an aerosol. Colony forming unit count after 24 hours showed the efficacy of the DDS against *P. aeruginosa*. MTT assays also performed after 24 hours reveal that the human cells survived the infection and could successfully be treated. Further experiments with the application of the DDS as a dry powder are planned.

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An *in vitro* reconstructed normal human corneal tissue model: applications to dry eye, wound healing, and ophthalmic drug delivery

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Current methods to investigate corneal wound healing (CWH), pathogenesis of dry eye disease (DED) [1], and permeation of topically applied ophthalmics utilize cell cultures or animals [2]. This study evaluated the utility of the EpiCorneal human tissue model for CWH, DED, and drug permeation.

The EpiCorneal tissue model is comprised of normal human corneal epithelial cells that are cultured at the air-liquid interface. Corneal wounds were introduced by abrasion or chemicals (1N NaOH). Wounded tissues were cultured in the presence or absence of human corneal keratocytes (HCK) or EGFR inhibitor (erlotinib, 10 uM). A DED model was generated by placing EpiCorneal tissues under desiccating stress conditions (DSC, 40% RH, 40°C, and 5% CO_2) that stimulate morphological, cellular, and molecular changes relevant to dry eye. Corneal permeability was evaluated using compounds with a wide range of properties: hydrophilic and hydrophobic, low and high molecular weight dyes, ophthalmic related drugs at seven different formulations [3].

CWH was analyzed by transepithelial electrical resistance (TEER), histology, confocal microscopy, and gene expression. TEER recovered to 933.7/502.4 Ω*cm^2 in the presence/or absence of HCK in 4 days post-wounded cultures. mRNA expression was analyzed using a 96-gene wound healing microarray. 13 genes (including collagen, integrin, chemokine, and protein kinase families) were up-regulated in the EpiCorneal tissues 24 h post-abrasion in the absence of HCK and 16 genes (including WNT, FGF, small GTPases, chemokine, and integrin families) were up-regulated in the presence of HCK, but not in control cultures. DED was analyzed by TEER, histology, tissue viability, mucins and tight junction (TJ) protein expression. Dramatic reduction in tissue thickness was observed after 48 h in DSC that coincided with decreased expression of mucins, increased TEER and atypical expression of TJ proteins. Topical application (25 µl/tissue) of lubricant gel drops (GenTeal, Alcon) improved tissue morphology and barrier function.

Out of seven formulations of Latanaprost eye drops tested, Xalatan (containing 0.02% BAC) had the highest permeation (Papp = 8.81 cm·s-1) and Monoprost (preservative-free) had the lowest (Papp = 1.15 cm·s-1). Formulations containing Poloxamer 407 had higher Papp (6.05 and 6.27) when compared to formulations without surfactants (1.69 to 2.57). Tissue integrity and viability were maintained in all experiments as evidenced by Lucifer Yellow and MTT results.

The reconstructed *in vitro* organotypic human corneal tissue structurally and functionally reproduces CWH and DED, and its permeability resembles that of the *in vivo* human cornea. This model is anticipated to be a useful tool to study molecular mechanisms of ocular surface damage, DED, and to evaluate new corneal drug formulations.

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The use of metabolomics *in vivo* as read-across tool: a case study with phenoxy carboxylic acid herbicides

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The purpose of this presentation is to provide a case study of how metabolomics *in vivo* can be used to address chemical grouping and read across from a biological perspective. To demonstrate this, we selected MCPP as the target substance and as source substances MCPA and 2,4-DP and evaluated the plasma metabolome data from 28 day repeated dose toxicity studies against the BASF data base MetaMap®Tox.

The 28-day metabolome evaluation of the source substances indicates the liver and the kidney as the target organs. The metabolome evaluation of the target substance provides the same information. The overall comparison of the metabolome data indicate that 2,4-DP is the best source substance. Using the information of the 90-day study of this compound, it would have been predicted that MCPP would have shown decreased food consumption and body weight gain at 2,500 ppm. The target organs are the liver (weight increase and clinical-pathology changes), as well as the kidney (weight increase and clinical-pathology changes). A moderate reduction of red-blood cell parameters would also be expected at this dose level. The NOEL would have been expected to be below the value of 2,4-DP, i.e. < 500 ppm and more likely in the range of that of MCPA, i.e. at least 150 ppm.

From a qualitative point of view, these predictions are very similar to the results of the actual 90-day study in rats performed with the target substance (reduced food consumption and body weight gain, target organs: liver and kidney – weight increases with concomitant clinical-pathology changes, reduced red blood cells values). From a quantitative point of view, the predicted NOAEL of 150 ppm is in the range of that of the actual study (NOEL 75 ppm, NOAEL below 500 ppm). Consequently, the 90-day rat toxicity study of the target substance could have been waived and substituted by the 90-day results of 2,4-DP. The NOAEL would have been correctly assessed as < 500 ppm, and using MCPA's values, as at least 150 ppm.

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Practical application of human iPS cells for chemical toxicity assessment

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A number of animals have been widely used for chemical toxicity assessment. *In vitro* toxicity testing method is one of the most challenging fields of toxicology for human safety. Human induced pluripotent stem cells (iPSC) are very attractive source to make various human cells, such as cardiomyocytes and neurons, and are expected to provide a novel testing systems with high predictability, instead of animal use.

Here we have developed a testing method using human iPSC-derived cardiomyocytes (iPSC-CMs). Drug-induced lethal arrhythmia including Torsades de Pointes (TdP) have been evaluated by *in vitro* hERG channel assay and *in vivo* QT assay. Since human iPSC-CMs are known to express multiple ion channels including potassium, calcium, and sodium channels, we focused on iPS-CMs as an "all-in-one" next-generation testing method. We have established standardized protocol to measure field potential (FP) of iPS-CM sheet with multi-electrode array (MEA) system and found FP duration prolongation and EAD (Early afterdepolarization) as indices for evaluation of proarrhythmia risk. We next shared our MEA protocols and pilot experimental data and with international consortium. Based on our collaboration, international validation study is now in progress.

We have also tried to apply the iPSC technology for developmental neurotoxicity (DNT) assessment. We selected several positive compounds and searched endpoints for DNT assessment using iPSCs. We found that inhibition of neural differentiation is one of a potential metrics for DNT assessment. It is necessary to validate this protocol for further analysis.

In the symposium, we will present our strategy for the fit-for-purpose cells and the standardized protocols and discuss a potential testing methods using human iPSC. Human iPSC innovative technology would contribute to chemical toxicity assessment and facilitate 3R alternatives.

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CON4EI: EpiOcular Eye Irritation Test (EIT)

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Assessment of the acute eye irritation potential is part of the international regulatory requirements for testing of chemicals. The objective of the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project is to develop tiered testing strategies for eye irritation assessment for all drivers of classification. For this, a set of 80 reference chemicals (38 liquids and 42 solids) was tested with eight different alternative methods. Here, the results obtained with reconstructed human cornea-like epithelium (RHCE) EpiOcular and the EpiOcular Eye Irritation Test (EIT) – adopted as OECD TG 492 – are shown.

The primary aim of this study was an evaluation of the performance of the test method to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling (Category 1 and 2). In addition, the predictive capacity in terms of *in vivo* driver of classification was investigated. In a second step, it was investigated whether the EpiOcular EIT can be used as part of a tiered-testing strategy for eye irritation assessment. The chemicals were tested in two independent runs by MatTek IVLSL.

For the EpiOcular EIT, a sensitivity of 96.9% and specificity of 86.7% with an accuracy of 95% was obtained overall and for both runs separately (100% concordance). The results of this study seem promising with regard to the evaluation of inclusion of this test method in an integrated testing strategy for eye irritation assessment.

This research is funded by CEFIC-LRI. We acknowledge Cosmetics Europe for their contribution in chemical selection.

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Development, optimization and validation 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 (MD) extracts are dilute solutions with low irritation potential, therefore the validated RhE-methods needed to be modified to reflect needs of ISO 10993.

A protocol employing RhE EpiDerm was optimized in 2013 using known irritants and spiked polymers [1] (TIV, 2013).

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, nine EU and USA laboratories were trained in the use of the protocol in the preparation for the validation. All laboratories produced data with almost 100% agreement of predictions for the selected references.

Two of the laboratories 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 either 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. Round Robin validation of the Skin irritation test *in vitro* for assessment of Medical devices extracts has started in March 2016.

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Increasing biological relevance *in vitro*: from single microtissues to micro-physiological systems

Jens Kelm

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Cell-based assays have become an inherent part in drug discovery and development to predict the *in vivo* response to biologicals and chemicals. Cells are used throughout the whole developmental chain as a test system: (i) drug target validation, (ii) primary and secondary screening set up's, (iii) lead optimization and (iv) toxicological profiling. Current standard technology is based on the culture of mammalian cells, either primary or cell lines, as monolayers in plastic dishes. However, to gain the maximum benefit of *in vitro* cell cultures for drug de-risking, cells have to be maintained in a format which reflects *in vivo* cellular functionality, either animal or human, as closely as possible. To further increase in the value of *in vitro* models, technologies which allow direct cell-cell and inter-tissue communication are essential. Therefore, advanced 3D cell culture models are gaining momentum as the development of new therapeutics is a time and cost intensive process profiting from better drug de-risking. With this objective, scalable and automation-compatible tissue engineering strategies are being exploited to further improve the predictive power of cell-based assays. Here microtissue technology is being presented which allows on the one hand high throughput efficacy and safety testing and on the other flexible design of microphysiological systems.

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A hiPSC-based test platform (DropTech®) for toxicity assessment of small molecule compounds in early stage drug discovery

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Safety related failures due to toxicities and side effects of clinical candidates are one of the major causes for high attrition rates in development phases. Optimizing preclinical toxicity studies towards more physiologically relevant *in vitro* models, with a special focus on organ specific toxicity, in particular for cardiac, neuronal and hepatocellular tissues, is of key importance. Induced pluripotent stem cells (iPSC) generated from somatic cells and differentiated into target tissue are considered as powerful tools with clear advantages over animal models and have the potential to compensate for the lack of availability of primary cells of human origin for *in vitro* testing purposes. We generated an embryonic stem cell test (EST) based on human iPSCs, as a model to assess toxicity *in vitro*. Similar to murine variants of the test, three endpoints are assessed in order to predict the embryotoxic specific potential of test substances. Readouts include inhibition of differentiation into beating cardiomyocytes or neuronal rosettes, cytotoxic effects on iPS cells and the cytotoxic effects on fibroblasts. We used quantitative morphological assessment of contracting cardiomyocytes or neuronal rosette formation as the readout for differentiation as well as highly predictive protein markers specific to developing cardiac or neuronal tissue. Using this platform, we reliably predicted the toxic potential of a panel of standard compounds suggested by the European Centre for the Validation of Alternative Methods (ECVAM).

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Impact of new technologies on malignant tumor management and therapy

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Diagnosis and treatment of malignant diseases in Austria are well embedded in a broadly accessible healthcare system providing standardized patient care and treatment for everybody. Nevertheless, cancer therapy, which is highly cost intensive, currently lacks efficient treatment strategies that are developed through close interaction between clinicians, scientists and social workers as is seen in Scandinavian comprehensive cancer centers. A major concern in the management of cancer patients is the gap between molecular understanding of cancer derived from modern cancer research and its application for diagnostic or therapeutic means. To bridge this gap, tumor tissue is cultured and compound screens are performed to elucidate the efficacy of individual drug as well as drug combinations. We will use large selections of fully annotated tumor patient samples of different genetic backgrounds, tumor stages and grades, also including patient matched metastases. In addition, we will use orbitrap mass spectroscopy together with QuantN package to quantify the proteome of each FFPE sample (6000 proteins) and correlate it retrospectively with patient survival to discover new biomarkers important for diagnosis, prognosis and therapy. We will validate biomarkers by immunohistochemistry in large patient cohorts or by targeting these biomarkers with antibodies that can be visualized *in-vivo* by PET/MRI imaging techniques in collaboration with the Nuclear Medicine Facility at the MUW. In summary, our laboratory aims to overcome existing diagnostic shortcomings by bridging proteomics in molecular pathology with functional *in-vivo* imaging techniques to define novel targets and to obtain novel biomarkers dependent on the respective tumor entity for clinical application.

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Group housing of rats and rabbits – thinking out of the box

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In their natural habitats, rats and rabbits live in large social groups. Furthermore, rabbits have a far reaching locomotion pattern due to their jumping gait, which is restricted in most conventional cage housing systems. Living together in a large social community within an enriched environment has been shown to stimulate self-motivated activity of experimental animals. Several experimental designs and settings using rats or rabbits benefit from extensive voluntary movement of the animals, e.g. musculoskeletal or pain models with behavioral readouts. In order to minimize artificial housing we firstly established a 54 m² floor housing unit to socialize 65 female New Zealand White (NZW) rabbits for long-term (> 3 months) experimental housing. After 9 groups and a total of 484 rabbits we résumé that group housed rabbits have a strong general condition with less claw, skin or teeth symptoms. They recover more quickly after surgical interventions and have an improved motor function and BMI. Furthermore, they are easier to handle when housed in a group compared to conventional cage housing in pairs. However, the housing system requires higher initial capital cost and is particularly suitable for long-term experiments. Secondly, for rat group housing we developed a modular system where one housing unit for groups of up to 48 rats comprises different, interconnected functional areas. In this rat colony cage, we continuously registered body weight and movement profiles between areas including jumping activity and stair walking automatically. Compared to pair-housed, age, strain and weight matched rats in conventional Type-IV makrolon cages the colony housed rats exhibited higher BMI, increased exploratory behavior and showed improved cooperativity during handling. Our experiences and the data presented here suggest that the rat colony cage improves animal welfare by allowing socialization in colonies and establishment of functional areas. Furthermore, automated online activity and body weight tracking enables continuous quantification of spontaneous motion, which may serve as objective measures of disease or stress related animal behavior in various models and after experimental interventions. Finally, health monitoring of single rats is enriched in an objective manner.

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The 3Rs in relation to REACH

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The REACH Regulation has various options on how non-animal approaches could be used to inform on (eco)toxicological properties of substances. There are obligations on registrants to consider use of alternatives to vertebrate animal tests where these would be adequate to meet the (standard) information requirements. REACH information requirements increase with increasing tonnage levels, and that the information requirements describe the information which is required and are not per se requirements for animal studies. This means that it may be possible to cover any specific information requirement with adequate alternative methods. The General "adaptation" rules in Annex XI of the REACH Regulation describe these possibilities. These include the use of: existing non-GLP and non-guideline data; historical human data; weight of evidence (WoE); (Q)SAR predictions; *in vitro* methods; grouping of substances and read across; and substance-tailored exposure-driven testing. A statistical analysis of registrations by ECHA shows that registrants make often make use of the possibilities of Annex XI. Requirements for acceptance are described at Annex XI and in ECHA Guidance. In general, the main principles are that information from use of alternatives is equivalent to that of the (standard) information requirement, the results are adequate for the purpose of classification and labelling and/or risk assessment, documentation is adequate and reliable, and, depending on the nature of the approach, the scientific validity of the methods has been established and the applicability domain is appropriate. A combination of various methods in a WoE or read-across adaptation could provide adequately high confidence to the prediction and these approaches are encouraged to be developed.

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The current status of non-animal test methods and prospects for Asian cooperation

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The International Cooperation on Alternative Test Methods (ICATM) was originally established through an agreement signed in 2009 by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and Health Canada. In March 2011, a Memorandum of Cooperation (MOC) was updated to include the Korean Center for the Validation of Alternative Methods (KoCVAM).

ICATM promotes enhanced international cooperation and coordination on the scientific validation of new methods for testing toxicity that either reduce or eliminate the use of laboratory animals. JaCVAM and KoCVAM have established a particularly close relationship in which joint meetings are held each year to promote validation of alternative test methods developed by Korean researchers. And in addition to JaCVAM activities, I personally am involved in providing support for validation studies on skin irritation and skin corrosion tests that utilize an epidermal model (EpiTRI) developed in the Taiwan as well as in planning training courses on alternative test methods to be held in Shanghai, China.

Other recent developments include the signing of a MOC between the Japanese Society for Alternatives to Animal Experiments (JSAAE) and the Korean Society for Alternatives to Animal Experiments (KSAAE) as well as one between the European Society for Alternatives to Animal Testing (EUSAAT) and the American Society for Cellular and Computational Toxicology (ASCCT). For more than ten years, the JSAAE has continued to promote mutual interaction at the annual meetings. In the future, we are planning to collaborate with the Chinese Society for Toxicological Alternative and Translational Toxicology (TATT) and the Chinese Society of Toxicity Testing and Alternatives (TTA) to encourage activities related to alternatives to animal testing in Asia.

Finally, the Asian Congress 2016 on Alternatives and Animal Use in the Life Sciences will be held jointly with the 29th JSAAE Annual meeting from November 15 to 18, 2016, at the Karatsu Civic Hall in Karatsu, Saga, and Kyushu University in Fukuoka, Japan, under the sponsorship of JSAAE and the Alternatives Congress Trust (ACT). 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 a region where the concept of the Three Rs is just now achieving penetration.

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Effects of anti-tumor drugs bevacizumab and cisplatin on cancer cell invaded human *ex-vivo* lung tissue in an organotypic model as an alternative to animal experiments

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Cancer is a major public health problem worldwide with statistics showing that every fourth death in the European Union is attributed to this disease. With a share of 28% of all cancer-related deaths, malignant cancers within the lung are among the leading causes, both due to the high occurrence of malignancy among lung cancers as well as metastasis formation.

Choosing the optimal preclinical model for drug testing is a crucial part in avoiding poor efficacy and prediction. Currently, most drugs are tested in subcutaneous xenograft models that fail to consider the importance of the human tumor microenvironment. This lack of prediction and neglect of tumorigenesis as a dynamic and multifactorial process represent drawbacks of animal models. Thus, three-dimensional models are needed that more closely mimic the *in vivo* situation without animal experiments.

AdGFP-transduced human breast cancer cell line MDA-MB-231 was cultivated in co-culture with human Precision-Cut Lung Slices (PCLS) over one week. Immunohistology of Ki67 and GFP signals were used to visualize proliferating MDA-MB-231 cells and to evaluate the changes in proliferation rate over time. Confocal time-lapse microscopy allowed for detailed observations of the dynamic cancer cell behaviour in their natural environment. Tumor-promoting and neoangiogenesis markers were determined by the cytokines GM-CSF and VEGF, respectively. To assess the role of the human microenvironment on anti-cancer effects of bevacizumab and cisplatin, cancer cell invaded PCLS and PCLS processed from primary tumor material were treated with the drugs for up to 72 h. The aforementioned methods were applied by analogy to common 2D culture of the cancer cells in order to compare cell behavior.

Cancer cells integrated in the human lung tissue and proliferated there. The proliferation rate of cancer cells within the human tissue decreased from 85% after 48 h of incubation to 35% after 144 h, thus mimicking the reduced proliferation rate that is observed *in vivo*. Changes in the morphological dynamics of cancer cells could be observed as three distinct phases during the process of tissue invasion, contrasting with static morphology of 2D cultures. The GM-CSF and VEGF-release over time correlated with the MDA-MB-231 growth curves in human tissue. Cancer cells within their natural environment were more resilient against anti-cancer drugs such as cisplatin in comparison to 2D cultured cells. Treatment with bevacizumab suppressed VEGF-release in cancer cell-invaded tissue up to 16fold, using 200 µg/mL. Cisplatin treatment led to a decline in viability and reduced cancer cell number after application of 50 µM cisplatin for 72 h by up to 51%. Adequate reduction of viability after cisplatin treatment was also seen in PCLS taken directly from primary tumor material.

This novel model is able to mimic cancer cell growth, proliferation and morphological dynamics within the microenvironment of human lung tissue. Pharmacological intervention with established drugs modulated the tumor growth, proliferation, as well as tumor-promoting and angiogenesis markers, respectively. In accordance with the 3R principle, we therefore propose this model as a novel option for preclinical testing of anti-tumor drugs in human tissue without the use of animal models.

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Establishing a culture of care by implementing AUGUST – a new and strong initiative at Aarhus University

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Implementing the 3Rs is of utmost importance both in terms of refining animal welfare and respecting animal lives, but also to keep improving scientific quality. At Aarhus University (AU) it has been focus of attention, particularly since the implementation of the EU Directive 2010/63 [1]. At that point, The Faculty of Health Sciences established a firm organisation with the animal welfare body having direct reference to the Head of Faculty, and with a connecting thread downwards in the institution with the goal to reach out both to scientific staff as well as veterinary staff, animal technicians and caretakers. This structure has a positive influence on implementation of the 3Rs, and particularly one initiative has caused a ripple effect in the organisation, namely the initiative to highlight the challenges of translating preclinical science to the clinic as a way of promoting reduction, refinement and replacement. The initiative has become such a success that the Department of Clinical Medicine now strives for a permanent anchoring of AUGUST [2] (Aarhus University Group for Understanding Systematic Reviews and Meta-analyses in Translational Preclinical Science) to conduct systematic reviews (SR) and thereby be a benefit to both 3R initiatives per se as well as the scientific quality.

In brief, during the last decade scientific evidence has underscored the fact that preclinical studies are challenged by a considerably poor translational success [3]. Even though the causative factor for lack of translation seems to be multifaceted, the lack of tradition to perform systematic reviews and meta-analyses within the field of preclinical animal studies is striking [4]. SR has several advantages and means that researchers approach their specific scientific field in a more transparent, thorough and structured way, by use of the same level of rigor to review already published research results, which should be used when carrying out research projects per se [5]. By conducting a SR researchers achieve an in-depth overview of their field of science and are able to qualify already published work as well as take basic steps to avoid obvious sources of bias in their own experiments. Thus it prompts responsible conduction of research and a more evidence-based use of animals with high quality research as the output. In terms of the 3Rs, the vision is that this "thorough evidence-based, high quality research approach" will create an awareness of how we treat our animals on a daily basis, lead to more focus on how distress, pain and suffering are alleviated as well as cause new and innovative discussions on whether the research could be performed differently – for instance partly complemented by a replacement approach. Surely, the formula for this ambition is among other things a very supportive and dedicated leadership, economic resources and committed, visionary and tireless employees at all levels, who strive to gather and insist on an interdisciplinary cooperation at all levels in the organisation.

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Novel aerosol exposure devices for *in-vitro* toxicity testing at the air/liquid interface

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In state-of-the-art health risk assessment, it is becoming increasingly important to accurately characterize the biological activities of active airborne substances, gases and complex mixtures in the human respiratory system. This can be achieved using *in vitro* exposure technologies for mammalian cellular systems from the respiratory tract. The exposure of mammalian cells or tissues to airborne substances is frequently performed under submerged conditions. In doing so, the test substances are dosed into the culture medium. This procedure results in an undesired interaction of the formerly airborne substances with the medium, causing limitations for authentic analysis. Furthermore, assessment of the applied dose proves to be difficult using this technique.

In response to the scientific need to expose in physiologically relevant conditions, cutting-edge exposure systems have been specifically designed and engineered to enable direct exposure of mammalian cells or tissue at the air/liquid interface. For this purpose the cell systems are cultivated on microporous membranes and later exposed while not being covered with culture medium. This approach allows for more credible and authentic results than achieved by submerged exposure due to a closer replication of the human physiology.

A typical *in vitro* exposure system consists of 4 major component groups: the aerosol generator or gas source, dilution systems, exposure modules and dosimetry tools. For a reliable product assessment it is essential that all components match the requirements of exposure the air/liquid interface.

Novel tailor-made system solutions are driven by 2 factors: the requirement for increased throughput by exposures such in 96-well sized membrane inserts as well the need for automated exposure stations which feature online dosimetric tools and possibilities to enhance particle deposition.

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Fast and label-free tracking of cell behavior in non-animal models

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Introduction

Besides being ethically questionable, animal testing is highly cost intensive and time consuming. Therefore, increasing effort is put into evaluating alternative non-animal methods for studying diseases and testing products – resulting in more and more elaborated tissue models and a broad variety of cell analysing systems. However, analysis of these models is not trivial and there is an increasing demand for handy, fast and easy-to-use cell detection and characterization methods.

Raman spectroscopy (RS) is a highly sensitive analytical method for marker-free and non-invasive identification and analysis of single cells. As RS works within physiological environment and does not require any chemical staining or antibody-based markers, the examined cells remain entirely vital and undisturbed. Here, we provide evidence that RS is a suitable tool for the analysis of biological samples within 2D and 3D setups, also allowing quality control of cell based products.

Methods

In a first approach, Raman spectra were taken from PFA fixed human blood monocytes infected with the intracellular pathogen *Chlamydia pneumoniae* to detect cellular infection. Furthermore, single living MOLM-13 human tumor cells were cultivated in microwells, treated with doxorubicine to induce apoptosis, and analyzed using Raman spectroscopy. In a last application, living and PFA fixed human skin grafts were cultivated with human fibroblasts, keratinocytes and melanocytes for 10 days and analysed using RS. For each sample Raman spectra from at least 60 cells were acquired.

Results

Raman Spectroscopy was able to discriminate cell types and states in all described experiments. In monocytes, RS revealed different molecular fingerprints for infected and non-infected cells, whereat the main differences could be associated with changes in lipid and fatty acid content. In case of MOLM-13 cells, RS allowed detection and monitoring of doxorubicine induced cellular reactions. In addition, changes in Raman spectra could be associated with apoptotic processes. Last but not least, RS was suitable to detect and discriminate different cell types in human skin grafts in a depth of up to 200 µm, enabling quality control of these cell based products.

Discussion

Taken together, we could provide evidence that Raman Spectroscopy is a fast and easy, yet highly precise tool to analyze biological samples, providing valuable information about the entire metabolome of individual cells as well as their type, state and fate. RS can be used in a large variety of different approaches, allowing the analysis of single cells and their reaction upon drugs or toxins, but also the monitoring of cellular reactions within tissue models or scaffolds. In addition, RS works under physiological conditions and without the necessity of any labelling agents or specific markers. All these properties make RS a suitable and handy tool to analyze samples from various innovative non-animal technologies – helping to establish alternatives on animal testing.

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International trend of 3Rs: Asian advancement

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Since 1959 Russell and Burch published a book "The Principles of Humane Experimental Technique", international activities to promote 3Rs are increasing. There were various European groups established such as FRAME, ATLA etc. In United States of America, the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) was founded in 1981. CIOMS published "The International Guiding Principles for Biomedical Research Involving Animals" released in 1985 as a scientific academic group. The first International Standard for animal welfare was published in 1996 as "ISO 10993-2 Animal Welfare Requirement" for the Biological Evaluation of Medical Devices.

In 1993, The First World Congress on Alternatives and Animal Use in the Life Sciences was held at Baltimore, USA. Since then The World Congress was continuously held around the world and the World Congress may be the center of international activities to promote 3Rs currently.

Japanese Society of Alternatives to Animal Experiments (SAAE) was founded in 1988 as an academic society for Alternatives in Japan which is the root of Asian advancement of 3Rs. When The 6th World Congress on Alternatives and Animal Use in the Life Sciences in Tokyo, Japan is held as a first World Congress in Asia, there were three satellite meetings were held at Beijing, Seoul and Kyoto. The 6th World Congress stimulated Asian scientists to advance 3Rs activities and Korean Society of Alternatives to Animal Experiments was founded in 2007 as an academic society of 3Rs. Laboratory Animal Science groups in various Asian countries soon followed this trend and they organized many groups of 3Rs within their groups or in their countries.

The success of the 6th World Congress in Tokyo may pushed JSAAE and other Asian groups of 3Rs, and consequently Asian Congress 2016 on Alternatives and Animals Use in the Life Science will be held in Kyushu. World wide participants in particular Asian specialists in 3Rs will be gathering from more than 10 countries to discuss about the advancement of 3Rs in the world and Asia. The leading scientific members in 3Rs will focus the methods of Replacement which are already or will be validated internationally on their scope and they intend to further advancement of the newly designed Replacement methods which are originally developed within Asian region. Asian advancement of Alternatives to Animal Experiments and Use may lead to the future World Congress held in Asian region.

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From bedside to bench – adding human context to *in vitro* models

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A better understanding of the mechanisms of disease would enable an earlier diagnosis, better prediction of the clinical outcome and the development of targeted therapies.

Animal models are still widely used in biomedical research, though it is increasingly recognised that animals are not a good model for human diseases; effective translation from laboratory to clinics is problematic. In view of similar translational problems – animal studies often fail to predict drug efficacy and toxicity in humans – toxicologists make efforts towards a better understanding of the mechanisms of toxicity, rather than simply observing its effects. The toxicological concept of adverse outcome pathways (AOPs) provides a tool for such a knowledge-based human health risk assessment. The AOP construct describes the sequence of causally linked events starting at molecular level, leading through different levels of biological organisation up to an adverse health outcome on organism level. An AOP Knowledge Base (AOP KB) is being implemented to facilitate the contribution to, and evaluation of, current pathway information collected by a wide range of experts, thus providing a basis for the collaboration between scientists from various disciplines. This paradigm change in toxicology could also provide guidance for new approaches in disease modelling and drug discovery. Perturbed cellular response pathways are equally part of toxicological and disease pathways. Collaboration between clinicians and toxicologists and the incorporation of clinical data into the adverse outcome pathway framework would be beneficial for both sides.

Currently there are several human-based cell models available, but each of them has its drawbacks and none truly reflects the interaction between the various cells among themselves and with the immune system. Patient samples could be used as benchmark to help evaluating whether *in vitro* data can be used to extrapolate to disease mechanisms *in vivo*.

The boundaries between individual disciplines need to be opened and health care professionals, academia and industry should collaborate in a common effort to better understand the biological mechanisms underlying disease and how to best model these mechanisms *in vitro*.

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The drinking water as application route for pain management in bone-linked mice models – Combining a refinement and a basic research study

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In order to avoid unnecessary stress and strain in small rodents effective, safe pain management plays a crucial role. Analgesia administration via injections requires repeated handling and restraint, especially in short acting opioids, and induces stress for the animal. A promising alternative is the application via the drinking water. Tramadol is a potent and widely used pain medication during scientific experiments in small rodents. However, studies that address the efficiency are scarce and different recommendations exist according the dosage that differ from potentially under- to overdosing. While underdosing can cause a lack of sufficient pain relief, overdosing can lead to unwanted side effects like daze and reduced wellbeing of the animal. Here we evaluated two commonly used pain management protocols, Tramadol (two concentrations) and Buphrenorphin administered with the drinking water, for their efficiency and side effects on experimental readout in a mouse osteotomy model. Therefore, we distinguish between (i) clinical parameters of reduced wellbeing and pain e.g. Mouse Grimace Scale, clinical scoring, body weight, water and food uptake, (ii) model specific pain parameters e.g. weight bearing, locomotor activity and hypersensitivity (Hotplate) and (iii) behavioural parameters of wellbeing e.g. nest building activity. To evaluate the influence of the pain management on the bone healing and the experimental readout, we perform µCT analysis and histological staining of the osteotomized bone to gain highly important data for experimenters working in the bone research field.

In order to enhance the knowledge on refinement options in basic research studies and to effectively reduce lab animal usage, we conceived a refinement study embedded in a basic research study in the mouse osteotomy model to show the possibility to combine both studies to reach a wider community with results in the field of refinement studies.

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ReThink3R – innovation strategies for young scientists – report of the first workshops

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University education is an important stage where future scientists should become aware of the possibilities and urgencies in the 3R field apart from traditional career options. We performed two Design Thinking workshops dealing with the implementation of the 3R in various coherences that were carried out at the Berlin School for Regenerative Therapies and the Dahlem Research School in Berlin. The workshops are designed for PhD-students and open to all other interested scientists working with animals or in the field of 3R. Many scientists are actually willing to work on the 3R, but due to time and money limitations as well as conventional research structures, the feasibility to reflect the topic is missing. Therefore, we offer them an open-minded atmosphere, time to intensively think about and discuss the problems with implementing the 3R within their own work. Moreover, the participants have the opportunity to join forces with an interdisciplinary team to create ideas for improvements and solutions to identified problems. We use Design Thinking methods to make the teamwork more efficient, user-oriented, and creative. Design Thinking is an innovation method that combines both analytical and creative methods to find practical solutions in an iterative process. Important aspects of the process are interdisciplinary teamwork, "thinking outside the box", a flexible work environment, visualisation and empathy. A central role within the process is the exploration of the complexity inherent to the challenge posed at the beginning. Within our workshops, we stated the following challenge: "Design an institute that keeps animal suffering to a minimum". During the understanding phase aspects coming up were e.g. a lack of communication and teamwork, hierarchical structures, time limitations due to application writings and statistics, problems in animal handling and experimental planning. By interviewing different stakeholders the participants get to know different perspectives and insights leading to the definition of a concrete underlying problem. All gathered information and different perspectives are incorporated into the solution process consisting of the collection of numerous ideas followed by prototyping and testing of a selected idea. Our workshops resulted in four very different innovative prototypes: 1) A research institute that improves the communication between animal keepers and scientists by different approaches, 2) an animal handling training package with robot animals and modern glasses that guide through SOPs and acquire videos during animal handling, 3) an online program for applications for animal experiments including support in statistics, finding alternative methods, and sharing of animals and 4) a grant for alternative methods to systemic investigations in animals including the guarantee for an article in a high-impact journal and a credit-system for grant-applicants that facilitates support and training for developing alternative methods in a specialized institute.

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 teamwork, an open-mindset and creative confidence – facilitating a change within the scientific community including awareness for the variety of the 3Rs and motivation to work on the 3R in future research careers.

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AniMatch, an innovative web-based platform to share organs and tissues – status update and challenges

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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. 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 programs for the sharing of organs and tissues of animals killed." (Article 18). In 2013, 1.3 million animals were killed for scientific purposes, used for organ/tissue collection under anesthesia and for educational purposes.

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. The platform was launched in October 2015 and within the last months we reported first users in Germany, which gave us the opportunity to identify optimization potential in functionality and usability. Furthermore, we presented the idea of AniMatch on different meetings and workshops and discussed challenges with animal welfare officers as well as researchers. The overall number of people is convinced of the resource-sparing and morally sustainable approach of AniMatch. Challenges are seen in the additional effort needed as well as in the lack of incentives. To tackle these we will facilitate the process of searching and offering by introducing advanced filtering as well as the option to create templates for offers. In addition, the design, structure and proof-of-concept for a payment system is on the way, making it possible to work cost-effective for both, offering and receiving parties.

The platform will be further tested and be provided to interested institutions in Germany as well as Europe. Besides the moral exculpation for 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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Hand1-Luc Embryonic stem cell test (Hand1-Luc EST): A reporter gene assay using engineered mouse ES cells to evaluate embryotoxicity *in vitro*

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The embryonic stem cell test (EST) is a promising alternative method for evaluating developmental toxicity. It has been developed to respond to the needs of the 3Rs (replace, refine, reduce) in 1997 by the team of Dr Horst Spielmann. In this test, embryotoxicity of chemicals is evaluated by measuring cytotoxicity and differentiation toxicity using mouse ES cells. Differentiation toxicity is analyzed by microscopically counting the beating of embryonic bodies after 10 days of culture. However, improvements were necessary to reduce the laborious manipulations involved and the time required to obtain results. We have previously reported that heart and neural crest derivatives expressed transcript 1 (Hand1), a transcription factor essential for mammalian heart development was a quantitative and objective molecular endpoint for predicting embryotoxicity. We have previously obtained stable transformant of ES cells with promoter of Hand1 gene upstream of luciferase reporter gene. We managed to establish a 96-well plate-based new EST evaluating the embryotoxicity of chemicals with luciferase reporter assay after treatment with test chemicals during cardiomyocyte differentiation. We named it Hand1-Luc EST. Cytotoxicity was expressed as the concentration of chemical that reduces the viability of cells to 50% of the control level against the vehicle control (ES-IC_{50}). The differentiation toxicity was expressed as the concentration of the test chemical that reduces the luminescence by 50% (ES-ID_{50}). The prediction model, based on 71 chemicals data obtained in the lead lab, is using ES-IC_{50} and ES-ID_{50} values along with the maximum dose (MD), this dose being the highest concentration of chemical that dissolves in the assay medium.

From February 2013 to February 2016, the protocol undertook the validation process. Members of International Cooperation on Alternative Test Methods (ICATM) along with experts in the domain provided us with precious advice that allowed us to significantly improve the protocol. Three participant laboratories took part in the validation. After protocol changes, determination of criteria, analysis adjustment (curve fitting) and the revision of the prediction model, we managed to obtain a high reproducibility. Indeed, both within – and between – laboratory reproducibilities were higher than 75%, limit value fixed in the study plan. Furthermore, concerning the predictivity of the test, although we only got an accuracy of 60.6%, the positive predicted value was very high (80.8%) (results based on 71 chemical's data). All those results lead us to think that Hand1-Luc EST is a powerful screening test to detect strong embryotoxicants.

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Cellular and non-cellular elements of the pulmonary air-blood barrier

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The fate of inhaled xenobiotics is of growing interest, both regarding the on-purpose delivery of aerosol medicines as well the unintended inhalation of chemicals and (nano)materials. Depending on the deposition in the central or peripheral lung, the bronchial and alveolar epithelium, respectively, represent the essential cellular barrier that prevents immediate translocation to the bloodstream. According to their physiological function, the epithelial cells of either region show remarkable differences: While bronchial epithelial cells are columnar in shape and ciliated, alveolar epithelial cells are cuboidal (AT-2) or squamous (AT-1) and not ciliated. As regards barrier and transport function, AT-1 cells are of major interest, because they cover up to 90% oft the more than 100m^2 large alveolar surface area.

In addition, however, the air-interface oft the pulmonary epithelium is never naked, but covered by some protective layer. The bronchial epithelium is covered by mucus, which is a natural hydrogel formed by mucins secreted from goblet cell. Being continuously cleared by the beating oft he cilia, pulmonary mucus acts both as a diffusional barrier as well as a dynamic barrier. In the alveolar region, mucus is absent, but the epithelium is still covered by a thin layer oft the pulmonary surfactant, which is produced by the AT-2 cells and contains besides phospholipids some unique lung surfactant proteins.

In order to understand the fate of inhaled aerosol particles, predictive *in-vitro* models are extremely useful and represent an important alternative to animal testing. While human bronchial (e.g. Calu-3) or AT-2 like (e.g. A549) cell lines are quite well established, we recently succeeded in establishing a human AT-1 like cell line (hAELVi) by Lenti-virus mediated transfection with some mildly proliferating genes. Most importantly, hAELVi cells maintain the capacity to form tight intercellular junctions, leading to high trans-epithelial electrical resistance (TEER > 1000 Ohm x cm^2) and significant barrier properties for small molecules [1]. The interaction of nanoparticles with the pulmonary surfactant was examined in terms of a detailed lipidomic and proteomic analysis of the formed corona, which is clearly different to the plasma corona [2]. As regards trachebronchial mucus, which acts as a significant barrier in the upper airways, we could demonstrate a foam-like structure with characteristic pores and different macro- and micro-rheological properties [3].

To realistically study the fate of inhaled molecules and particles, advanced *in vitro* models of the air-blood barriers will have to feature both cellular as well as non-cellular elements.

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Combating infectious diseases: novel approaches and *in vitro* models

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Between the site of action of a drug and the site of its administration to the patient, there are always some biological barriers. For non-invasive ("needle-free") delivery, the body's outer epithelia – intestines, skin and lungs – are of particular importance. In addition, the efficient delivery of antiinfectives faces some peculiar barriers, such as biofilms, host cell membranes and the bacterial cellular envelope itself. This presentation will highlight the potential of *in vitro* models to evaluate the safety and efficacy of new therapies and thus to facilitate their translation into the clinic.

In colitis patients, a significantly increased particle accumulation in inflamed mucosal areas could be demonstrated by confocal laser endoscopy, correlating with the diagnosed degree of the inflammation For the treatment of inflammatory bowel diseases (IBD) we could demonstrate an improved efficacy of anti-inflammatory drugs, paralleled by a reduction of adverse side effects due to systemic drug absorption, both in various mouse models as well as in some advanced human cell culture models. To target intracellular intestinal infection we have designed bacteriomimetic nanocarriers by decorating their surface with Yersinia invasin. When loading such carriers with gentamicin, a significant reduction of intracellular bacteria could be achieved while the free drug was ineffective.

Based on the finding that nanoparticles applied to the skin do not penetrate the stratum corneum but accumulate in hair follicles, we hypothesized that this route could possibly allow the non-invasive delivery of antigens. By encapsulating a model antigen (ovalbumin) along with some innovative adjuvant in biodegradable polymer nanoparticles and applying such formulation to the back of shaved mice, we found encouraging evidence that transfollicular immunization through the intact skin is possible, thereby stimulating antigen-specific T cells without the need of using any needles, chemical or physical penetration enhancers.

While the lung is a most attractive route for both local and systemic drug delivery by way of inhalation, its peculiar cellular and non-cellular barriers ask for a thorough understanding of the relevant nano-bio interactions. Besides cellular uptake and penetration, the interaction with bronchotracheal mucus and alveolar surfactant has implication for designing the shape and surface properties of aerosolized nanocarriers. Encouraging examples include the improved delivery of novel antiinfectives against biofilm-forming Pseudomonas aeruginosa, as well as the successful delivery of nuclease-encoding mRNA for genome editing in Sp-B deficient mice.

Comprising not only one, but two lipid membranes on either side of a polyglycan wall, the gram negative cellular envelope is likely to provide a substantially different permeability barrier to drug molecules than the mammalian cell membrane. Apart from the existence of specific transporter and efflux systems, improving the intracellular bioavailability of antiinfectives may therefore perhaps obey other rules than e.g. improving systemic oral bioavailability. Based on this hypothesiss, we have started to develop a new permeability assay based on filter membranes coated with by bacterial cell wall lipids. Interestingly enough, permeability data of test compounds do not correlate with those across filter membranes coated with mammalian lipids.

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Clickertraining – a cognitive enrichment for laboratory mice?

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The development of new refinement strategies for laboratory mice is a challenging task that contributes towards fulfilling the 3R-principle (Replacement, Reduction, Refinement) of laboratory animal science. In 3R – discussions, the main emphasis of interest is often on replacement and reduction as these two aspects may lead to a decrease of the laboratory animal number. But even with the best efforts, there are thousands of animals that are integral parts of research. Research in the field of refinement substantially benefits those animals still kept for scientific purposes. This study focuses on a cognitive enrichment program for laboratory mice.

The Directive 2010/63 points out that the "establishments shall set up habituation and training programmes suitable for the animals" (Article 3.7). In accordance with that, it has been shown that handling programs can efficiently decrease anxiety towards the investigator in laboratory rodents. The goal of this study is to introduce clicker training for mice as a specific handling program. Clicker training is a form of positive reinforcement training using a conditioned secondary reinforcment – the click sound of a clicker – to be very accurate in strengthening a specific behaviour.

Clicker training is well known from the field of companion animals and has made its way into laboratory animal science where it has been successfully implemented with laboratory monkeys. By introducing clicker training into the keeping of mice, we enable mice to experience a cognitive enrichment. Enhancing the ability of mice to cope successfully with environmental challenges contributes to their welfare.

By developing a reliable protocol that is easily integrated into the daily routine of keeping laboratory mice, we can significantly improve the mice's life-time experience of welfare.

Those animals can efficiently profit from intense research in the field of refinement.

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Litter loss as a neglected welfare problem?

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The implementation of Russell and Burch's three R's of Replacement, Reduction and Refinement led to a myriad of published literature on animal-friendly alternatives. But refinement as the one R that really has the potential to ameliorate the kept mice's welfare is often neglected. In the field of refinement during the perinatal period till weaning, only few empirical investigations were performed. Postnatal litter loss reaches numbers between 0-50% and thus seems to be a neglected issue in laboratory mouse breeding. This gives reasonable cause for further investigation. Existing research recognizes the critical role played by the correlation between environmental enrichment of the breeding cage and maternal behaviour.

The present study investigates the impact of different amounts of environmental enrichment in breeding cages on infant survival rate and development. It especially attempts to reveal the conditions which are associated with high litter losses. To ensure a close monitoring, infants were counted daily. In addition, the infants were weighed on postnatal day three and postnatal day twenty-four.

In impoverished breeding cages, with just soiled bedding material without any other enrichment, infant survival was significantly lower than in standard enriched breeding cages. Standard enriched breeding cages showed less divergence concerning infant survival rate compared to breeding cages with overstuffed environmental enrichment. A change to standard environment enrichment on postnatal day one did not prevent the infant losses. We hypothesize that the high infant losses may be caused by high peripartal stress of the dam. Peripartal stress due to poor environmental enrichment has a sustained negative impact on maternal behaviour.

The subsequent implementation of the gained knowledge will be one further step to fulfilling the 3R-principle. It has the power to refine the dams' wellbeing which consequently leads to a reduction of bred animals.

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Three Rs in endocrine disruptor assessment – Refining sampling, reducing numbers of tested animals and replacing *in vivo* testing

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The increasing awareness of the endocrine effects of chemical compounds used in agrochemical, pharmaceutical, cosmetic products etc. has led to a growing number of animals being employed in chemical testing and environmental monitoring / evaluations. The OECD Guidelines recommend fishes as toxicological models for endocrine disruption assessment (OECD TG 229, 230, 234).

Activation of estrogen receptors is commonly regarded as the major endocrine disruption. Investigation of the consequences of this kind of interference with the endocrine system currently demands extensive animal tests that are both invasive and destructive. The prediction of endocrine disrupting effects finally depends on *in vivo* approaches including the cybernetic control of the hypothalamic-pituitary axis, which means that the complete vertebrate organism needs to be considered [1]. The central toxicological endpoint for the detection of estrogenic endocrine disruption is the exogenous induction of the estrogen-dependent protein vitellogenin (VTG). Evaluation of this endpoint is routinely performed under *in vivo* or *ex-vivo* conditions, either by exposing fishes or primary hepatocytes to test substances. The *in vivo* assessment commonly relies on blood and/or homogenates that usually require sacrificing the test animals [2].

A highly sensitive vitellogenin ELISA enables the measurement of VTG in the epidermal mucus, a non-invasive, animal friendly sample matrix. This allows for repeated sampling and recording of individual changes, so that the number of tested animals can be drastically reduced. Moreover, mucus sampling represents a clear refinement in terms of care for test organisms.

For screening purposes, the *in vivo* testing strategy was complemented with an *in vitro* approach. VTG induction can be measured in an estrogen-sensitive fish stem cell line using with the vitellogenin ELISA for cyprinids. This allows for endocrine disruption testing on vertebrates to be based on lower tier investigations. The number of *in vivo* tests can thus be reduced while upholding the principles of responsible care.

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

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

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

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

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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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Prediction of relative developmental toxicity of antifungal compounds using the ES-D3 cell differentiation assay, combined with the BeWo transport model

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Toxicological hazard and risk assessment nowadays largely rely on animal testing. For economic and ethical reasons, the development and validation of reliable alternative methods to *in vivo* toxicity testing are urgently needed. However, these toxicology tests do not take into account the kinetics of a compound. As placental transfer may be a key parameter in developmental toxicity testing, we used the BeWo transport model, consisting of BeWo b30 cells grown on transwell inserts, mimicking the placental barrier, to determine relative placental transport velocity. Relative transport rates were obtained using antipyrine as a reference compound. To determine developmental toxicity of 5 reference antifungal compounds we determined the inhibitory effect on the differentiation of the mouse embryonic stem cells (ES-D3 cells) into beating cardiomyocytes in the differentiation assay. We then compared the *in vitro* and *in vivo* developmental toxicity potencies of these compounds by assessing the correlation between *in vitro* BMC_{50} values and *in vivo* BMD_{10} values ($R^2 = 0.57$). When the ES-D3 cell differentiation data were combined with the relative transport rates obtained from the BeWo model, the correlation with the *in vivo* data significantly improved with R^2 being 0.95. In conclusion, we show that the ES-D3 cell differentiation assay is able to better predict the *in vivo* developmental toxicity ranking of antifungal compounds when combined with the BeWo transport model, than as a stand-alone assay.

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Tests for the safety evaluation of a Chinese herbal medicine formula used in the treatment of atopic dermatitis

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Background
There are considerable interests in Chinese herbal medicine (CHM) as an alternative treatment for Atopic Dermatitis (AD), a frequent recidivating inflammatory skin disease of which the prevalence is increasing in the last few decades but without definitive treatment so far. A palatable and well tolerated formula (PentaHerbs Formulation, PHF), in which five CHMs (Amur Corktree Bark (Cortex phellodendri), Tree Peony Bark (Cortex moutan), Common Mint (Herba menthae), Honeysuckle Flower (Flos lonicerae), Swordlike Atractylodes Rhizome (Rhizoma atratylodis)) were combined and further extracted, reduces topical corticosteroid use and improves quality of life in children with moderate-to-severe AD [1]. No obvious adverse effects were observed in the clinical studies [1]. However, more scientific evidences are required to confirm the safety of CHM, particularly when used for pregnant women.

Objectives
The ECVAM-validated embryonic stem cell test (EST) was applied to evaluate and predict the embryotoxicity of PHF and its five individual CHMs. The EpiDerm protocol used for skin irritation testing of medical devices [4] was used with minor modifications.

Methods
Chromatography (TLC and/or HPLC) was applied to authenticate the quality of PHF and the individual CHMs. The mouse embryonic stem cell line (D3) and the mouse fibroblast cell line (3T3) were used to study cell differentiation and cytotoxicity. Endpoints assessed after 10 days of culture were the inhibition of differentiation into beating cardiomyocytes (ID50D3), the cytotoxic effects on embryonic stem cells (IC50D3) and fibroblasts (IC503T3), respectively. Applying 7 concentrations of each CHM (46.4 to 4640 µg/ml) for 18 hours, 1% Triton X-100 served as positive control, ultrapure water as negative control and an untreated control were included in the skin irritation test.

Results
All test CHMs contained their main active compounds, which were confirmed by the chemical authentication. Amur Corktree Bark induced weak embryotoxicity while the other 4 CHMs (Tree Peony Bark, Common Mint, Honeysuckle Flower, Swordlike Atractylodes Rhizome) and the formula PHF are non-embryotoxic in the EST test [3] The viability of the EPI-200 skin tissues were within the range of 87.5%-101.1% under the exposures to PHF and each CHM, not indicating skin irritation.

Conclusions
According to our EST results, we suggest the potentially embryotoxic CHM to be eliminated from PHF or replaced by another CHM, although the skin irritation test results show high probability that PHF and its 5 CHMs will not produce skin irritation when applied to normal human skin. At its present form PHF should not be used during pregnancy, further studies are needed to confirm its safety and to support its application as an adjunctive treatment for AD during pregnancy.

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Qualitative reasoning about biological processes and systems: Computational approaches to reduce animal testing

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Precision medicine proposes to improve healthcare through the application of genetic information but critical gaps remain in the translation between theory and practice. Mathematics is considered the language of physics; physics, the language of chemistry; and chemistry, the language of biology. We must also understand the language's punctuation and grammar, i.e. the processes and systems in biology that amplify and modulate the molecular aspects of its component languages. We apply qualitative reasoning to enable progress to be made in light of imperfect information and knowledge.

Computational Modeling: Biological pathways represent topological networks of interacting macromolecular entities. The biochemist sees enzymes and receptors; the chemist sees substrates, products and discrete kinetic parameters; the molecular biologist sees expression levels and polymorphisms. The biological function of the pathway requires integration of all these characteristics, thus providing separable features that critically distinguish between its activity in human and in an animal model. We have applied both Petri net and stochastic activity network analysis to enable qualitative modeling of pathway behavior involving variation of all these characteristics, and using pathway, *in vivo*, as opposed to *in vitro*, discrete experimental data. This modeling approach allows for the comparison between topological and functional equivalence that is critical to evaluate alternative approaches to animal models. Examples of studies in blood coagulation and in menopause models will be shown.

Knowledge Modeling: Our development of a comprehensive and objective model of the disease process also serves as a data model for virtual data integration centered about the progression of a human from pre-disease through diagnosis, treatment and outcome. This disease-agnostic knowledge structure is used to evaluate the existing definitions in a specific disease and/or patient population and has been implemented into an ontology and computational platform. This model has been applied in oncology, heart failure, respiratory disease, MS, brain aneurysms, etc and has revealed a general problem. Almost all diseases can be categorized as complex disorders or syndromes and this significantly impacts and limits the accuracy of diagnosis, treatment decisions and outcome. Notably, this lack of adequate stratification and delineation of discrete disease processes has a major impact on establishing the suitability of an animal model for testing and evaluating drug efficacy and safety. This knowledge/informatic-based modeling provides critical insight into reducing the inappropriate utilization of animal models and points to critical limitations in current clinical data modeling. Examples in several disease areas will be presented.

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Real world medicine and real world patients: Something that animal testing can never approximate

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There are many good reasons to question the value of animal testing that range from ethical considerations to their potential for predicting human response and these questions support the need to develop alternative approaches for drug development. The predictability issue focuses on whether the transition from animal studies to clinical trials in humans is successful but in reality this is only part of a problem that raises much larger concerns about the use of animal models. The gap between the animal models and real world medicine and real world patients is even greater and significantly argues against their potential to be the bridge that crosses it but these issues are rarely discussed.

The focus on improving success in clinical trials, based on safety and efficacy testing in animals, fails to address the true complexity that exists in actual disease management:

1) Clinical trials test the efficacy and safety of a drug in a selected, homogeneous patient population that meets criteria to optimize drug performance and rarely, if ever, approximates patients that physicians treat in the real world. It has been noted in many studies that all patients exhibit 5 or more co-morbidities and may be taking as many as 10 medications concurrently, all which impact the effectiveness and safety of the newly approved treatment. Animal models do not accurately represent such patients, in part because pharma does not yet deal with this reality. In addition, each patient is impacted by clinical history as well as environment and lifestyle exposures

2) The design and implementation of studies in animals and clinical trials in humans requires adherence to strict protocols to enable statistical analysis of data with respect to variation of a minimum of variables. Physicians in the real world are not required to and typically do not follow protocols or even guidelines. Guidelines, whether consensus-based or evidence-based, for use in diagnosis or for treatment, change frequently in almost every disease area. As a result, the application of strict protocols testing in animal models and subsequently in clinical trials does not reflect actual clinical practice.

3) The Institute of Medicine (US) has established that at least 10% of all diagnoses in medicine are wrong. This number is actually grossly underestimated when one considers that almost all diseases are syndromes or complex disorders and that disease stratification is critical to identify the right drug for the right patient. Animal studies cannot overcome this deficiency in our understanding of the natural history of disease and pharma has not yet begun to deal with true understanding of the basis and complexity of the disease process.

I will present examples from three disease areas: pediatric Acute Respiratory Disease (pARDS), heart failure and breast cancer.

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Focus on severe suffering: an update on SPCA activity to end severe suffering

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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.

In the last 12 months we have continues our "multi-strand" approach to this important issue.

A comprehensive web resource for the research community, providing guidance and resources to help end severe suffering: http://www.rspca.org.uk/severesuffering.

A series of downloadable "Road Map" resources to guide establishments through the process of ending severe suffering.

Publication of our fourth expert working group report on reducing suffering in animal "models" of rheumatoid arthritis.

Formation of our fifth expert working group on animal "models" of spinal cord injury.

Our first International meeting "Focus on severe suffering" was held in Brussels in June 2016 with 150 delegates from 24 countries.

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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The impact of ethanol *in vitro* and *in vivo*: a comparative study

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The presence of a toxic substance in an ecosystem affects most of its organisms. The response of the exposed organisms depends on their metabolic rate, physiology, anatomy etc. However, comparative studies on such effects are rare. Taking this into consideration, the aim of the present study was to test the effect of ethanol from cells to vertebrates. Ethanol was chosen as the toxic factor, as it has been characterized as teratogen and psychoactive substance in many species. Zebrafish embryos and their 7 days post-fertilization (dpf) larvae (lower vertebrate), Daphnia magna (invertebrate) and the RTgill-W1 fish cells were the model systems which were exposed to ethanol concentrations of 0,5-5% (v/v). Zebrafish embryos and RTgill-W1 fish cells were exposed for 96 hours in ethanol, D. magna individuals for 48 hours and 7 dpf larvae for 25 min. Lethal concentrations (LC), effect concentrations that result in a nonlethal response (EC), no observed effect levels (NOEL) and LC_{50} and IC_{50} values were calculated. The locomotor activity of the zebrafish larvae and D. magna individuals was recorded using an observation chamber (DanioVision). The results of the study indicate that the toxicological response to ethanol is species specific, with D. magna individuals having the lowest LC_{50}. The IC_{50} and LC_{50} values were comparable between *in vivo* and *in vitro* trials. Also, a dose dependent effect was observed in the locomotor activity of both zebrafish and D. magna individuals. This comparative outcome reinforces the knowledge of the impact of ethanol in the tested organisms. Also, it is important under the prism of the implementation of the 3Rs principles. Given that replacement is the "R" most often considered, it is important to know how different taxonomic level organisms respond under the effect of the same toxic factor.

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Assessment of the phototoxicity of three different TiO_2 nano-forms using reconstructed human tissue model EpiDerm

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Absorption of the solar light by photo-sensitive substances and consequent formation of reactive oxygen species (ROS) and other photo-products may lead to the cellular damage as well as to responses of the immune system. Taken that into the consideration, the determination of phototoxicity of substances absorbing UV and visible spectra of the solar light (VIS) belongs therefore to the basic toxicology tests.

One of the methods used for the determination of phototoxicity is a test based on the use of 3D *in vitro* reconstructed human skin tissue model-EpiDerm™, the EpiDerm H3D-PT. This test was developed and pre-validated by organization ZEBET already in 1997 [1,2]. The main objective of this work was to verify and determine the phototoxicity and phototoxic potential of the selected reference substances and three different types of TiO2 nanoparticles using the EpiDerm™ H3D-PT.

We firstly evaluated and standardized the measurement conditions of the sunlight simulator SOL-500 and verified the sensitivity of the EpiDerm™ tissues towards UV/VIS light. Next, we evaluated correct prediction of phototoxicity of the EpiDerm™ H3D-PT using six reference substances, of which four were known phototoxins (chlorpromazine hydrochloride, two types of bergamot oil and anthracene) and two compounds were UV-absorbing, but without phototoxic potential (cinnamaldehyde, p-aminobenzoic acid). Finally, we have used this method to predict the phototoxicity of three different types of titanium dioxide (P25 AEROXID, Eusolex T-2000, TIG-115).

Based on the results obtained in this work, we conclude that the EpiDerm™ H3D-PT is a reliable test for the detection of phototoxicity and prediction of the phototoxic potential of selected substances. This conclusion is supported by the fact that during the measurements we obtained the same or better results as published by Liebsch et al. [1,2]. Phototoxicity of TiO_2 has not been demonstrated in any of the three samples tested. This is because TiO_2 nanoparticles do not penetrate deep enough into the epidermis to cause cytotoxicity by irradiation with UVA/VIS.

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Bringing a human heart & fat on a chip: microphysiological platforms as *in vitro* models of cardiac and adipose tissue

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Drug discovery and development to date has relied on animal models, which are useful, but fail to resemble human physiology. The discovery of human induced pluripotent stem (iPS) cells has led to the emergence of a new paradigm of drug screening using human disease-specific organ-models. One promising approach to produce these systems is employing microfluidic devices, which can simulate 3D tissue structure and function. Using microfabrication techniques we have developed two microphysiological platforms (MPSs) that incorporate *in vitro* models of human cardiac and adipose tissue. Both MPSs consist of three functional components: a tissue culture chamber mimicking geometrical organ-specific *in vivo* properties; "vasculature-like" media channels enabling a precise and computationally predictable delivery of compounds (nutrients, drugs); "endothelial-like" barriers protecting the tissues from shear forces while allowing diffusive transport. Both organ-chips are able to create physiological micro-tissues that are viable and functional for multiple weeks. The developed chips are the first systems that combine human genetic background, physiologically relevant tissue structure and "vasculature-like" perfusion. Pharmacological studies on the heart-chip show IC_{50}/EC_{50} values more consistent with data from primary tissue references compared to cellular scale studies. Both MPSs are extremely versatile and can be applied for drug toxicity screening and fundamental research.

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The role of the National Committees (NC) and Animal Welfare Bodies (AWB) under Directive 2010/63/EU

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Within the terms of Directive 2010/63/EU [1], all Member States are required to establish a National Committee for the protection of animals used for scientific purposes (Article 49) and to ensure that each establishment (user, breeder and supplier) maintains an Animal Welfare Body (Articles 26 and 27).

The main functions of the National Committee are to advise 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. These committees shall also exchange information on the operation of animal-welfare bodies and project evaluation and share best practice within the Union.

Animal welfare bodies are expected within the establishment to provide advice on welfare and care, the Three Rs, review management and operational processes, follow the development and outcome of projects and advise on rehoming. This is a key structure in developing and maintaining a good Culture of Care within the establishment.

The EU has endorsed guidance [2] for AWBs and NCs and although good progress has been made in developing the roles of the AWBs and National Committees, there is some way to go before the benefits of the respective objectives of the Directive are realised.

The remaining challenges and potential solutions to ensure optimum operational animal science and welfare benefit will be explored.

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Morphological analysis of *in vitro* skin models generated by hair follicle-derived and skin-derived keratinocytes and fibroblasts

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Human skin models are increasingly used as an alternative to animal experiments. Most of the currently established *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. During the past years, the potential of hair follicle-derived cells for the generation of skin models has been shown [1]. Furthermore, plucking hairs is a non-invasive and less painful method compared to invasive methods like skin biopsies to generate patient-derived cells. In this project, we compared the morphology and ultrastructure of *in vitro* skin models generated using keratinocytes and fibroblasts that were isolated and generated either from outer root sheath cells of plucked hair follicles or from juvenile foreskin.

The hair follicle-derived keratinocytes (HFDK) and fibroblasts (HFDF) were isolated by direct outgrowth on cell culture inserts which were coated with a feeder layer of postmitotic fibroblasts on the basal side of the insert membrane. After 2-3 weeks, hair follicle-derived cells were harvested and either further cultivated with feeder cells in specific serum-enriched cell culture medium to obtain keratinocytes or using the same culture medium without feeder cells to obtain fibroblasts. Normal human skin-derived epidermal keratinocytes (NHEK) and dermal fibroblasts (NHDF) were used for comparison. Skin models were generated according to previously published procedures [2]. The following cell combinations were used: NHDF and NHEK, NHDF and HFDK, HFDF and NHEK, as well as HFDF and HFDK. After a cultivation period of two weeks, the skin models were subjected to light microscopy and electron microscopy, followed by semi-quantitative, quantitative as well as qualitative assessment of the thickness and number of all epidermal strata, mitotic cells in the stratum basale and spinosum and keratohyalin granules in the stratum granulosum.

Light microscopy and ultrastructure analysis showed that skin models generated from hair follicle-derived cells resembled native human skin slightly better than skin models that were composed using skin-derived cells. All strata of stratified soft-cornified epidermis were present in all generated skin models. In the skin models generated from hair follicle-derived fibroblast and/or keratinocytes hemidesmosome and basal membrane expression was more pronounced compared to conventional skin models. Overall, the results of the ultrastructure analysis show the potential of using hair follicle-derived cells for generating *in vitro* skin models.

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Development of an *in vitro* inhalation toxicity test with potential regulatory applicability

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Knowledge of acute inhalation toxicity and irritation potential is important for establishing safe handling, packaging, labeling, transport and emergency response procedures for chemicals. The US EPA High Production Volume Chemical Challenge, and the EU REACH programs have further increased the need for inhalation toxicity information [1,2,3].

A UN treaty endorsed by the US, EU and others outlines a "Globally Harmonized System" (GHS) of Classification and Labeling of Chemicals [4]. The GHS specifies 5 inhalation toxicity categories. The EPA has established a separate system that uses 4 toxicity categories.

Acute inhalation toxicity tests currently accepted within the GHS and EPA systems involve *in vivo* 4 hr rat inhalation LC_{50} tests (OECD TG 403/436). In the current work, a newly developed *in vitro* toxicity test was evaluated in comparison to the established *in vivo* tests [5]. The *in vitro* test exposes an organotypic human airway tissue model (EpiAirway) to test chemicals for 3 hrs, followed by measurement of tissue viability (IC75). 64 chemicals covering a broad range of toxicity classes, chemical structures and physical properties were evaluated. Results show that the *in vivo* and *in vitro* tests had 100% concordance for identifying highly toxic chemicals (GHS CAT 1-2 and EPA CAT I-II). However, the *in vivo* tests had only 35.3% (EPA system) or 73.1% (GHS system) sensitivity for identifying less toxic respiratory irritants. Numerous human respiratory irritants including acids, bases, aldehydes, amines and others were not classified as respiratory toxins/irritants by the *in vivo* tests. The *in vitro* airway model was very good (sensitivity of 81.1 - 85.1%) for distinguishing respiratory toxins and irritants (corresponding to GHS 1-3, EPA, I-III) from non-toxins, non-irritants (corresponding to GHS 4-5, EPA IV). Overall accuracy of the *in vitro* test was 81.2 - 86.4%. There were no false negative GHS CAT 1-2 or EPA CAT I-II predictions using the *in vitro* test.

These data suggest that tests based on lethality in animals, while good for predicting highly toxic chemicals, produce a high percentage of false negative predictions for moderately/slightly toxic or irritating chemicals. The *in vitro* test using an organotypic human airway model EpiAirway was equal to current animal tests for predicting highly toxic inhaled chemicals, and better than animal tests for predicting moderately/slightly toxic respiratory irritants. The new *in vitro* testing approach should provide improved protection of human health compared to the current animal tests.

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Establishment of 2D and 3D cell culture assays using RT-gillW1 cells

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In vitro models are widely used in many areas of life sciences and cytotoxic assays are well established, especially for mammalian cells. In the context of the three R's many cell lines have been introduced from a wide variety of organisms during the last decades. Up to date, more than 4,000 cell lines are listed at the American Type Culture Collection (ATCC). Astonishingly, only 31 of these cell lines are of fish origin. The challenging aspect in fish cell culture is that only the basic rules known from mammalian cell culture apply. In particular, culture environment and passaging guidelines differ strongly according to the type and origin of the certain cell line. Furthermore, in cytotoxic assays the response may vary due to differences in metabolic activity, serum composition, exposure time, incubation temperature etc. Therefore, this study focused on the establishment and comparison of basic cytotoxic assays on 2D and 3D fish cell culture. RTgill-W1 epithelial cells were used as cell culture model following the culture recommendations of the ATCC. In the 2D cell culture the characterization of growth behavior and proliferation (Alamar blue and neutral red uptake assay) as well as mode of action studies (cell cycle analysis and apoptosis/necrosis assay) were successfully established. Finally, 3D spheroids were formed by three different techniques. Furthermore, the proliferation assays used in 2D were adapted for spheroid culture. The establishment of these methods sets the grounds for performing *in vitro* cytotoxic assays in rainbow trout cells and thus reducing the animal use in experimental set-ups.

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A microfluidic trapping device for electronic monitoring of 3D spheroids

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Reliable *in vitro* models are urgently required in order to reduce the costs in the assessment of new drugs and to follow the REACh's guidelines concerning the reduction of animal-based testing methods, which have been raising many ethical issues.

So far, the most commonly used approach to study tissues *in vitro* has relied on 2D monolayer tissue cultures, however they have the major disadvantage of not reproducing the communication network that maintains the specificity and homeostasis of tissue in organs. Recent advancements in the field of biology have demonstrated that, by allowing cells to grow and interact with their surroundings, thus in 3D, it is possible to achieve more physiologically relevant *in vitro* cell models. Indeed, 3D cell cultures closely retain tissue-specific architecture based on cell-cell and cell-extracellular matrix interactions, thus accurately mimic both *in vivo* mechanical and biochemical characteristics [1,2]. By improving the quantitative and physiological accuracy of *in vitro* systems, 3D models can have a profound impact on the screening of newly formulated drugs [3].

In this scenario, mono and multicellular spheroids have been recognised as a promising technology for the development of 3D *in vitro* culture models. A major advantage of cell spheroids is their ease of fabrication protocols, as it is not required the use of any external scaffolds to form [1].

Among the currently available techniques, only few systems are suitable to monitor spheroids' electrical properties, i.e. TEER (trans-epithelial/endothelial electric resistance), effectively [4]. In the last years, our group has intensively worked on a new generation of organic electrochemical transistor (OECTs) that can accurately sense highly resistant cell barrier as well as low resistant and adherence cells (coverage). Here we show for the first time a microfluidic spheroid trapping device integrated with an OECT to obtain an easy-to-use platform to monitor the electrical properties of spheroids.

By using an impedance-based approach, it is possible to determine both the TEER and the electrical capacitance of the spheroid. The microfluidic device consists of a single microchannel in which a "bottleneck" structure is used to trap the spheroid at the desired location. The trapped spheroid affects the ionic flux of the OECT, thus the transistor output current response. By using a simple equivalent circuit to model the system, TEER and capacitance of the spheroid can be measured. We have already performed calibration of the fully assembled platform through the use of polystyrene microbeads with different diameters, i.e. 60 and 160 µm, to simulate controlled blockage of the microchannel. The device validation experiments show that it is possible to record changes in the ionic flow due to the presence of the polystyrene bead inside the microfluidic trap. We are currently working on the development of spheroids with a co-culture of MDCK II/stromal cells, obtained through the hanging drop technology. Our final aim is to perform short term and long term TEER monitoring of the spheroids by using our newly developed trapping microfluidic platform.

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DropTech® – an automated stem cell technology platform based on hanging drops for production and testing

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Test animals for the assessment of potential toxic effect of compounds and pharmaceuticals are still involved in current legislative guidelines. However, cell-based screening assays are becoming more and more important, valuable, and reliable tools for pre-clinical compound development and approval. Main limitations of these assays currently are the application of animal cells as well as the usually manual and thus laborious procedures. Hence, the focus of our work was the automation of all assay steps by introducing a modular platform ready for high throughput and the adaption of this platform to the manipulation of human induced pluripotent stem cells (hiPSCs). Core task for the DropTech® platform is the supply of homogeneous and physiological hiPSCs as foundation stones for reliable assays. Since state of the art cultivation techniques are still based on non-physiological 2D formats (i.e. flasks, Petri dishes) or on bulk suspension cultures (i.e. Spinner flasks) lacking in high medium consumption and non-controllable micro environments of cells or cell aggregates, a drop-based cultivation is highly favorable. In either hanging drops (HDs) or U-/V-bottom well plates the initial seeding density in micro volumes is controllable and each drop/well acts as a single bioreactor. The applicability of the platform is shown by the automation of the Embryonic Stem Cell Test (EST), a validated assay for the assessment of embryo- and cytotoxicity. It has been validated by the European Centre for the Validation of Alternative Methods (ECVAM) as an alternative to animal testing. We show here that both workflows (embryotoxicity as well as cytotoxicity workflow), using murine embryonic and human induced pluripotent stem cells, are fully automatable using the DropTech® platform. Additionally, we introduce a novel readout system, which is capable of non-contact contraction detection in differentiated cardiac cluster in HDs. We showed that the DropTech® platform serves the 3R principle and has the potential to refine, reduce, and refine animal testing.

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Extending the limits of the hCLAT assay – test of the sensitization potential of oxidative hair dyes

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Repeated exposure to sensitizing chemicals can lead to allergic contact dermatitis (ACD). In order to avoid the risk of ACD, all ingredients of cosmetic products must be toxicologically assessed before entering the market. The human cell-line activation test (h-CLAT) has been developed as an *in vitro* alternative to animal testing which mimics the activation/maturation process of Langerhans cells, the dendritic cells of human skin, to predict whether a chemical is a sensitizer or not. Upon contact with a sensitizer two surface proteins, CD54 and CD86, become overexpressed in cultured THP-1 cells, which serve as a surrogate for the Langerhans cells. After labelling the cells with specific, fluorescein isothiocyanate (FITC, emission maximum 488 nm)-tagged antibodies against the respective CDs, their expression levels can be detected and quantified via flow cytometry (FC).

However, the method reveals serious limits when chemicals are tested which generate fluorescence signals based solely on their chemical properties when excited with the FC laser, irrespective of any FITC labelling of the treated cells. This is the case e.g. for oxidative hair dyes. If they fluoresce in the same spectrum as FITC, this will result in fluorescence interference which, in the worst case, can completely superimpose a signal possibly caused by CD54 and 86 expression.

A possible option to overcome this limit is to use fluorochromes with different spectral properties compared to FITC, e.g. allophycocyanin (APC, emission maximum 680 nm), in order to avoid fluorescence interferences. Following the newest version of the draft OECD test guideline for the h-CLAT [1], alternative fluorchromes are allowed as far as it is proven that they provide similar results as the FITC-labelled antibodies.

For this reason we have performed a proficiency exercise to compare the results generated with APC- and FITC-labelled antibodies. The 10 proficiency chemicals listed in the draft guideline were tested in two independent runs according to the h-CLAT standard operation procedure (SOP, [2]). After chemical treatment the THP-1 cells were split into one fraction incubated with the FITC-, and another one incubated with the APC antibodies. The fluorescence signals generated with both fluorochromes were then corrected and analyzed as described in the SOP. All proficiency chemicals were predicted correctly according to their *in vivo* skin sensitization potential, irrespective of the fluorochromes used. The mean fluorescence intensity data generated with FITC and APC for all chemicals in all concentrations measured were strongly positively correlated. APC signal intensity was generally lower than FITC signal intensity, but always high enough to correctly identify the sensitizing chemicals. Based on these results oxidative hair dyes, which could not be tested with FITC-labelled antibodies in the first instance, were re-tested with APC, leading to reliable classifications.

In summary, by using antibodies with alternative fluorochromes it is possible to analyze the skin-sensitizing potential of autofluorescent oxidative hair dyes by circumventing fluorescence interferences between dye and fluorochrome-tagged antibodies. With this strategy the limits of the h-CLAT can be extended towards chemicals of challenging physical-chemical properties.

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The influence of *in vitro* complexity on detectable inflammatory responses induced by gold nanoparticles

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Context

Nanoparticles released into the environment or administered for biomedical applications can pose potential health risks to the human respiratory tract by inducing severe inflammation.

Objective

The aim of this study was to compare the inflammatory response of four *in vitro* models of the human lung epithelium, differing for composition and/or culturing substrate, when exposed to gold nanoparticles (AuNPs), selected as case study.

Method

Three *in vitro* models of lung adenocarcinoma (A549) cells, a cell line representative of the alveolar respiratory region, and a commercially available 3D culture (MucilAir™) mimicking the upper airway epithelium, were tested. Such models were exposed to AuNPs for 3, 6 and 24 h. AuNPs internalization was investigated by confocal and electron microscopy, while Enzyme-Linked Immunosorbent Assays (ELISAs) were used for quantifying the secretion of the inflammatory mediator IL-6. Finally, a microfluidic setup was developed in-house for studying whether the inflammatory cytokines secreted by epithelial cells could trigger monocytes recruitment.

Results

Our results demonstrated that AuNPs were internalized only in conventional submerged cultures. Nevertheless, AuNPs internalization did not trigger a significant IL-6 secretion from such cultures. Conversely, significant amounts of cytokines were detected in all the other *in vitro* models, whereas significant monocyte activation was triggered only by supernatants derived from AuNPs-treated models comprising more than one cell type.

Conclusions

Our data demonstrates that cell cultures complexity, as well as culturing substrates, deeply influence the detectable cellular responses to AuNPs, and advocate for the adoption of tissue-mimetic *in vitro* models of the human respiratory tract as a suitable models for nanomaterial exposure research.

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Characterization of reconstructed human skin containing Langerhans cells to monitor molecular events in skin sensitization

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The sensitising potential of xenobiotics is determined using animal tests preferably by the local lymph node assay. Alternative approaches are limited since monocultures of skin cells do not reflect cutaneous penetration, biotransformation, and cutaneous cross-talk in the biological environment. In this study, we integrated *in vitro*-generated immature MUTZ-3-derived Langerhans-like cells (MUTZ-LCs) or monocyte-derived LC-like cells (MoLCs) into reconstructed human skin (RHS), consistent of a stratified epidermis formed by primary keratinocytes on a dermal compartment with collagen-embedded primary fibroblasts. LC-like cells were mainly localized in the epidermal compartment and distributed homogenously in accordance with native human skin. Topical application of the strong contact sensitizer 2,4-dinitrochlorobenzene (DNCB) induced IL-6 and IL-8 secretion in RHS with LC-like cells, whereas no change was observed in reference models. Increased gene expression of CD83, PD-L1, and CXCR4 in the dermal compartment indicated LC maturation. Importantly, exposure to DNCB enhanced mobility of the LC-like cells from epidermal to dermal compartments. In response to the moderate sensitizer isoeugenol and irritant sodium dodecyl sulphate, the obtained response was less pronounced. In summary, we integrated immature and functional MUTZ-LCs and MoLCs into RHS and provide a unique comparative experimental setting to monitor early events during skin sensitization. This study fosters the 3R approach by developing and characterizing human cell-based test systems for advanced and potentially individualized hazard assessment of skin sensitization.

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A stem cell based metabolic activation system – Potential for replacing rat liver S9 fraction

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The *in vitro* investigation of substances that become toxic after metabolic activation employ rat liver S9 fraction as the source for liver enzymes. According to standard protocols, isolation of S9 fraction with enhanced enzymatic content requires poisoning of animals prior to liver extraction, for instance by treatment with Aroclor-1254 or with a combination of sodium phenobarbital and beta-naphthoflavone [1]. As a consequence, large numbers of animals suffer for the production of a supplement for *in vitro* tests. This is controversial in terms of the ethical use of animals in testing (3Rs). Moreover, S9 fraction isolation constitutes a very time consuming and costly process, since rats have to be maintained under GLP/GMP conditions until reaching maturity and considering that the poisoning-extraction-purification process requires trained personnel. Thus, replacement of S9 fraction for an *in vitro* system capable of metabolic activation would be extremely beneficial in toxicological testing, both in terms of animal welfare and with regards to production costs. We hereby show the preliminary results of an alternative method for metabolic activation of toxicants, namely by the *ex-vivo* induction of hepatocyte cultures mixed with a stem cell line isolated from the KOI carp (*Cyprinus carpio haematopterus*) brain (KCB), so that the resulting co-culture would be long lasting and present liver enzymatic activity. The ability of this culture to produce liver enzymes was assessed by gene expression analysis using RT-qPCR. Several enzymes involved in the metabolic activation of xenobiotics have been investigated, namely CYP1A, CYP1B, CYP 3A, CYP 2K; CYP 1B2, CYP 1C1, GSTA (glutathione-S-transferase alpha) and MGST1α (microsomal glutathione-S-transferase). All investigated enzymes have been found to be expressed in the mixed cultures, while KCB cultures without hepatocytes express only CYP 3A, possibly due to its function in brain steroidogenic metabolism. Morphological features of the attached cells present in the mixed hepatocyte / KCB cultures strongly differed from those observed in KCB monolayers, suggesting that the primary cells either grow embedded in the KCB layers or trigger biochemical pathways that modify the normal physiology of the stem cells, probably both. The mixed cultures could be maintained for over 7 days maintaining hybrid characteristics. To investigate the metabolic potential of these mixed cultures, the activity of CYP1A1 has been measured by means of the EROD assay. The preliminary results show a clear activity of this enzyme in the co-cultures, suggesting that phase I metabolic activation, at least by CYP1A1, is performed by the cultivated cells. The next stage in establishing the system is the *in vitro* metabolic activation of substances by the cultured cells and/or by cell homogenates or fractions. Two substances which require metabolic activation (2-aminoanthracene, cyclophosphamide) will be employed in genotoxicity testing both in prokaryotic (umuCD assay) and eukaryotic (micronucleus assay) systems, and the results will be compared with negative (i.e. no metabolic activation) and positive (in the presence of rat liver S9 fraction) controls.

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Replacement and non animal methods: the importance to be the change

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"Replacement and non animal methods" are a reality that needs to be addressed seriously and correctly by governments, academia, scientists and media. There is a clear and growing awareness about the need to stop doing and financing animal experiments in science and research and to avoid using animal testing for safety or efficacy, seeking but too often failing, to extrapolate information on humans from animals. Furthermore, there is a clear connection between human health and how humans treat animals and the environment. Science aims to understand and explain Nature but has also a role in the society, that is to inform and guide the changes needed to drive human progress and improvement, and therefore also help the use and development of non animal methods and alternatives to animal experiments and to move research towards a new era, an era without any use of animals, and individual scientists have a pivotal role to drive this change. How this change is happening now? Is for scientists to investigate and report.

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A Course in replacement alternatives: to give a good example

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Replacement Alternatives and non animal methods need to be developed, but most of all need to be used. Academia and industry therefore need to be kept up to date about the latest methods and technologies. It is clear therefore that advanced courses need to be developed and financed in order to train scientists, researchers and laboratory heads on the latest development in Replacement Alternatives in order to change to non-animal based science and methods to perform both basic research and help to design goal-targeted experiments without the use of animals to respond to precise scientific question of human and scientific relevance. A brief analysis of funding for Alternatives in Italy and in other countries in EU, as training of scientists on Alternatives, as well as research focussed on developing and using replacement alternatives in research will be made, with the conclusion that in all the EU countries the financial contribution for Replacement Alternatives is very low comparing to animal-based research. The Italian Regional Authory of Tuscany made possible to create and implement a Course on Alternatives in Italy exclusively focussed on 1 of the "3Rs", the most important and the final goal of the Directive 2010/63/EU the R of Replacement Alternatives to animal experiments and non-animals methods. The Course focus was about methods and technologies aimed to Replace the use of animals in research and education. 4 editions of the Course took place open to students, graduates, post-docs, researches and lecturers, with an introduction to the topics and questionnaires to assess learning objectives. The Course, open to 20 individuals, was run in 3 days full-time and it was organised with morning lectures and afternoon practical training of selected methods. The Course was very successful and it is proposed as a model for more Courses around Europe with collaborations with other Universities, institutions and industry. From the Course many conclusions were reached which would be useful to address also at policy level, one of the most important being that laboratory heads should attend to these courses as a way to implement more instruments and advanced research methods in their labs, methods that are often unknown to scientists not familiar with the Alternatives Replacement field. To this regard it is proposed that more Alternatives Replacement Courses are need to fill the gap between Replacement Alternative methods and final users in all EU and more collaboration is needed between Course organisers from different countries in EU, academia and industry to organise more Courses: therefore TheAlternatives calls for collaborations to this aim to organise more courses to fill the gap all around EU.

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The Danish 3R-Center

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The Danish 3R-Center has supported ten 3R research projects since 2014 covering replacement, reduction and refinement. Results from some of the projects will soon be published.

The center has recently launched teaching material on the 3Rs for high school students. The material consists of quizzes, a film and four articles. The teaching material is currently being transformed so it can be used in primary school as well.

In order to reinforce the activities of the Danish 3R-Center and assess their effect, the center commissioned a study of stakeholders' knowledge and experience of the 3Rs in Denmark.

The results from this survey point at some future action points that the Danish 3R Center can pursue in their future activities.

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Stereotypic mice: To use or not to use, that is the question

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During caring and breeding of FVB/NJ mice we recognized a high prevalence of mice performing various types of stereotypic behavior. Laboratory housing conditions are standardized to a high level, but standardization can interfere with animal's behavioral needs and thus lead to coping responses, such as stereotypies. Abnormal repetitive behaviors like stereotypies arise when kept in adverse circumstances with a lack of physical complexity, restrictive space and social deprivation [1]. Stereotypies are defined as "repetitive behavior induced by frustration, repeated attempts to cope and/or [brain] dysfunction" that are "demonstrably caused by deficits in captive housing" [2]. Performing stereotypies influences the scientific outcome [3]. It is known that environmental enrichment affects the development of stereotypic behavior [4] and the prevalence can be even decreased [5]. Hence, housing conditions highly influence animal's behavior and lead to structural brain changes [6,7]. In the sense of refinement in animal experimentation it is of paramount importance to understand and interpret behavioral changes. In order to assess what impact the performance of stereotypic behavior has for animal welfare and severity assessment we reared 35 singly-housed FVB/NJ mice in standard cages for 11 weeks. Animals were characterized by a behavioral protocol based on the SHIRPA-protocol [8] and open field tests and fecal corticosterone and brain dopamine and serotonin levels were measured. At the end of the study organs were weighed. Overall 54.29% of FVB/NJ mice developed stereotypic behavior divided in 3 types: circuit running (14.29%), back flipping (28.57%) and wire gnawing (11.43%). Averagely, FVB/NJ mice developed stereotypies at age of 8 weeks. Stereotypic animals showed less explorative behavior during the first open field test, which was abolished during the second test 1 week later. Moreover, stereotypies were associated with a significantly higher activity and motility. Stereotypic animals had higher brain dopamine levels and there were no differences in brain serotonin contents. We measured lower fecal corticosterone levels in stereotypic mice in week 11. Moreover, relative weight of heart, liver and spleen were significantly higher in stereotypic animals. No evidence for a distress-dependent regulation of stereotypies was found. The brain contents of the endogenous opioids serotonin and dopamine lead to the assumption that stereotypies are not an indicator of poor animal wellbeing. Nevertheless, the preliminary results lead to the recommendation not to use stereotypic mice in metabolic and behavioural studies as a consequence of the unsteady reproducibility and the expected variation in metabolic and immunological parameters.

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Rupintrivir reduces rhinovirus infection in human Precision-Cut Lung Slices

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Human rhinovirus (HRV) as a main cause of respiratory tract infections in humans is a risk factor of exacerbations in patients suffering from severe lung diseases, for instance asthma. The virus encounters the airway mucosa and invades into host epithelial cells. Upon replication, new virions are assembled and released from cells without inducing a direct cytopathic effect. The infection of epithelial cells by HRV induces an innate immune response against the invading virus. Thus, diverse signalling pathways are activated, leading to secretion of pro-inflammatory cytokines and chemokines in order to mediate the anti-viral host response. For this study a model of HRV1B infection in human Precision-Cut Lung Slices (PCLS) was established. The aim of the study was to investigate the host immune response and intervention with pharmacological treatments.

Human PCLS containing cross-sectioned airways were prepared from agarose filled lung lobes by slicing the tissue into about 300 µm thick tissue slices. PCLS were inoculated with HRV1B, UV-inactivated HRV1B, medium or HRV1B in the presence of therapeutic intervention with Rupintrivir or Pleconaril for 2h at 33°C. Slices were post-incubated for 24 h and 72 h with or without single administration of Rupintrivir or Pleconaril. Tissue vitality was determined using LIVE/DEAD staining and measurement of lactate dehydrogenase. Pro-inflammatory and virus-specific cytokine release was determined by MSD. Viral load of supernatant and homogenates of slices was titrated by serial dilution plating on HeLa cells and analysis of the cytopathic effect (CPE) using TCID50 assay.

HRV1B infection induced expression of replication-dependent and independent cytokines. Pro-inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interferon-inducible T-cell α chemoattractant (I-TAC), interleukin-6 (IL-6), regulated on activation, normal T cell expressed and secreted (RANTES), interferon-γ (IFNγ), interferon γ-induced protein 10 (IP-10) and interferon-α2a (IFNα2a) were significantly upregulated by HRV1B infection. HRV1B infection didn't show an obvious cytopathic effect confirmed by maintenance of tissue vitality as indicated by LIVE/DEAD staining of PCLS. A release of virions from HRV1B infected human PCLS to supernatant was not observed, however, replicating virus was detected in immunohistochemically stained tissue, recorded by confocal laser scanning microscopy, confirmed an active infection after 24 h. Cytokine release and TCID50 were reduced after pharmacological intervention by Rupintrivir. Treatment with Pleconaril during inoculation and a single dose treatment immediately after inoculation period did not show a significant reduction of pro-inflammatory cytokine release.

The infection of *ex vivo* lung tissue with HRV1B induced pro-inflammatory and viral-specific immune responses, which can be regulated by pharmacological intervention. Future experiments will focus on the induction of an asthmatic phenotype in PCLS and a subsequent infection with HRV1B to investigate mechanisms of viral-induced asthma exacerbations.

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Biotechnological chimeras and experimentation: Legislative lacunae legerdemain?

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This paper seeks to achieve a number of tasks: first, to chart a brief history of the creation, regulation and patenting of biotech inventions in the US and Europe and secondly, to examine critically the role that morality has played and may play in this domain. It will show that ethical and moral concerns are in a state of disarray and when they are applied, they are reactive, unpredictable, applied from a non-principled perspective and are fundamentally ad hoc. This leaves biotechnological inventions in a vulnerable gap where despite advancements in animal welfare and rights, a genetic tweak or two may take such "inventions" outside of a protected realm. Law is often more about semantics than justice and not being specifically named in legislation can have grim consequences. Legal protection invariably trails technological expansion and political expediency can lead to inertia about law reform. Principled legislation, rather than living laboratory inventions, should come first. This should be carried out in a deliberative and participatory setting where the citizenry and not unelected highly specialised biotechnologists get to decide about our genetic future and, indeed, our treatment of genetically engineered or biotechnological beings or "inventions".

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Simulating chemical kinetics and dynamics for the prediction of toxicity

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The EURL ECVAM Toxicokinetics (TK) Strategy was set up to promote a better use of TK data in the framework of the 3Rs to reduce, refine, and ultimately replace animal testing in the assessment of systemic toxicity. The strategy addresses kinetic modelling and good modelling practices. So far a number of biokinetic / biodynamic models have enabled the simulation of kinetic processes and dynamic interactions underlying toxicity, providing a basis to perform *in vitro* to *in vivo* extrapolations (IVIVE). This objective was set up to facilitate the acceptance and use of biokinetic models (such as the Virtual Cell Based Assay, Physiologically Based Kinetic Models) in the risk assessment process; for instance to improve extrapolation from *in vitro* effects to an actual (external) exposure dose by applying reverse dosimetry. Furthermore, information on kinetics and dynamics properties, Mode-of-Action, dose-response and toxicity of chemicals as well as Adverse Outcome Pathways or simple collection of ADME properties should be collected in a harmonized way. Integrating these types of information will be valuable for regulatory anchoring to develop guidance on human exposure, ADME/TK/TD data on how to generate, use, interpret these data in a regulatory setting, applying IVIVE, or in Read Across for a better risk assessment of chemicals.

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Uncertainties and complexities of the 2 years rodent cancer bioassay – hitchhiking OECD work on systematic description of *in vitro* testing strategies to explain limitations of *in vivo* reference data

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For developing new *in vitro* based IATAs for carcinogenicity [1] it will be necessary to systematically describe and agree on the complexities and uncertainties for the reference standard, i.e. the largely laboratory animal based testing and evaluation approach. This is to

– define what type of effects should be predicted by the new IATA (sufficient/ limited evidence in humans/ animals, GHS category 1A/ 1B/ 2, specific or multi-site neoplasms etc.) and
– select the most suitable reference data/ evaluations and
– define a benchmark for the minimum performance of the new *in vitro* IATA. Correlation of *in vitro* IATA results with reference standard results is limited by complexity and other uncertainties of reference standard data/evaluations.

As a basis to explore how this can be constructed, the draft OECD Guidance document on the reporting of defined approaches to be used within integrated approaches to testing and assessment [ENV/JM/HA (2015)7, Jan 2016] was used to assess suitability as a framework for such a characterisation. The uncertainties and complexities around the current definition of "sufficient evidence of carcinogenicity in animals, including point of departure for risk assessment" were specifically analysed for each of the four elements defining a testing and assessment approach: 1) the endpoint addressed, 2) the rationale underlying the construction and interpretation of the approach, 3) the description of the individual information sources constituting the defined approach, 4) the data interpretation procedure applied. The format appears suitable to support, structure and substantiate the discussion on the complexity and potential multitude of approaches for integrating and interpreting data from standard animal testing approaches, and ultimately appears to be conceptually similar to the challenge of integrating relevant *in vitro* and *in silico* data. The systematic analysis will be further discussed and developed within the OECD expert group on non-genotoxic-carcinogenicity IATA development.

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The human placenta *ex vivo* model: Analysis of ampicillin and cefotaxime transfer from the maternal to the fetal circulation

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Background
Ampicillin (AMP) and cefotaxime (CTX) are commonly used to treat maternal infections during pregnancy. Prevention of intrauterine and consecutive fetal infections may be aimed when treating pregnant women with antibiotics. The efficacy is related to sufficient drug concentration in the fetus. However, thus far, the achieved concentrations of antibiotics in the fetal circulation following standard therapy of the mother are unknown.

Aim
The aim of this study was, to evaluate the maternal blood concentrations following i.v. treatment and the placental transfer of AMP and CTX by *ex-vivo* placenta perfusion.

Methods
Sampling of maternal blood and perfusion studies were monitored over 4 h and concentrations of both antibiotics were measured by LC-MS. 30 min following i.v. treatment with 2 g AMP or 2 g CTX, serum blood levels of pregnant women reached a maximum of 50 µg/ml AMP and 30 µg/ml CTX. During placenta perfusion with 100 µg/ml AMP or CTX, a continuous transfer of both drugs into the fetal circulation was observed. Fetal drug levels corresponding to the minimum inhibitory concentration for efficient treatment of streptococci were obtained 30 min after beginning of perfusion and were maintained for the whole perfusion time.

Conclusion
The results demonstrate the usefulness of the human *ex vivo* placenta perfusion system for the analysis of desired short term transfer of drugs from the mother to the fetus. Other models, especially in animals, may not be able to provide such exact kinetics for prediction of transfer.

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IONOSENSE POC: integration of OECTs with live cells an a 12-well plate for *in-vitro* diagnostics

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Relevant, and physiologically accurate *in vitro* models can greatly advance drug discovery and enable human-centric diagnostics, with the ultimate goal of pushing animal testing to a last resort used in final stages of compound testing. Diagnostics and drug discovery represent two large market areas, on whom the pressure to move away from animal testing is considerable. At present however, *in vitro* models are found to be severely lacking and not predictive of the *in vivo* system they attempt to mimic, and are thus not cost effective. We have developed a platform based on organic devices with low-cost production techniques. It is compatible with a combined optical/electrical approach for readout of traditional markers such as protein expression along with electrical measurements such as transepithelial/endothelial resistance (TEER). The technology developed has been adapted to meet complex requirements of companies in the various fields of pharmacology, toxicology and cosmetics.

We have previously shown that the organic electrochemical transistor (OECT) can be used to great benefit for both *in vivo* and *in vitro* applications requiring direct interfacing with tissue [1,2]. The OECT has been shown to be a high fidelity method for recording ionic fluxes in biological systems and is thus an excellent technique for establishing a baseline signal in healthy tissue, and by corollary detecting changes which occur in the normal flow of ions related to toxicity events, or disease states. Advantages over traditional cell monitoring methods of these polymer based devices, include increased signal transduction, biocompatibility, mechanical properties akin to tissue, chemical tuneability and optical transparency.

As the community wishes to transition to high-throughput systems, we have integrated our sensors in a 12-well plate format that enables parallel measurements and remains suitable for other techniques such as microscopy. We have designed a device for a demonstrator prototype; optimizing geometry and placement of electrical contacts. Taking advantage of the optical transparency of the active material used in our devices (PEDOT:PSS) we demonstrated simultaneous electronic and optical monitoring of epithelial cells *in vitro* [3]. In order to streamline the fabrication process, we used novel photolithography resists compatible with organic materials that also increase device-to-device reproducibility. Used as active devices, the transistors enhance the quality of the recorded signal and the sensitivity of the measurement. Collectively, these attributes in a single platform will contribute greatly towards the accuracy and efficiency of *in-vitro* toxicology and diagnostic.

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An ethical challenge to the normalisation of animal experimentation

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Each year millions of nonhuman animal subjects are used in experiments. The types of experiments they are subjected to are wide-ranging and no species of nonhuman animal is safe from being used. Because the nonhuman animal subjects involved have no choice but to be exposed to experiments that cause them suffering, harm and often death it follows that such experiments require strong moral justification. This is the subject of a recent report by the working group of the Oxford Centre for Animal Ethics (UK), which offers a wide-ranging assessment of whether experiments that use nonhuman animal subjects can be justified morally. The report was commissioned by the British Union for the Abolition of Vivisection (now Cruelty Free International) to provide independent academic research on the subject and is one of the very few reports to focus on the ethical dimension and to do so from a variety of disciplines, including philosophy, science, history, theology, law, critical animal studies, and sociology. This paper presents an overview and discussion of that report.

The normalisation of animal experiments is based in the assumption that human needs, wants or desires should have absolute or near absolute priority in moral calculations about whether or not they should take place and on these grounds nonhuman animal experiments have become established and routine. This normalisation is propped up by an overconfidence in the value of nonhuman animal experiments as a scientific technique and is augmented by a range of regulations and controls that often do very little to protect nonhuman animal subjects. But this normalisation contradicts what is now known about the extent and range of how nonhuman animals suffer and can be harmed, and is based on the idea that nonhuman animals are commodities who can be used as a means to human ends. In countering the normalisation of nonhuman animal experiments the report argues for their de-normalisation and de-institutionalisation. This paper presents the challenges offered in report, challenges that seek to counter the idea that humans should always have absolute priority in our moral thinking, that nonhuman animals exist to serve the interests and wants of humans and that humans should be distinguished and separated from other animals in terms of the hierarchical binary "them" and "us" in which the nonhuman animal "Other" is denigrated. The paper closes with the report's pressing appeal for the full institutionalisation of ethical research techniques that counter moral anthropocentrism by recognising fully that individual nonhuman animals have worth in themselves with the consequent urgent switch of funding to non-animal replacement techniques.

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Development and standardization of a predictive and bio-relevant wound healing assay for preclinical development

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Despite limitations in predictability, animal-based testing still plays a pivotal role during preclinical development to evaluate safety and efficacy of novel therapeutic systems. This is especially relevant for the development of novel wound therapeutics due to an increasing clinical need based on chronicity as well as on severe wound infections. Besides ethical concerns, major differences in anatomy, immune response and in particular the wound healing process impede the transferability of animal-derived data to the situation in the human body, which significantly skew the interpretation of the obtained results.

As *in vitro* alternative, simple cell migration tests are applied using human cells ("scratch wound" assay), which are based on introduction of a "wound" by manually disrupting an intact cell monolayer using pipette tips. However, these assays can only be utilized for the assessment of liquids as other therapeutics like wound dressings would harm the cell monolayer integrity upon removal. Further, excessive hydration conditions due to total immersion of the assay are far away from approximating a physiological wound moisture situation.

To address these issues, we introduce a novel concept of a cell-based wound assay allowing for rapid and predictive assessment of wound therapeutics independent of their physical state. Our assay provides a bio-relevant culture environment simulating human *in vivo* skin hydration conditions (basolateral diffusion from the dermal compartment and air-exposition of the skin surface). To allow for removal of the therapeutic system for monitoring over longer time intervals, a novel cell seeding approach based on capillary forces was introduced. In addition, we considered other key aspects, which might influence cell response and consequently affect the interpretation of the performance of the tested systems to further enhance the predictive value of this novel wound assay. In a systematic study, we evaluated different wounding procedures and discovered a significant correlation of wounding and cytokine expression. Based on our findings, we established a precisely standardized protocol for the novel wound assay. In a proof-of-concept study, the applicability of the assay could be demonstrated by investigation of unloaded and drug-loaded electrospun wound dressings and their mediating effect on cell migration. The observed findings regarding the diversity in cell responsiveness dependent on the applied treatment strategy (no treatment, unloaded or drug-loaded dressing) could successfully corroborate the discriminative power of this wound healing assay.

Based on these data, the novel cell-based wound healing assay has the potential to serve as a powerful and predictive tool for preclinical testing of novel therapeutics for wound application, thus contributing to the reduction of animal testing during preclinical development.

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Utilization of Hommunculus platform for evaluation of cytochrome P450 activity in "liver-on-a-chip"

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The cytochrome P450 substrate-inhibitor panel has been designed for preclinical evaluation of drugs on a 3D human histotypical cell model using Hommunculus "liver-on-a-chip" technology. The concentrations of substrates and inhibitors have been optimized to ensure reliable detection of major metabolites by means of HPLC-mass-spectroscopy. Selected specific substrate-inhibitor pairs bupropion/2-phenyl-2-(1-piperidinyl) propane to evaluate CYP2B6B activity, tolbutamide/sulfaphenazole for CYP2C9, omeprazole/(+)-N-benzylnirvanol for CYP2C19, testosterone/ketoconazole for CYP3A4 provide drugs metabolic pathway determination. Developed "liver-on-a-chip" cell model reflects human hepatocytes metabolism *in vivo* in comparison with animal models that have inter- species variability of CYP450 isoforms expression and activity profiles. The cytochrome P450 substrate-inhibitor panel for preclinical evaluation of drugs' *in vitro* biotransformation has been validated. The experimental evaluation of the developed protocol has been performed by testing two model drugs, warfarin and dasatinib. The results obtained correspond to the reported mechanisms of both drugs *in vitro* biotransformation in a "liver-on-a-chip": biotransformation of dasatinib is catalyzed by CYP3A4, and that of warfarin by CYP2C9. An existing possibility for dasatinib and warfarin interaction with drugs metabolized CYP2C19 isoform is demonstrated.

Reference

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3D vasculature-on-a-chip: development of a perfused human coronary artery endothelial microvessel model for vascular research and toxicological risk assessment *in vitro*

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Lifestyle (e.g. diets, smoking, drugs) and genetic predispositions can favor and accelerate the development of atherosclerosis that may ultimately lead to cardiovascular adverse events. In line with the "3R" principles and Tox21 strategy encouraging the development of cellular alternatives to animal testing, various *in vitro* models have been established to study vascular functions and disorders such as monocyte/platelet-endothelial cell adhesion and transendothelial migration, critical steps in atherogenesis, or angiogenesis to cite a few. These processes are in general modeled separately and in static conditions using endothelial cells organized in 2D cell monolayer. However, the 3D geometry of vessels (e.g. curvature, stenosis) directly impacts hemodynamic flow patterns, and therefore forces acting on the blood vessel wall. Mechano-transduction signals combined with signals induced by soluble mediators present in blood (e.g. oxidants, cytokines) lead to changes in vascular cells acquiring molecular profiles rather pro- or anti-atherogenic. We envision the development of a 3D vasculature model on a microfluidic chip enabling to study vascular functions with dynamic and integrated processes better mimicking the *in vivo* situation. As a first step, we present, here, the establishment of a perfused micro-vessel using primary human coronary artery endothelial cells in OrganoPlate™. The development steps include the optimization of cell culture conditions in 3D and the quantification of barrier formation and integrity through cell structure staining and imaging, and the perfusion of fluorescent-labeled beads, respectively. The development of innovative 3D vasculature models on a chip will open new avenues for vascular research and toxicological risk assessment *in vitro*.

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A microfluidic-based easy-to-use cardiac tissue model for drug screening applications

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Since the introduction of microfluidic technology to the field of tissue engineering, a range of highly sophisticated tissue- and organ-like *in vitro* systems have been developed [1]. Such approaches hold the promise to be more clinically relevant compared to conventional 2D cell culture, and have the potential to replace animal testing during the early stages of drug development. In this project we focus on the development of an easy-to-use cardiac tissue model by generating spheroids from mouse cardiovascular progenitor cells and incorporating them into a microfluidic device.

Through lactate-based metabolic cell type selection [2] in combination with the application of up to three distinct laminar streams, a spatially controlled tissue-like structure containing the main cardiac cell types, cardiomyocytes, smooth muscle cells and endothelial cells can be obtained. The common origin of the cells gives rise to coherent intercellular communication, which is critical for reliable drug efficiency and toxicity studies in an *in vitro* tissue model.

So far our group successfully accomplished the site-specific differentiation and metabolic selection of cardiomyocytes and smooth muscle cells from a single spheroid, and the maintenance of the culture for up to 27 days. Furthermore, we achieved the refinement of standard analysis protocols; such as qPCR for mRNA and miRNA, immunofluorescence staining, and contraction monitoring methods, to be used on our device. Next steps include the integration of impedance and Raman-spectroscopy based sensors on the system for non-invasive cell monitoring. With the integration of those elements, we will have developed not only a cardiac tissue model but also a multiplexable platform with real time monitoring capabilities suitable for drug screening applications.

Financial support from the City of Vienna project Tissue Engineering International (MA 23 - Projekt 14-06) is gratefully acknowledged.

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The Lush Prize – an overview

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Now in its fifth year, the Lush Prize has awarded over £1 million in prize funding, aiming to bring forward the day when safety testing takes place without animals and uses only humane and scientifically robust 21st century test methods.

This presentation will provide an overview of the success of the prize to date; the different categories of prize available, as well as showcasing award winning work in Science, Training, Public Awareness, Lobbying, prize winning Young Researcher projects, as well as the exciting "Black Box" Prize, awarded for the first time in 2015 for breakthrough research on the Adverse Outcome Pathway (AOP) for skin sensitisation.

For more information, visit http://www.lushprize.org

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Is Balb/c 3T3 cell line suitable model for phototoxicity testing?

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As animal use on cosmetic purpose testing is banned in Europe, for identifying phototoxic hazards an *in vitro* method, 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU) has been validated. This test uses the mouse fibroblast cell line Balb/c 3T3 [1]. However, animal cells like Balb/c 3T3 may differ in response to toxins as the shorter lifespan of animals means less powerful the cytoprotective and regenerative pathways compared to human ones. Further, Balb/c 3T3 cells are embryo cells [2] that may be more sensitive compared to human skin cells that are predetermined to protect against environmental assault. In our recent study on natural polyphenols phototoxic potential [3], we used a validated 3T3 NRU test. During experiments we had a problem with Balb/c 3T3 cells attachment on cultivation plates especially during washing steps and following incubation in phosphate buffered saline (PBS), recommended buffer in the validated protocol. On the purpose to demonstrate and highlight the above problem, we compared surface attachment of the Balb/c 3T3 and human keratinocyte cell line (HaCaT).

Four types of plate surface (normal and collagen coated NUNC® and TPP® plates) and three types of washing solutions PBS, PBS supplemented with glucose (1 mg/ml; PBS-Glu) and Earle's Balanced Salt Solution supplemented with glucose (1 mg/ml; EBSS) were tested. Twenty-four hours after seeding, cells were washed twice with the respective solution, then the solution was applied and immediately or after 10, 20, 30 or 40 minutes replaced with serum free medium. After 24 h amount of viable cells was evaluated by neutral red incorporation. Results showed that Balb/c 3T3 cells were very susceptible to detachment from all tested types of surface during washing steps as cell viability decreased about 30-50% compared to untreated cells. The problem intensified with the incubation period in all used washing solutions (PBS, PBS-Glu, EBSS). In contrast there was minimal effect of the washing steps and buffer treatment on HaCaT viability (up to 10%).

In conclusion, Balb/c 3T3 cells seem to be not suitable for phototoxicity testing as many cells are lost during washing steps and different cells should be used. HaCaT represent a more suitable alternative for phototoxicity testing than Balb/c 3T3 as they are less affected during the test procedure, are derived from human skin and allow distinguishing phototoxic and non-phototoxic compounds similarly to Balb/c 3T3 cells [3].

This work was supported by GACR 15-10897S and the Institutional Support of Palacký University in Olomouc RVO 61989592.

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New possibilities in mathematical toxicology

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Bayesian Networks have recently been advocated to predict hazard and potency class of chemicals in the context of skin sensitization by using only animal-free assays. We have used developments in probability and statistics to both simplify and generalize these initial results. Our new approach provides robust procedures for constructing Bayesian Networks, avoiding earlier pitfalls involving data imputation, loss of information and cross-validation while maintaining (or improving) the accuracy of hazard and potency prediction.

Importantly, our methods use Machine learning, avoiding need for mechanistic knowledge of the biological processes in question. This gives us hope that Bayesian Networks may be successful in general toxicity prediction using only Machine Learning and animal-free assays. In this spirit preliminary results for liver carcinogenicity, where the underlying biological mechanisms are much less understood, will be discussed.

By extending this probabilistic thinking, we have also developed a rigorous framework to derive optimal integrated testing strategies for toxicity assessment using animal-free test alone. We combine a population model (accounting for individual-level differences in exposure and in reaction to that exposure) with an explicit cost structure (including both testing and misclassification costs) to derive optimal integrated testing strategies based on the powerful mathematical machinery of Markov Decision Problems. It turns out that, even in the simplest set-ups, optimal policies turn are typically adaptive. In other words, our mathematics demonstrates that one-size-fits-all testing policies cannot possibly be optimal.

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Anticancer effects of co-administration of daunorubicin and resveratrol in MOLT-4, U266 B1 and RAJI cell lines

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Resveratrol (RES) is a naturally occurring compound with strong anti-oxidant effects that combats cancer via several mechanisms. It has been found to inhibit P-glycoprotein and protect cells from chemotherapeutics adverse effects.

Daunorubicin (DAN) is mostly used in the treatment of leukaemia and some solid tumour such as glioma. In the current study, we evaluated the effect of co-administration of RES and DAN (RES/DAN) in MOLT-4, U266 B1 and Raji cell lines. MTT assay was used to investigate cell viability at different concentrations of RES, DAN and RES/DAN. Also, to elucidate the mechanism of cell death, flow cytometry study of Annexin V/PI staining was used. Our results from MTT assay showed that RES and DAN induce cell death. IC_{50} for RES (20 μM for MOLT-4, 73 μM for U266 B1 and 47 μM for Raji cell lines) and IC_{50} for DAN (0.5 μM for MOLT-4, 0.5 μM for U266 B1 and 0.6 μM for Raji cell lines) were calculated. Flow cytometry study using Annexin V/Pi showed induction of apoptosis following RES, DAN and RES/DAN. The effects of RES/DAN were significantly more marked as compared to DAN and RES ($p < 0.001$). Taken together, RES and DAN showed synergistic effects in induction of apoptosis in leukaemia cell lines.

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Predicting adverse immune responses to biologics

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There are currently no reliable human *in vitro* assays which test for immunogenicity, sensitivity, efficacy and allergic reactions of biologics that are equivalent to *in vivo* animal testing. Here we describe a novel test named Skimune™, a non-artificial (non-3D) human *in vitro* test which can be used as diagnostic tool to predict allergicor hypersensitivity reactions to monoclonal antibodies (Skimune™mAb) and small molecule drugs (Skimune™Pharm). The test contributes to the NC3R's initiative and offers either an alternative or a reduction to the use of animal models. The test gives a predictive readout of skin damage (graded I-IV) which also correlates with inflammatory cytokine release and T cell proliferation responses. The data from the different assays is integrated to provide a precise report of the potential risk of the test compound to induce adverse reactions and thus allows the study of immune responses in the presence of drugs, chemicals or cosmetics.

To validate the test as an effective tool for predicting allergic responses to new therapeutics, before testing in man, various antibody formulations were tested on a normal skin biopsy in the presence and absence of autologous lymphocytes, results gave a Pearson Correlation = 0.96, p value = 0.0001 in correlation to clinical data. Additionally, an analogue to TGN (TGN1412, which caused the problem in the Northwick Park trial) has been tested in the Skimune™ model and shows the test could have predicted the serious life threatening cytokine storm which affected healthy volunteers in the 2006 Northwick Park trial. The Skimune™ technologies can also test the efficacy of novel immunomodulatory drugs or be used for comparison studies for biosimilars.

Additionally a number of small molecule drugs have been tested with promising results. The test allows for the improved development of therapeutic drugs and compounds by the early detection of allergic reactions and immune responses and therefore aid in the safety profiling and a reduction in the cost of potentially adverse reactions being picked up before Phase I clinical trials.

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The use of human (non-3D equivalent) skin assays (Skimune™) for the detection of adverse reactions, potency and efficacy

Ana Ribeiro [1,], Shaheda Ahmed [2], Xiao Wang [3], Michael Fielding [1], Andrew Kerry [1], Iain Dickinson [3], Revathty Munuswamy [1], Ian Kimber [3] and Anne Dickinson [1,2]*

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The current most favorable method to test compounds for allergenicity is the mouse local lymph node assay (LLNA). However, recent changes in EU legislation have banned animal testing on cosmetics. A number of alternative predictive test methods for the identification of compounds with the potential to cause skin sensitization are available but are inappropriate for assessment of relative potency. Alcyomics has developed human *in vitro* skin explant assays (Skimune™) as an alternative to the use of animal models. They assays can be used as diagnostic tools for the pharmaceutical, cosmetic and chemical industries for the testing of drugs, novel compounds or monoclonal antibodies for potential allergic or hypersensitivity reactions. The Skimune™ assays have been evaluated against the LLNA, with 95% concordance ($P < 0.001$ sensitivity 95%, specificity 95%) and human sensitisation data ($P < 0.001$ sensitivity 96%, specificity 95%) showing that it is a reliable tool for safety, potency and toxicity testing and also successfully identifies chemicals which have been shown to be negative in the LLNA but positive in man e.g. nickel sulphate.

The Skimune™ technologies can also test the efficacy of novel immunomodulatory drugs or monoclonal antibodies, as well as potential allergic responses, before use in Phase I clinical trials. We have shown that the Skimune™ assays could have predicted and therefore prevented the TGN1412, Northwick Park incidence. The Skimune™ assays bridge the gap between animal-to-man studies and overcome interspecies barriers which often prevent detection of adverse effects during safety testing. The assays use a human autologous system to test for sensitivity and adverse reactions, in which activity is measured as histopathological grading of skin damage, caused by induced immune responses, which correlate with T cell proliferation and IFN-γ production. The data demonstrate that the Skimune™ technologies provide novel and reliable approaches to the determination of skin sensitization, potency assessment, drug or monoclonal antibody evaluation and efficacy testing and can be used as a first step in the risk assessment process.

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Exposure-based chemical safety assessment workflow integrating alternative methods: An ab initio case study for repeated-dose toxicity

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Alternative methods are gaining increasing importance for the hazard and risk assessment of chemicals. There are not replacing traditional toxicological methods one by one, but are considered in integrated approaches such as IATA (Integrated Assessment and Testing Approaches). Within the SEURAT-1 ("Safety Evaluation Ultimately Replacing Animal Testing") research initiative (http://www.seurat-1.eu), which aimed at finding alternative approaches for the safety assessment of cosmetics-related chemicals for repeated-dose toxicity, a conceptual framework for safety assessment was developed [1] and proof-of-concept case studies for applied safety assessment undertaken. Based on the *ab initio* case study assessing the safe use, regarding repeated-dose toxicity, of a cosmetic ingredient in a (hypothetical) exposure scenario, a general workflow was devised. The idea is to structure the evaluation and generation of information and data in a logic sequence for an integrated exposure-driven safety assessment, based on non-animal data. The workflow includes considerations of Threshold of Toxicological Concern (TTC) and read-across approaches. If not applicable, the *ab initio* assessment proceeds with the use of *in silico* profilers and *in vitro* data generation. Special emphasis is given to physiologically-based kinetic (PBK) modelling to identify target organs and internal concentrations. Integrating the obtained information, a mode-of-action hypothesis can be formulated to build a weight of evidence, in order to make a final conclusion on the safety of the substance for the specific use. The workflow focusses on alternative approaches and highlights the challenges in integrating multiple data streams for safety assessment decisions. It goes beyond a weight of evidence by constructing a decision logic with a step-wise look at different levels of information, emphasising the consideration of the exposure scenario from the start, with sequential refinements of the internal exposure and kinetics (*in vitro* to *in vivo* extrapolation – IVIVE) modelling.

The funding from the European Community's 7th Framework program (FP7/2007-2013) and from Cosmetics Europe is gratefully acknowledged.

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SEURAT-1: A cluster of European projects that have changed the landscape for 21st Century European Toxicology

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The SEURAT-1 (Safety Evaluation Ultimately Replacing Animal Testing) initiative, funded by the European Commission and Cosmetics Europe from 2011-2015, aimed to develop alternatives to repeated dose toxicity testing with a focus on cosmetics-related substances, stimulated by European legislation such as the Cosmetics Regulation enforcing a complete ban of animal testing on cosmetics ingredients and products within the EU. The six individual research and development projects of SEURAT-1 were: HeMiBio creating a hepatic microfluidic bioreactor; DETECTIVE developing biomarkers of repeated dose toxicity using *in vitro* systems; SCR&Tox developing stem cells technologies for toxicology; COSMOS taking forward databases and *in silico* approaches; NOTOX predicting long-term toxic effects using computer models based on systems characterised on organotypic cultures; as well as ToxBank supporting integrated data analysis and the servicing of alternative testing methods in toxicology. The coordination and support action COACH coordinated the overarching working groups and cross-cluster case studies.

The SEURAT-1 projects have developed a number of transferable technologies from computational approaches, cell based assays to simulations of organ function. These are suitable to provide toxicological information for new and existing chemicals and will ultimately inform risk assessment. The methods from SEURAT-1 have been brought together in the "proof-of-concept" case studies: a Threshold of Toxicological Concern (TTC) approach, different read-across scenarios and an *ab initio* approach to risk assessment. They have used *in silico* and *in vitro* technologies to provide a platform for integration applying the SEURAT-1 Conceptual Framework through Adverse Outcome Pathways. The realisation is an increasing gain in international acceptance of these alternative methods. SEURAT-1 has fostered international collaboration with the US EPA and gained successful engagement with stakeholders. Overall SEURAT-1 has provided an excellent launch pad for upcoming initiatives to drive forward utilising the technologies and philosophy developed for an innovative and more efficient safety assessment of chemicals.

The funding from the European Community's 7th Framework program (FP7/2007-2013) and from Cosmetics Europe is gratefully acknowledged.

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Applicability of new approach methodologies to support read-across – conclusions from a case study evaluated according to the ECHA read-across assessment framework

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By grouping similar chemicals together, chemical properties as well as toxicity can be read across from data-rich substances to similar substances with no data. Category building and read-across approaches play an increasing role in the safety assessment of chemicals, also for regulatory submissions such as REACH dossiers. The definition and justification of the similarity of the chemicals in an appropriate category is key for the read-across prediction. In most cases, in particular in view of REACH, the similarity is based on chemical structures, however biological similarity, the impact of kinetics and (bio)transformations have to be considered equally. Uncertainties in the categorisation and read-across process need to be identified to conclude on the reliability of the read-across prediction. The Read Across-Assessment Framework (RAAF) has been developed by the European Chemicals Agency (ECHA) to guide through a structured evaluation of (regulatory) read-across justifications.

New approach methodologies (NAM) including new types of *in vitro* assays, high throughput/content screening and *-omics* technologies may offer valuable support to the read-across argumentations and to reduce existing uncertainties. In this context, the read-across case studies carried out in the EU research initiative SEURAT-1 (http://www.seurat-1.eu) covered different scenarios of chemical similarity and aimed at including NAM as strengthening evidence [1]. In the presented work, the ECHA RAAF and its Assessment Elements were used to analyse systematically the argumentation and the contribution of NAM to reduce uncertainties in one of the SEURAT-1 read-across case studies. Major sources of uncertainties were highlighted, the support by NAM of the read-across hypothesis was demonstrated, as well as further needs for new methods and opportunities to use NAM, e.g. through targeted testing.

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Systematic reviews of animal studies equal the implementation of the 3Rs

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Systematic reviews of animal studies can contribute to a more evidence-based choice of animal models, the implementation of the 3Rs and more translational transparency. In my presentation I will explain what systematic reviews are and the work SYRCLE (http://www.syrcle.nl) does. I will present many practical examples. Systematic reviews can be considered an animal free innovation (Replacement), as new information is derived from already published data, without doing any new animal studies. Moreover, knowledge gaps are made transparent and unnecessary duplication can be prevented (Reduction). In addition, much new information on welfare and Refinement is obtained. As a best practices approach for ethical evaluations and funding bodies I will make a plea for including (steps of) systematic reviews, to ensure that all available information on a certain topic has been found, critically evaluated and summarized.

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ITS for skin sensitization potential – 1 out of 2 or 2 out of 3?

David Roberts [1] *and Grace Patlewicz* [2]

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Three *in vitro* methods, aiming to represent different key events (KE) in the Adverse Outcome Pathway (AOP) for skin sensitization have gained regulatory acceptance or are close to OECD adopted test guidelines (OECD TG). These are the DPRA (for detection of ability to bind covalently to protein nucleophiles, KE1), the KeratinoSens™ assay (addressing keratinocyte activation, KE2) and the h-CLAT assay (addressing dendritic cell activation, KE3). Currently a 2 out of 3 integrated testing strategy (ITS) based on these three assays is receiving much attention. In this strategy, if a chemical is positive in any two or these assays it is classed as a sensitizer, and if it is negative in any two of these assays it is classed as a non-sensitizer. In this presentation we report an analysis of data on over 200 chemicals, covering both directly reactive sensitizers and chemicals (pre- and pro-haptens) that require activation), for which LLNA data, human sensitization data and *in vitro* data are available. Our analysis reveals that a simpler and more economical 1 out of 2 strategy can detect sensitizers more effectively than a 2 out of 3 strategy. In the 1 out of 2 strategy, either the DPRA or the KeratinoSens™ assay, but not both, can be used together with h-CLAT: a positive in either assay classifies the chemical as a sensitizer and negative in both assays classifies a chemical as a non-sensitizer.

The views expressed are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

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Mechanism-based modeling for non-animal prediction of skin sensitization potency

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Since the pioneering SAR work of Landsteiner and Jacobs 80 years ago, demonstrating for the first time the relationship between chemical reactivity and ability to sensitize [1], much progress has been made in gaining deeper understanding of the nature of the relationship and towards putting it on a quantitative basis in the form of quantitative mechanistic models (QMMs) covering LLNA data, guinea pig data and human data. This presentation will discuss the extent to which sensitization potency can now be predicted and what further needs to be done to extend and improve predictive capability.

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Potency ranking of skin sensitizers using the Reconstituted human Epidermis (RhE) IL-18 test and the Genomic Allergen Rapid Detection (GARD) test

Erwin L. Roggen [1], Els Adriaens [2], Eric Andres [3], Daniel Bouchard [4], Sun-A Cho [5], Emanuela Corsini [6], George DeGeorge [7], Horst Fuchs [8], Pierre-Jacques Ferret [9], Sue Gibbs [10], Patrick Hayden [11], Olivia Larne [12], Hanna Hofman Huether [13], Silvia Letasiova [14], Malin Lindsted [15] and Jean-Philippe Therrien [16]

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IL-18 production by keratinocytes is a potentially useful endpoint for determination of contact sensitization potential of low molecular weight chemicals. Potency classification of skin sensitizers relates to the irritant potential of the chemical in a RhE model. 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. This test addresses Key Event (KE) 2 of the Adverse Outcome Pathway (AOP) for skin sensitization. The GARD assay is based on a predictive biomarker signature of 200 transcripts differentially regulated in a myeloid cell line when stimulated with sensitizing compounds, as compared to non-sensitizing compounds. The GARD Prediction Signature (GPS) participates in signaling pathways involved in cytoprotective mechanisms and dendritic cell maturation. Both test methods more accurately (> 95%) discriminate contact sensitizers from non-sensitizers as compared to current Test Guidelines (TG) (hazard identification) (< 80%).

The available data suggest a potential use of the RhE IL-18 (KE 2) and the GARDskin (KE 3) test methods in the identification of skin sensitizers. While the GARDskin can be considered as a stand-alone test for hazard identification of hydrophilic substances, the RhE IL-18 potency test may have added value when hydrophobic or water-unstable substances have to be tested. In addition, the latter method in combination with e.g. impedance spectroscopy may provide quantitative information about the amount of substance reaching the viable cell layers in the RhE, and the rate by which this happens.

For chemicals with human data available, the potency information acquired with the RhE IL-18 and the GARD test methods is more reliable than the data acquired with the LLNA. This conclusion is based on RhE IL-18 data and a study by the US interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) concluding that 22% of human sensitizers were not detected by the LLNA, while, overall, 48% were placed in the wrong GHS sub-category. The GARDskin is currently being improved for CLP 1B classification.

Integrated in a testing and assessment strategy, the test methods have the potential to meet the regulatory needs and data requirements related to skin sensitization assessment in the context of the REACH legislation (Regulation 1907/2006/EC), the EU Cosmetics Regulation (1223/2009), the EU Regulation on classification, labelling and packaging of substances and mixtures (1272/2008), the EU Legislation on Plant Protection Products (1107/2009) and the EU Regulation on Biocidal Products (528/2012).

The RhE IL-18 and the GARDskin test methods are in the process of being formally validated.

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EuroMix: using (Q)SARs, TTC, molecular docking simulation and read-across as a first tier in mixture toxicity risk assessment

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Every day, we are exposed to mixtures of multiple chemicals via food intake, inhalation and dermal contact. Whether exposure to these mixtures poses a health risk depends on how the effects of different chemicals in the mixture combine, and whether there is any synergism or antagonism between them. The number of different combinations of chemicals in mixtures or co-exposures is infinite, making testing of all possible combinations practically impossible and ethically unacceptable if it involves animal testing.

The overall objective of the EU project EuroMix (https://www.euromixproject.eu/) is to establish and to disseminate new, efficient, validated test strategies for risk assessment of mixtures, and do this by using, among others, *in silico* and *in vitro* methods. The EuroMix strategy to risk assessment of mixtures follows a tiered approach, where hazard assessment in the first tier is done by applying *in silico* tools including QSARs, Read Across, and the TTC concept [1]. Exposure assessment via multiple exposure routes is performed by using the MCRA tool [2,3]. The EuroMix approach aims to extend, generalize and refine the EFSA Cumulative Assessment Group (CAG) concept [4], and make it possible to apply the CAG approach also to substances outside the EFSA determined grouping of pesticides, like environmental contaminants, food additives, industrial chemicals etc.

To achieve this goal existing (Quantitative) Structure Activity Relationships ((Q)SARs) are used in a first assessment tier to determine to which CAG(s) a substance should (potentially) belong. Existing (Q)SARs (among others DEREK, OECD QSAR Toolbox profiles, MultiCASE) are evaluated to assess whether a substance can be categorized into the CAGs *liver toxicity*, *developmental toxicity* and *endocrine disruption*. A decision strategy using multiple (Q)SARs will be presented for the example of liver toxicity. For the CAG endocrine disruption molecular docking simulations are performed in the project to predict the approximated binding free energy to the estrogen and androgen receptors.

The individual substances are subsequently prioritized on their contribution to the overall risk of the mixture or co-exposure, by assigning Relative Potency Factors (RPF) to each substance. The RPF is expressed as the simple ratio of the (effect specific) NOAEL of a substance relative to the NOAEL of a known substance in the mixture causing the same toxicological effect. Full dose additivity is thereby assumed in this first tier for substances in the same CAG. If toxicological data is available for a substance, effect specific NOAELs are used. If no data is available, Read Across is used in the first tier of the mixture assessment strategy to estimate a NOAEL. If no valid read across structures are available, the TTC concept is used to generate a plausible worst case NOAEL for the substance in the mixture. For the CAG endocrine disruption binding energy estimates from the docking models give a first estimate of the Relative Potency Factor.

These first tier estimates of both CAG membership and RPFs are ideally confirmed/refined in the following tiers where *in vitro* testing information is generated in CAG / mechanism specific assays. These further tiers in the EuroMix mixture assessment strategy will not be elaborated in this presentation.

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From organoid technology to Multi-Organ-Chip development for substance testing

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Cell organoids generated from primary stem cells or iPS cells by self-assembly provide a powerful tool to model human developmental processes and diseases. In recent years, several organ parts and even complete organs have been developed, among them liver, gut and kidney organoids. Recently we developed a human bone marrow organoid containing the hematopoietic stem cell niche for long-term cultivation of HSCs. Based on hydroxyapatite coated zirconium oxide scaffold human hematopoietic stem cells can be cultivated for up to 8 weeks for the first time maintaining their stem cell phenotype in this bona fide *in vitro* culture system. The developed unique artificial niche facilitates the expansion of HSCs and will serve as a tool to elucidate the etiology and therapy of hematopoietic diseases.

Combinations of several organoids on an interconnected, perfused Multi-Organ-Chip platform will further enhance the significance of such organoid culture systems offering a promising potential for 3R strategies. Current examples of our Multi-Organ-Chip combinations will be presented, first successful applications in substance testing will be shown, and next hurdles to further optimize the Multi-Organ-Chip culture system will be discussed as well.

In general, the evolving Multi-Organ-Chip technology provides an essential tool to reduce and replace laboratory animal testing, since the technology enables a more reliable prediction of human outcomes of tested drugs or substances in comparison to present test systems.

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BioVaSc-TERM – a platform technology to engineer human barrier models

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Introduction

In vitro test systems gain increasing importance to improve predictivity and to reduce animal experiments. Of special interest are barrier tissues that guard into the human body. These barriers are formed by highly specialized tissues such as skin, airways and intestine. However, to recapitulate these tissues, researchers are currently restricted by a lack of suitable supporting scaffolds. In this study, we present a biological scaffold based on decellularized porcine jejunum segments (BioVaSc®-TERM) that offers a natural environment for cell growth and differentiation. Employing the scaffold, human barrier models of the skin, the airways, and the intestine are generated that mimic the natural histological architecture and functionality and can be used for efficacy and safety testing of drugs, biologicals and vaccines.

Materials and methods

Following explantation and decellularization, the BioVaSc® was fixed between two metal cylinders. For airway and skin models, primary fibroblasts were seeded on the former luminal side of the jejunum. After 1 days and 5 days, respectively, primary epithelial cells were added and cultured for further 20 days and 11 days under air-liquid interface. For intestinal models, Caco-2 cells were seeded on the BioVaSc® and cultured for 14 days.

Results and discussion

Preserving the complex composition and three-dimensional ultrastructure of the extracellular matrix, the BioVaSc® allows generating tissue models close to the *in vivo* situation [1]. Skin equivalents comprise anatomical and cellular characteristics of native human skin. Fibroblasts migrate into the scaffold forming a homogeneously populated dermal compartment. Keratinocytes develop 8-10 viable layers. Immunofluorescence staining confirmed the close mimicry to native skin, thus enabling to study efficacy testing of drugs [2]. By reseeding the former vessels of the porcine jejunum with human endothelial cells and the subsequent perfusion using bioreactor systems, the scaffold can be used to generate vascularized skin equivalents [3]. The BioVaSc® is also suitable to create three-dimensional airway test systems [4]. They consist of a connective tissue department seeded with fibroblasts and a polarized ciliated respiratory epithelium that is anchored to the lamina propria-like connective tissue. Immunohistochemical staining revealed the similarity to the native tissue and that the epithelium produces major gel-forming respiratory mucins. Thus, the airway models comprise features to investigate biocompatibility, distribution and metabolization of inhalable drugs. Histological assessment of the intestinal models confirmed a confluent monolayer of cubic epithelial cells on the BioVaSc®. Cell polarization was shown by SEM analysis showing the typical microvilli covering the apical cell membrane of the intestinal epithelium [5].

Conclusion

The presented models show tissue specific barrier properties, such as stratification of the skin, mucociliary phenotype of the airways, and polarization of the intestinal epithelium. More physiological skin, airway and intestinal tissue models based on the BioVaSc® facilitate the *in vitro* generation of human barrier models that might help to gain more predictable and reliable data in pre-clinical research for drugs, biologicals and vaccines and represent a useful tool to reduce, refine and replace animal studies.

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Animal welfare requirements – significant room for improvement

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Following years of negotiations the EU Directive on the protection of animals used for scientific purposes (2010/63/EU) entered into force in November 2010.

Eurogroup for Animals and its members were historically involved leading to the adoption of the legislation and remain committed to work with the European Commission and engage with all key stakeholders. Implementation and enforcement of the directive is imperative. Therefore Eurogroup has carried out a survey with member state authorities to gain relevant information on inspections, project evaluation and authorisation, non-human primate breeding strategies and the National Committees in the member states. Eurogroup's members passionately communicate and highlight incorrect transposition and implementation of the Directive in their member state. In Germany in particular, the Deutscher Tierschutzbund have actively voiced their views on incorrect transposition of the Directive.

The Directive is currently under review. It may be poorly timed regarding the late transposition of the Directive by some member states which means limited experience. Moreover no EU statistics have been published under the new Directive and the Commission report on the implementation of the directive, processing information provided by the member states is only due in 2019. However it is an essential moment to put forward our views on where the directive requires improvement for alternatives, non-human primates and the authorisation process. In addition, member states should be allowed the possibility to implement higher standards where animal welfare benefits exist.

Furthermore, the directive is integral to all EU chemical legislations involving the use of animals for scientific purposes. With internationally accepted non animal approaches now part of the EU chemical legislation REACH, it is an imperative moment to have this taken up horizontally in other legislations which include the biocides and plant protection products legislation.

This presentation will give an overview of the work taken place over the past year and the future recommendations for moving forward.

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Classification and Reporting of Severity – an interactive workshop to examine the process and challenges of achieving consensus

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Directive 2010/63/EU introduced the requirement for the classification of procedures (Article 15) during the application for project authorisation to use animals in scientific procedures. It also introduced the requirement to report the actual severity experienced by each animal used in a procedure. Both these processes provide opportunity to refine the adverse effects of procedures.

Consistency of assignment of severity categories across Member States is a key requirement. The examples given in Annex IX are limited in number and have little descriptive power to aid assignment. Additionally, the examples given relate to the procedure and do not attempt to assess the outcome, such as adverse effects that may occur.

The session will commence with an introduction to the severity framework.

Using models developed within the EU guidance document and the FELASA/ECLAM/ESLAV Working Group on severity, each group of participants will identify the components within the procedures which may cause pain, suffering, distress or lasting harm, define the adverse effects associated with these, identify actions to mitigate the adverse effects, identify appropriate end points and finally assign a prospective severity classification.

Each group will define what clinical welfare assessment criteria should be used.

This will be followed by a session using an audience response system (Turning Point) to consider examples of actual severity assessment.

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A High Accuracy QSAR based on rabbit data to predict the human skin and eye irritation potential of individual constituents and mixtures

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Background

To evaluate the potential for skin and eye irritation of a substance normally experimental tests (*in vitro*/*in vivo*) must be performed. Currently, this means an *in vitro* test first, but if inconclusive or for some reason not feasible, it is followed or replaced by OECD 404 and 405 *in vivo* studies to indicate the potential for skin and eye irritation/corrosion of the substance [1,2]. With the EU ban on animal testing of finished cosmetic products and cosmetic ingredients in 2004 and 2009 respectively, and with REACH in 2007, the validation of alternative approaches such as *in vitro* methods and the use of Read-Across approaches have progressed. Consequently, testing in animals for these endpoints has been reduced, but not totally avoided.

NC3Rs CRACK-IT QSARs Mix Challenge

To date QSARs have proven to be inadequate for routine determination of skin and eye irritancy. The QSARs Mix Challenge, sponsored by Shell, was initiated in 2015 under the NC3Rs CRACK-IT programme with the aim of using QSARs to address the 3Rs issues associated with assessing skin and eye irritation endpoints in animal studies, so that these can ultimately be replaced by *in silico* models, ultimately to examine the effects of mixtures [3]. The Challenge was completed in March 2016 with the development and validation of the Skin and Eye Irritation modules of "iSafeRabbit" – the High Accuracy QSAR (HA-QSAR) [4]. The models have been validated as per the five OECD principles for QSAR models [5].

What to expect from this talk?

The iSafeRabbit models predict the irritation potential of substances by determining if the input dose is able to cause cytotoxicity, thereby inducing erythema and/or oedema in the case of skin model, and corneal opacity for the eye model. The dose is the input to a series of calculations (derived from ecotoxicology) to determine if the concentration in the viable epidermis (for the skin model) or stroma (for the eye model) reaches a cytotoxic concentration. Moreover, iSafeRabbit v1.1 has been designed to calculate irritation of mixtures for constituents falling within the chemical groups that define the applicability domain of the model. The first half of this talk will describe the model development strategy for both iSafeRabbit modules.

Both iSafeRabbit modules satisfy the five OECD principles for the QSAR models making them fit for regulatory purposes. iSafeRabbit v1.1 models have been so far validated for 15 chemical families of which only 2 families fall outside the model's applicability domain. Of classes within applicability domain, the iSafeRabbit predictions have a true positive/negative rate of 95% using validated experimental data as reference. The results were also compared on the same validation set with other existing tools including OECD QSAR Toolbox, DEREK and the Danish QSAR Database; the iSafeRabbit results were found to have the highest predictive power. This validation work will be the focus of the second half of this talk.

The talk will conclude with a summary of how the authors intend to extend this work to develop the future versions of iSafeRabbit.

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OECD projects on Integrated Approaches to Testing and Assessment (IATA): a combination of mechanistic information and alternative methods to improve predictive toxicology

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Integrated approaches to testing and assessment (IATA) follow an iterative process to answer a precise question in a specific regulatory context, taking into account the acceptable level of uncertainty associated with the decision context. They are pragmatic and science-based approaches that rely on the principle that before new toxicity testing is performed, relevant existing and available toxicological information is retrieved and considered in a weight of evidence (WoE) assessment to inform regulatory decision-making and guide the targeted generation of new data. IATA can include a combination of methods and can be informed by integrating results from one or many methodological approaches ((Q)SAR, read-across, *in chemico*, *in vitro*, *ex vivo*, *in vivo*) or omic technologies (e.g. toxicogenomics). There is a range of IATA – from more flexible, non-formalised judgment based approaches (e.g. grouping and read-across) to more structured, prescriptive, rule based approaches (e.g. Integrated Testing Strategy (ITS)). This presentation highlights the main milestones achieved since the presentation of a roadmap to a framework for the development and use of IATA that was presented to the 50th Joint Meeting (June 2013) and explores how it plays a central role in OECD work to improve predictive toxicology by using mechanistic information and alternative methods in an integrated way to address both testing and assessment needs. After that, I will briefly outline the available OECD projects related to defining a framework, developing case studies and building IATA.

While an IATA necessarily includes a degree of expert judgement (e.g., in the choice of information sources and their weighting) some elements within an IATA can be standardised (i.e., rule-based). For certain endpoints (e.g., skin corrosion and irritation), progress has been made in the development of defined approaches to testing and assessment [1]. Defined approaches consist of a fixed data interpretation procedure (DIP) used to interpret data from a defined set of information elements (i.e., a formalised decision-making approach). When such approaches are clearly defined they can also be harmonised to enable consistency in how information is used in regulatory decision making. Based on OECD work done in this filed, I will present the guidance document developed to provide principles for describing and evaluating defined approaches to testing and assessment. I will also present the templates for reporting of defined approaches and individual information sources and illustrate their value by presenting skin sensitisation case studies.

The vision is that the outcomes from appropriate combinations of alternative to animal methods that target key events (KEs) along well defined toxicity pathways (e.g. adverse outcome pathways) should ideally provide sufficient information for hazard and risk assessments with no or minimal *in vivo* testing. OECD member countries continue to work on IATA projects and discuss case studies and this experience will eventually inform future guidance development that will further enhance predictive toxicology.

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Online TEER measurements in Homunculus system for orally administered drugs testing

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Human-on-chip and organ-on-chip technologies are developing rapidly last five years. There are few available systems that allow to culture cells for a prolonged period for a substance toxicity and mechanism of action testing. Only few available systems are capable for cultivation of different cell types in closed environment, representing human organs. Different end-point analysis techniques are used to test cell function after experiments: qPCR, microscopy, microarray and sequencing, etc., but there are lack of online methods suitable for use with closed systems, cultivated for days to weeks.

Homunculus platform are known for ability to combine 2 to 6 different cell models in one chip with cultural media circulation and automatic media exchange [1]. Here we report development of system and first experimental results for online TEER measurements.

Models of biological barriers are extremely important for the investigation of physiological functions and mechanisms of transport, pathologies, development of novel drugs and their efficient delivery and therapy. The layers forming the barrier of epithelial and endothelial cells, mainly characterized by the ability to form tight intercellular contacts, separating the apical and basolateral side of the layer.

The cells form a layer between the respective compartments with selective permeability is controlled by diffusion through the paracellular route and transport – through intracellular. At the same time, the barrier is not static and can be modulated by various stimuli, leading to its closure or opening. One known method of evaluating the barrier and transport functions is to measure the permeability barrier to hydrophilic substrates with different molecular weights, for example, sucrose or dextrans. This method couldn't be used in Human-on-chip systems for online monitoring.

We develop and test noninvasive assessment of the barrier function measuring transendothelial or transepithelial electrical resistance of the cells in closed cell-chip for a prolonged period. We demonstrate that this system with combination with Homunculus allow to test absorption of orally administered drugs and monitor intercellular contacts during experiment without affecting the monolayer.

This work was supported by the Russian Scientific Foundation (Grant 16-19-10597)

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Live or dead is not enough: a large-scale mobility assay with *Daphnia magna*

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In many toxicological studies tested animals are recorded, as changes in their behavior can indicate a response to toxic substances. The manual evaluation of videos can be time consuming and biased, therefore large-scale tracking has come in handy. *Daphnia magna* STRAUS 1820 is an established model organism in ecotoxicological research due to its high sensitivity to toxic substances. Additionally, the species taxonomic level makes it a suitable organism for implying the 3R strategy; i.e. replacing vertebrates by invertebrates. However, Daphnia´s transparent body constitutes recording and data evaluation challenging due to a low contrast to the background. The aim of the present study was to develop a reliable system for quantifying the effects of ethanol as a reference substance on the swimming behavior of *D. magna* based on large-scale tracking. To that aim, middle sized *D. magna* individuals were exposed to ethanol solutions (concentrations: 0.5% - 4% (v/v)) in 24-well plates (1 individual/well) for 48 hours. The mobility assay was performed with the observation chamber DanioVision and the according tracking software EthoVision (Noldus Information Technology, Wageningen, The Netherlands). The mean velocity and the total distance moved were automatically measured from the acquired videos. The results of the present study indicate dose- and time-dependent effects of ethanol on the species' locomotor activity. This outcome as well as the automated individual large-scale tracking of *D. magna* were performed for the first time. A quantification of the species' locomotor activity is of great importance as it, additionally to the lethal concentration data, shows effects of sub-lethal concentrations.

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BB3R – Educating the next generation of scientists

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Funded by the German Government (BMBF) 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. In 2016 BB3R has accomplished the following goals:

The development of alternative methods for preclinical drug development and basic research is strongly enforced by BB3R´s principle investigators. For the expansion of research activities three junior professors have been appointed, started research and contribute to teaching. BMBF funded PhD students as well as those funded by the host institutions are supervised and qualified for management positions in professional areas related to the 3Rs. Specifically, the PhD students participate in annual Spring Schools. The 3rd Spring School with a focus on legal and ethical aspects (2017) will be opened for junior scientists beyond BB3R. The award-winning course "Alternative Approaches to Animal Testing" introduces under-graduate and graduate students into the ethical and legal challenges of animal tests. General professional qualification is offered by the Dahlem Research School.

National and international co-operations have been and still are built which have resulted in publications in high-ranking often cited journals as well as presentations of BB3R on conferences. In the consequence, scientists increasing in number apply for associated membership in BB3R.

Cooperation with the Collaborative Research Centre 1112, among others, allows the introduction of cutting-edge analytical tools in BB3R and vice versa strengthens testing in 3D disease models in CRC1112.Most importantly: Freie Universität Berlin has adopted animal protection as a focus points in future development. In 2015 the German Government has opened the German Centre for the Protection of Laboratory Animals Bf3R at the Federal Institute of Risk Assessment (BfR). This unique hub of 3R research in Berlin will be the basis for ensuring sustainability.

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A human neuronal *in vitro* model for application in biomedical research and in pharmacological and toxicological investigations

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Parkinson's Disease (PD) is characterized by the decline in striatal dopamine (DA) levels and a subsequent degeneration of nigrostriatal dopaminergic neurons. The identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), that evokes a phenotype in humans almost indistinguishable from that observed in idiopathic PD, allowed the establishment of experimental *in vivo* models involving primarily non-human primates and mice, which however boosted the demand for test animals. The application of *in vitro* neuronal models as potential substitution was for a long time confined to the use of primary neurons that contain a mixture of various neuronal cell types and require a steady supply of animals. Cell lines available so far allow the generation of neurons with an only inadequate neuronal phenotype.

In order to provide an alternative to the use of test animals in different areas of neurodegeneration research that reflects key aspects of the *in vivo* models, we established the LUHMES model, representing a human immortalized neuronal precursor cell line that allows the generation of a homogeneous population of post-mitotic neurons with a distinct DA phenotype [1,2]. Treatment of LUHMES with MPTP however does not evoke neurotoxicity as the pro-toxicant MPTP first requires the enzymatic activation by monoamine oxidase-B (MAO-B). This enzyme is preferentially localized in astrocytes and enables the formation of the active toxicant MPP^+. Incubation of LUHMES with MPP+ first leads to the degeneration of neurites, followed by the death of the cell. As an example for the potential limitations of a mono-culture, we applied the mixed lineage kinase inhibitor CEP1347. In the LUHMES monoculture, this compound protected from MPP^+ toxicity [1], while it failed to display a beneficial influence when applied in human PD patients [3]. Such observations indicate that, for certain applications, the LUHMES mono-culture model lacks the complexity required for an appropriate representation of the situation *in vivo*.

In order to respond to this limitation, we have generated different astrocyte models [4,5], derived either from immortalized mouse astrocytes, or from human stem cells, and established co-culture models of LUHMES and astrocytes [6]. In these models, the pro-toxicant MPTP is converted into the active toxicant MPP^+ by astrocytes, which subsequently leads to neuronal degeneration [7]. In continuation of the experiments with CEP1347, the influence of a battery of potential neuroprotective compounds was tested both in the mono- and in the co-culture models, and revealed that individual compounds displayed neuroprotective properties only in one, but not in both models.

To generate proinflammatory conditions, as regularly observed in PD, astrocytes in the co-culture model were in a next step activated by pro-inflammatory cytokines. While in the non-activated co-culture model the presence of glia protected neurons against toxic insult, the inflammatory activation of glia resulted in an active astrocyte-mediated neurodegeneration [8]. In summary, these observations clearly indicate the necessity to provide more sophisticated *in vitro* neuronal models (co-cultures, 3D) in order to gain their acceptance by the scientific community as an alternative to experiments involving animals.

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Skin penetration and tumor specific cellular uptake of tecto-dendrimer nanoparticles into a human cell-based model of non-melanoma skin cancer model

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Dendritic nanoparticles possess many characteristics, such as high loading capacity for guest molecules and tunable end groups facilitating surface functionalizing, that have brought them into the focus as a new treatment option for therapy-resistant diseases. High recurrence rates of cutaneous squamous cell carcinoma (cSCC) indicate limited efficacy of currently available treatment. cSCC poses a deadly risk for immunocompromised patients, since the initial carcinoma in-situ (actinic keratosis, Morbus Bowen) often progresses into invasive and metastatic tumour (cSCC) [1]. Nanoparticles can enhance poor skin penetration of topically applied agents [2], and tecto-dendrimer nanoparticles even induced selective toxicity to a melanoma cell line [3]. However the skin penetration and toxicity of the dendritic nanoparticle itself remains unknown. Here, we report on an investigation of skin penetration and cellular uptake of fluorescently labelled (FITC) tecto-dendrimer (G5.G2.5) nanoparticles [3] into reconstructed human skin and an organotypic cSCC model.

For visualization of the local distribution of tecto-dendrimers in the skin a new Fluorescence Lifetime Imaging Microscopy (FLIM) technique [4,5] was applied. This technique combines information from excited-state fluorescence decay curves of the fluorescing molecules with a sophisticated analysis method that uses multivariate analysis algorithm identifying unique fluorescence lifetime species.

Analysis of sections from reconstructed human skin showed no penetration of tecto-dendrimer nanoparticles into the skin. In sections from the organotypic model for cSCC, however, a penetration of the tecto-dendrimer nanoparticles into the viable epidermis of the skin was observed. The regions of concentrated uptake appeared to correlate with the occurrence of "tumor nest" within the organotypic cSCC model. Therefore, monolayer cell experiments were performed to confirm the selective uptake of tecto-dendrimer nanoparticles into tumor cells. While the tecto-dendrimer nanoparticles remain in the membrane of normal keratinocytes, squamous cell carcinoma (SCC) cells take up the particles into the cytoplasm via an active process. As both the SCC cell line and the organotypic model for cSCC show specific uptake of the tecto-dendrimer nanoparticles into tumor cells, there is a huge potential for these tecto-dendrimer nanoparticles in targeted delivery by topical application.

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A retrospective on 40 years of funding animalfree methods

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In 2016, our foundation Animalfree Research celebrates its 40th birthday. We have taken the opportunity to do a quality assessment of our former work, which includes literature studies and reviews as well as the development of approaches designed to abolish or reduce animal use and suffering. The assessment was done with the focus on the impact that our funded projects have had on the 3Rs and on animal welfare. Mandating an independent investigator for this work, we tried to answer the following questions:

- Have these approaches been successfully used?
- Have they contributed to improvements in animal welfare?
- Have they had a lasting impact on the way research is viewed and performed?

In many regards, these questions cannot be answered satisfactorily, in part because of the long time between performance and assessment and in part because there are inherent difficulties of such an assessment. An additional question is therefore:

- Are there aspects for future funding that could make our work more efficient?

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Computational profiling as a proxy for drug hazard prediction in pharmaceutical research

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Pharmaceutical research requires the assessment and constant monitoring of numerous parameters related to ADME and safety. In an early discovery phase, target and substance liabilities must be identified, to prioritize projects and lead candidates demonstrating the highest possible probability of success, but also to plan for critical experiments for early decision making.

Combined *in silico*/*in vitro* profiling strategies are most effective to identify critical liabilities in drug discovery. A stepwise approach is required to optimally support drug safety profiling. Examples will be provided in the fields of ADMET prediction, such as phospholipidosis and phototoxicity.

We will focus on the lead identification, optimization and the candidate selection phases. Specifically here, efficient property ranking and compound filtering tools must be employed to improve compound liabilities and contribute to a reduction in unnecessary experiments. These tools include off-target QSARs, ADME QSPRs and *in vitro* assays.

Drug promiscuity (adverse polypharmacology) is another important parameter to monitor. According to recent analyses, most drug candidates are active against more than six different biological targets [1]. Some interactions can lead to drug toxicity, so the kinase off-target promiscuity has been held responsible for genetic toxicity, but hERG potassium channel and the serotonin receptor 5HT2B have both been linked to severe cardiovascular side effects of drugs. Computational models for numerous off-targets allow for systematic prediction of drug-target interactions [2]. Target engagement in adverse pathways can be analyzed with pathway databases, supporting the toxicity hazard prediction and mode-of-toxicity evaluation of substances. Relevant application examples will be discussed, focusing on hazard prediction (e.g. genetic toxicity, cardiotoxicity).

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Retinal organotypic cultures as disease model for ophthalmic diseases – recently proposed models

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Retinal eye disease, like glaucoma, AMD or diabetic retinopathy, are a major health concern especially with the further aging population. For some of these diseases a therapy is available that slows down the degeneration. However, for none of the major eye diseases a cure is available. They all lead to irreversible blindness. Therefore, a lot of efforts are put into research to identify potential therapies. However, the evaluation of novel therapies requires the use of a large number of animals to determine possible effects. This evaluation includes the induction of a disease either via surgical intervention, toxic substances or by genetic manipulation in the living animal followed by treatment testing. Animals are always euthanized at the end of a trial in order to examine the ocular tissue. Therefore, alternative approaches are needed to reduce the number of animals. Over the last years, several organotypic models have been introduced to the scientific community. To further promote these models, we want to give an overview of currently available models and in addition introduce novel models for retinal diseases.

All presented models are based on organotypic cultures of either porcine, bovine, human, mouse or rat retina. An advantage of organ cultures over cell cultures is the fact that the complex structure of the retina is maintained. The best option for organ cultures are eyes of butchered animals, which are used for the food industry, like pig or bovine eyes. These eyes are not used by the food industry and discarded. By using these eyes for research one can drastically reduce the number of laboratory animals. Anyhow, the pig eye is morphologically and physiologically more similar to human eyes, than the eyes of mice, rats or rabbits. Another advantage of retinal organ cultures from porcine or bovine eyes is that a larger number of samples can be obtained from each eye compared to the smaller rat or mice eyes.

As with *in-vivo* experiments the retinal organotypic cultures can be used for any histological, Western blot, ELISA and qRT-PCR analysis. Even more, also live electrophysiological testing like electroretinogram (ERG) or multi electrode analysis (MEA) can be performed *ex-vivo*. Recently, also the use of optical coherence tomography (OCT) for retinal explants was introduced. Thus, all standard laboratory techniques can be performed in organotypic cultures. In addition, several degeneration models are already available as rodent organotyoic cultures. Newly introduced are porcine models for oxidative, glutamate and hypoxia stress. Thus, offering a multitude of damage models similar to the degeneration models *in-vivo*.

In conclusion, organotypic retina models offer high chances to drastically reduce the amount of animals used in retinal research. Especially by using eyes obtained from the food industry. In consequence, less bureaucracy is need. These models are cost efficient and less time intense than *in-vivo* models. Moreover, especially the pig eye is anatomically and molecularly closer to the human eye, thus even offering higher chances of a successful prescreening of potential novel therapies.

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Testing as a service – flexibility through seamless integration for cell based assays

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The reliable classification of new substances and the verification of efficacy and safety of drugs and biologicals sets strong requirements on methods and infrastructure. Reproducibility as well as transferability throughout laboratories is essential. Nevertheless, current testing procedures often lack standard principles of an efficient and lean process flow: process chains and information flow gets frequently interrupted; test evaluation and generation is redundant throughout labs – wasting money and obvious synergies. Transparency of critical test parameters across the lifespan of the related product is pure science fiction at the moment. One main reason is a lack of sufficient data structures and transferability between different tests and laboratories.

The use of automated systems is in great demand and might introduce a disruptive change how biochemical testing of substances is done nowadays. Nevertheless, there are a lot of barriers at the moment limiting the successful application of automated system. By the lack of flexibility and the demand for skilled computer scientists & engineers just the two main aspects stated by experts shall be mentioned.

The Fraunhofer IPA has a strong background on automated cell culture technologies. The expertise, gained in the successful "Tissue-Factory" light-house project, let us rethink the overall process chain and overcome established principles. We would like to introduce a new service orientated concept for verifying cell based assays that are commonly used. The concept has a strong link to current industry 4.0 concepts and applies a seamless integration throughout the value-added chain. The main idea is to provide maximal transparency through digitalization and the design of smart automates. The interface to the customer – pharmaceutical company, regulatory board or consumer – is the disruptive change with regard to state of the art attempts. Starting with the vision of full transparency down to the bench, the overall infrastructure and test processes need to be reconsidered.

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Perspectives to use the fish embryo tests for the prediction of acute fish toxicity

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Embryonic stages of fish are considered to sentinel less or no pain or distress and are therefore accepted as alternatives to testing of (adult) animals for the hazard assessment of chemicals. Hence, the use of embryos addresses the 3R principles. Most advanced at present for endpoints of regulatory concern is the prediction of acute fish toxicity – a hazard endpoint required by diverse regulations. Many reports have shown that on average acute toxicity of fish embryos and adult/juvenile fish exhibit a high correlation with similar sensitivity. However, for certain groups of chemicals, particularly those with a neuroacitive/-toxic mode of action fish embryos appear to exhibit a weak sensitivity. Our recent study conducted for the European Chemical Agency (ECHA), aimed at a comparison of fish embryo and acute fish toxicity restricted to data that fulfilled various quality criteria resulting in a limited dataset comprising 123 compounds. In principal the study confirmed previous analyses. I.e. an overall high correlation was observed. However, we also detected about 20 compounds with an at least 10fold weaker sensitivity in the fish embryo test. Most prominent was the enrichment of neurotoxic compounds. For the same mode of action also relatively high species variability in the acute fish toxicity test was observed. Furthermore, we analysed whether alternative endpoints of the fish embryo test or daphnia acute and algae toxicity exhibited similar sensitivity as the acute fish toxicity test. For all chemicals (at present 6, work in progress) with weak sensitivity in the fish embryo and with available comparative data the daphnia acute toxicity test was more sensitive than the acute fish toxicity. This indicates that using alternative endpoints and/or combining the fish embryo test with a modified threshold approach for the PNEC assessment could overcome limitations of the fish embryo test.

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Perspectives to use the fish embryo test for applications beyond acute toxicity prediction – chronic fish toxicity, endocrine disruption and developmental toxicity

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Embryonic stages of fish are considered to sentinel less pain or stress and are therefore accepted as alternatives to testing of (adult) animals for the hazard assessment of chemicals. Hence, the use of embryos addresses the 3R principles. Most advanced at present is the prediction of acute fish toxicity. They have been suggested as alternative models for the prediction or screening of diverse endpoints relevant for environmental and human toxicology. However, there is a potential to predict other hazard endpoint required by diverse regulations.

Given the complexity of the fish embryo model it allows to study an array of non-apical endpoints, that can be linked to AOP (adverse outcome pathway) and other endpoints of regulatory relevance such as chronic fish toxicity or developmental toxicity or endocrine disruption. The presentation will highlight various AOP-related endpoints in the zebrafish embryo model and how their analysis and quantitation can be facilitated. A focus will also be given on recent technological advances that enable users without computer programming skills to establish routines for automatic feature assessment. Examples will highlight analysis of embryonic movements (behaviour), thyroid hormone synthesis disruption and analysis of developmental toxicity. The latter particularly addresses the second-species paradigm and aims at avoiding the need to test for developmental toxicity in the rabbit in addition to the rat.

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Towards global harmonisation of 3Rs in biologicals

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The EPAA project on Biological products convened an international workshop in 2015 which achieved consensus to actively encourage deletion of the abnormal toxicity tests (ATTs), general safety tests (GSTs) and target animal batch safety tests (TABSTs) for vaccines from all relevant legal requirements and guidance documents, such as pharmacopoeia monographs, World Health Organisation recommendations, and World Organisation for Animal Health guidelines.

These tests have become obsolete through the introduction of GMP and, most importantly through the use of adequate and stringent QC measures in vaccine production. Advanced process understanding, in-process controls, validation of the manufacturing process and release testing complying with international standards are also part of modern vaccine development and can replace animal testing.

With respect to vaccine potency tests, workshop participants identified international convergence on the scientific principles of the use of appropriately validated *in vitro* assays in place of *in vivo* methods as overarching goal. In pursuing this goal, it was considered essential to include key regulators and manufacturers early on in the corresponding discussions. As an outcome of such discussions, collaborative studies to advance new assays should be initiated as appropriate.

The presentation aims to summarize the scientific background that led to the recommendation and other key conclusions from the published workshop report and to report on the important follow-up steps achieved at the level of the European Pharmacopeia and WHO in the meantime.

Reference

EPAA International Workshop Report. Modern science for better quality control of medicinal products “Towards global harmonisation of 3Rs in biologicals” 15-16 September 2015, publication pending.

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3D *in vitro* tissue models for infection studies with human pathogens

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Introduction

Studying host-pathogen encounters requires appropriate models that properly reflect the complexity of these interactions. While small animal models have been invaluable for the general understanding of the biology of infection, many pathogens are either unable to infect these model organisms or do not fully recapitulate the observed pathogenesis in humans. To overcome these issues, we have developed 3D *in vitro* tissue models that mimic the infection-relevant physiological organ context.

Materials and methods

Based on decellularized porcine gut scaffolds (BioVaSc-TERM®) that offer a natural environment for 3D cell growth and differentiation we generated human barrier models of the intestine, the airways and the skin. Infection studies were performed with the tissue-specific pathogens *Salmonella enterica*, *Bordetella pertussis* and *Trypanosoma brucei*, respectively.

We developed a human intestinal triple culture model, which depicts the human intestinal epithelium (Caco-2), the blood barrier (endothelial cells), and components of the immune system (Peripheral Blood Mononuclear Cells). Transmigration was examined by flow cytometry using fluorescence labelled Salmonella. For airway tissue model generation, primary human bronchial fibroblasts and human airway epithelial cells were seeded and cultured under airlift conditions. The differentiated test systems were treated with sterile-filtrated supernatants of *B. pertussis* and afterwards analyzed using transmission electron microscopy. The skin models were set up using primary human epidermal keratinocytes and human dermal fibroblasts isolated from human foreskin biopsies and cultured under airlift conditions. To investigate the interplay of factors from vector, host and parasite within the chancre we made use of infected tsetse flies to inject metacyclic trypanosomes in artificial human skin tissue.

Results

The human tissue models showed tissue-specific properties, such as the stratification of the skin, the mucociliary phenotype of the airways, and polarization of the intestinal epithelium. In the *S. Typhimurium* infected intestinal tissue model we observed a time-dependent increase of infected epithelial cells while the endothelium was not affected. Moreover, the infection led to the release of IL-8 into the vascular compartment and an activation of monocytes (CD14+) and natural killer cells (CD56+). Incubating the airway models with sterile-filtered culture supernatant of *B. pertussis*, we observed cytoplasmic vacuoles, cellular extrusions and impaired barrier integrity. The natural infection path through the tsetse fly *in vitro* demonstrated that the fly accepts the skin model as a host. Moreover, we were able to show that the sting leads to the transmission of trypanosomes. The trypanosomes were active inside the model for several days.

Conclusions

Understanding important steps of infection mechanisms of human obligate pathogens forms the basis to develop new preventive and therapeutic strategies to fight infectious diseases. Our complex 3D *in vitro* test systems are suitable to further investigate these mechanisms and can support the (further) development of therapy strategies and vaccines in the long run. Additionally, our human organotypic cultures support the reduction of animal testing.

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Refinement and reduction strategies in experimental stroke

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Stroke is a leading cause of death and invalidism worldwide. This study investigated the effect of post-stroke, direct AT-2 receptor stimulation with the non-peptide AT2R-agonist compound 21 (C21) on infarct size, survival and neurological outcome after middle cerebral artery occlusion (MCAO) in mice and looked for potential underlying mechanisms [1]. MCAO is one of the most frequently used animal models for stroke and provides opportunities for implementing refinement and reduction. First step is the study design and statistical planning. C57/BL6J or AT2R-knockout mice (AT2-KO) underwent MCAO for 30 min followed by reperfusion [1]. Starting 45 min after MCAO, mice were treated once daily for 4 days with either vehicle or C21 (0,03mg/kg i.p.) [1]. Neurological deficits were scored daily in accordance to Garcia et al. [2]. This provides the opportunity to define humane endpoints on behalf of refinement. Furthermore, infarct volumes were measured 96 h post-stroke by MRI. Calculation of infarct volume by MRI is mild and related to reduction, because it offers the possibility to measure, infarct volume at different time points without killing animals and save the brain for further investigations.

C21 significantly improved survival after MCAO when compared to vehicle treated mice [1]. C21 treatment had no impact on infarct size, but significantly attenuated neurological deficits [1]. Expression of brain-derived neurotrophic factor (BDNF), tyrosine kinase receptor B (TrkB) (receptor for BDNF) and growth–associated protein 43 (GAP-43) were significantly increased in the periinfarct cortex of C21-treated mice when compared to vehicle-treated mice [1]. Furthermore, the number of apoptotic neurons was significantly decreased in the peri-infarct cortex in mice treated with C21 compared to controls [1]. There were no effects of C21 on neurological outcome, infarct size and expression of BDNF or GAP-43 in AT2-KO mice [1]. From these data, it can be concluded that AT2R stimulation attenuates early mortality and neurological deficits after experimental stroke through neuroprotective mechanisms in an AT2R-specific way [1]. By using refinement and reduction strategies like good statistical planning and study design as well as using MRI and Garcia Score the number of animals could be reduced by half minimum and welfare of animals could be improved and led to valid scientific data.

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An improved reactive oxygen species assay for photosafety assessment of chemicals with limited aqueous solubility

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A reactive oxygen species (ROS) assay was developed for evaluating ROS generation from drug candidates under exposure to simulated sunlight. Validation study on the ROS assay was carried out by the Japan Pharmaceutical Manufacturers Association (JPMA) and our laboratory, supervised by the Japanese Center for the Validation of Alternative Methods (JaCVAM), and the ROS assay is a recommended photosafety assessment tool by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the Personal Care Products Council (PCPC). However, the ROS assay has limited applicability to monitoring ROS generation from poorly water-soluble chemicals. To overcome the solubility issues of the ROS assay, a micellar ROS (mROS) assay was developed for poorly-water soluble chemicals, and a micellar solution of 0.5% (v/v) Tween 20 was applied to the ROS assay system for enhancing solubilizing potency. In repeated mROS assay using positive and negative controls, the intra- and inter-day precision of the mROS assay was almost identical to those of the ROS assay, and the mROS assay had large separation bands between positive and negative controls. Thus, the mROS assay would have sufficient assay precision and robustness for monitoring ROS generation from irradiated chemicals. The ROS and mROS assays were applied to 83 chemicals (200 μM) for comparative purposes. Of all 83 chemicals, 25 and 2 chemicals were unevaluable in the ROS and mROS assay, respectively, due to their poor solubility, and the use of the micellar solution led to successful solubilzation of most poorly water-soluble chemicals. Based on the obtained ROS data, the prediction capacity of the mROS assay were almost similar to that of the ROS assay; however, the negative predictivity of the mROS assay was found to be lower than that of the ROS assay. False negative predictions on ibuprofen and indomethacin were observed in the mROS assay, whereas they were correctly determined to be positive in the ROS assay. Therefore, the ROS assay would provide more reliable photosafety predictions compared with the mROS assay. Considering the pros and cons of ROS and mROS assays, the strategic combined use of ROS and mROS assays was established, and the photosafety of 82 chemicals out of 83 chemicals could be evaluated. The strategic use of ROS assay systems also provided reliable photosafety evaluation on 34 cosmetic chemicals, and the complementary use of mROS assay would be of great help for evaluating photosafety of poorly-water soluble chemicals. In cosmetic industry, complex cosmetic ingredients, such as plant extracts and polymers, were widely used, and development of modified ROS assay systems in sample concentration was required for complex cosmetic ingredients because of their undefined molecular weight. The combined use of ROS and mROS assays (50 μg/ml) was applied to 20 plant extracts, and the obtained results were almost in agreement with their photosafety information in human use. In conclusion, the strategic use of ROS assay systems would provide reliable photosafety assessments on wide range of test chemicals in product development and contribute to the implementation of the 3Rs principle.

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Norecopa: providing global 3R-resources for animal research

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With the implementation of EU Directive 2010/63 and global interest in laboratory animal welfare and alternatives, the need for easy access to species-specific guidelines for animal research has never been greater.

Finding these guidelines in the ocean of scientific literature is not always easy. Searching for them is often hindered by the fact that many scientists do not highlight progress within the 3Rs in their publications, or do not publish all the small details of a procedure which are often critical for its success.

All stakeholders in animal research need easy access to guidelines, information centres, discussion forums, journals, key publications and databases containing information of relevance to the 3Rs.

Norecopa maintains databases relevant to the three Rs which have existed for 25 years. The newest addition to the database collection is a collaboration with the US Department of Agriculture's Animal Welfare Information Center (AWIC), called 3R Guide.

To make these resources readily available on a global basis, Norecopa has constructed a totally new website which was launched on 31 May 2016. An intelligent search engine has been specially developed for the site. This search engine returns hits from all of Norecopa's databases and webpages. A large number of filters allow users to select relevant material and avoid large numbers of irrelevant hits. All searches generate unique URLs, making it easy to document searches when planning a research project.

The website is available at: http://norecopa.no

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The effect of mesenchymal stem cells on breast cancer cell lines tested on the chick embryo chorioallantioc membrane: first results

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Introduction

Treatment of breast cancer involves resection of various extent, often followed by reconstruction of the breast [1]. The use of autologous fat as a filler material (lipofilling) is a relatively new technique in breast reconstruction. Cases of tumour recurrence after lipofilling however raised concerns about the oncologic safety of the procedure [2]. Various studies suggest that adipose tissue, and especially adipose-derived mesenchymal stem cells may have breast-cancer promoting effects [3-5]. The aim of our study was to evaluate the effects of ASCs and human adipose tissue on the breast cancer cell lines MCF7, MDA-MB-231 and BT474 using the chick embryo's chorioallantoic membrane (CAM) as an *in-vivo* model. The CAM essay has been suggested as an alternative to animal trials previously [6].

Methods

18 fertilised chicken eggs were incubated at 37°C. Eggs were cracked into sterile plastic shells on day 3 and xenografting was performed on day 10 of incubation. For xenografting a 5 mm silicone ring was placed on each CAM. 10 µl of Matrigel-cell suspension (10^5/µl) were pipetted into the centre of each ring. Per cell line 3 groups consisting of two CAMs with one spot each were formed. Group 1 served as control. Group 2 was added with 10 µl of human mesenchymal adipose derived stem cells (hMADS) on day 11. Group 3 was added with 25 µl of aspirated fat (lipoaspirate) from a healthy human donor. After harvest on day 13, grafts were formalin fixed and embedded in paraffin. For histo-morphologic analysis H/E staining was performed. 3 binary morphologic criteria were established: matrigel-remnants visible (y/n), tumour cells in CAM stroma (y/n) and tumour cells in adipose tissue (y/n). Number of tumour clusters in the CAM stroma was assessed.

Results

Matrigel remnants were most frequently present in the hMADs goup (5/6) followed by the controls (3/6) and lipoaspirate group (2/6). In all but one case tumour cells could be found in the CAM stroma. In the lipoaspirate group tumour cells invaded the adipose tissue in 4 of 6 cases. An average of 3.3 tumour clusters were present in the CAM stroma of the lipoaspirate group, while in controls and hMADs groups 4.3 and 6.2 cell clusters could be found, respectively. Concerning tumour cell entity, MCF7 and BT474 formed a similar amount of clusters (mean 6.7 and 6.5 respectively), while 10x fewer clusters were formed by MDA-MB-231 cells (0.7 on average).

Conclusion

According to these preliminary results, hMADS seem to have an effect on the growth pattern of breast cancer cell lines. This is reflected by the observation that matrigel remnants were found more often in breast cancer cell lines added with hMADs and by the higher number of tumour cell clusters in that very group. We aim to present our full data at the EUSAAT meeting.

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Assessing laboratory mouse welfare using animal-based measures

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Protocols to assess laboratory animal health and welfare are commonly used in experimental studies to record the effect of an experimental treatment, yet many of the welfare problems occur in the home cage and are irrespective of the type of research. Appropriate resources and management, such as suitable bedding, nesting material and husbandry routines reduce the risk of poor animal welfare. However, since animals react differently to the same environment depending on age, sex, genetic background, previous experiences etc., it is more relevant to assess welfare using animal-based measures. The same animal-based measures can then be applied in different types of animal facilities, allowing comparisons between them, or to track changes over time following modification in housing and management within the same facility. The aim of this project was to design and test a protocol for assessing the welfare of laboratory mice in their home cage using only animal-based measures [1]. The protocol, to be used as a benchmarking tool, assesses mouse welfare in the home cage and does not contain parameters related to experimental situations. The parameters used were selected to cover different aspects of animal welfare, namely feeding, housing, health and behaviour according to the 12 welfare criteria established by the Welfare Qualityâ project [2]. The selection of animal-based parameters was performed by scanning existing published, web-based and informal protocols, and by choosing parameters that matched these criteria, were feasible in practice and, if possible, were already validated indicators of mouse welfare. The parameters had to identify possible animal welfare problems and enable assessment directly in an animal room during cage cleaning procedures, without the need for extra equipment. The protocol was tested by four different assessors at three different animal facilities in Sweden during 2012, to evaluate inter-observer reliability, the feasibility of the parameters and the scales used to assess them. The results showed overall good inter-observer reliability, but the assessment of nest building performance showed only fair reliability. However, this could be improved by modification from a 5-point to a 3-point scoring scale. The assessment also identified several differences between facilities tested, such as prevalence of whisker and fur trimming, urination/defecation during handling and the status of the coat. The results confirm the sensitivity and thereby the potential usefulness of the protocol. Although it should be considered a living document that will be modified over time, as new validated and feasible parameters are identified.

Refinement actions to improve mouse welfare could be based on the results from such a welfare assessment scheme. Regular use of the protocol as part of a benchmarking programme would also contribute to improved knowledge on the effects of housing and husbandry on laboratory mouse welfare.

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Introducing the Round Table Discussion on "Implementing the concept of 'Integrated Approaches to Testing and Assessment IATA' into international regulatory testing"

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In 2016 the OECD has published a "Proposal for the governance of integrated approaches to testing and assessment of chemicals (IATA) activities at OECD", which takes into account the OECD DRAFT Guidance documents on integrated approaches on testing and assessment (IATA) for skin corrosion and irritation (OECD RG 404) and for eye irritation and corrosion (OECD TG 405). The two IATAs should replace the "testing and evaluation strategies", which are currently provided in the supplements to TG 404 and TG 405, which require adaptation to technical progress [1].

The new document furthermore provides consistent information on key performance characteristics of each of the individual information sources comprising the IATA and provides guidance on how to integrate information for decision making within the approach (including decisions on the need for further testing) and when integrating all existing and generated information on the corrosive and irritant hazard potential of chemicals for final decisions for classification and labelling.

Generally, one single non-animal method cannot cover the complexity of any given animal test. Therefore, fixed sets of *in vitro* (and *in chemico*) methods have been combined into testing strategies for skin and eye irritation and skin sensitisation testing with pre-defined prediction models for substance classification. Many of these methods have been adopted as OECD test guidelines. Various testing strategies have been successfully validated in extensive in-house and inter-laboratory studies, but they have not yet received formal acceptance for substance classification. Therefore, under the European REACH Regulation, data from testing strategies may generally only be used in so-called weight-of-evidence approaches. While the authorities may generally not question the sufficiency of animal testing data for registration, they may question the sufficiency of weight-of-evidence approaches and request further testing, which is most likely animal testing. This constitutes an imbalance between data from non-animal methods accepted for regulatory purposes and animal tests that is not to be justified on scientific grounds. To ensure that testing strategies for local tolerance testing truly serve to replace animal testing for the REACH 2018 deadline, when the majority of existing chemicals have to be registered, clarity on their regulatory acceptance as complete replacements is urgently required.

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25th Anniversary of the EUSAAT (European Society for Alternatives to Animal Testing) Congresses in Linz/Austria

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Upon initiative of local animal welfare activists, in 1991 the first congress on Alternatives to Animal Experiments was held in German at the Johannes Kepler University (JKU) in Linz (Austria) in cooperation with Austrian, German and Swiss government institutions, industry, and animal welfare NGOs. To ensure continuation of the annual 3Rs congresses Linz, in 1993 the Middle European Society for Alternatives to Animal Experiments (MEGAT = *Mitteleuropäische Gesellschaft für Alternativmethoden zu Tierversuchen*) was founded and ALTEX served as official journal of the society. The 3Rs congresses in Linz became quite popular and colleagues from other European countries joined, e.g. from the Czech Republic, Italy, Netherlands, Slovakia and Sweden. As a consequence, since 2006 the congresses have been held in English and in 2009 the name of the society was changed to EUSAAT, European Society for Alternatives to Animal Testing – the European 3Rs Society (www.eusaat.org).

As described above, during the past 25 years the MEGAT/EUSAAT congresses in Linz have been jointly organised by the four major stakeholders in the field of the 3Rs: academia – industry – government & animal welfare. During the Austrian Presidency of the EU Council, in 2006 the EUSAAT 3Rs congress in Linz was held as an official European event. During the last 25 years, the main topics of the EUSAAT conferences included ethical and legal issues of animal experimentation, *in vitro* pharmacology and (eco)toxicology, immunology, education and molecular modelling of diseases including transgenic models.

EUSAAT has always maintained close cooperation with the European Commission and its services. EUSAAT has particularly focused its activities on the challenges of EU Dir. 2010/63 "on the protection of animals used for scientific purposes", e.g. by serving as scientific platform via EUSAAT congresses and the ALTEX journal in order to meet the ban of animal experiments in the EU Cosmetics Directive in 2013 and for implementing the 3Rs principle into the EU REACH and CLP Directives for safety testing of chemicals. In 2014, EUSAAT was accepted to the ECVAM Stakeholder Forum ESTAF. EUSAAT members have served internationally as co-chairs of world congresses on alternatives, e.g. both co-chairs of WC9, which was held in 2014 in Prague, Dagmar Jirova and Horst Spielmann, are EUSAAT members.

On the occasion of the 25th anniversary the EUSAAT Board is pleased that due to the continuous support by members, stakeholders and sponsors EUSAAT has been able to establish official cooperation with 3Rs societies outside Europe, e.g. ASCCT in the USA and JSAAE in Japan, and that in 2015 and in 2016 the EUSAAT congresses are the major international 3Rs congresses.

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The CRISPR/Cas9 system: a game changer in the life sciences

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The CRISPR/Cas9 system is a powerful tool for genome editing. CRISPR/Cas9 can be used in embryonic stem cells (ESCs) or directly in fertilized oocytes for the generation of transgenic mice. The advantages of producing mutations in ESCs are easy selection of the desired mutations and the direct use of these cells in culture to investigate the effects of induced mutations. CRISPR/Cas9 mutagenesis is highly site specific and will replaced conventional shotgun mutagenesis approaches. The CRISPR/Cas9 technology can also be used to generate large structural variations. We applied the CRISPR technology in mouse ESCs and developed a 10-week protocol to efficiently produce deletions, inversions, and duplications in mice. We were able to rearrange targeted genomic intervals ranging from 1 kb to 1.6 Mb using the CRISPR/Cas system in mouse ESCs. Cells harboring these mutations can be used to generate highly chimeric animals by tetraploid ESCs aggregation. This allows the analysis of F0 animals and reduces "waste animals". The speed and efficiency of CRISPR/Cas9 in introducing mutations can be considered a game changer in genetics. Variants or unknown clinical significance can now be tested quickly *in vitro* or *in vivo*. This is of particular importance for extremely rare diseases/variants for which additional patients are difficult to find.

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Replacing animal testing by genotoxicity and carcinogenic cell transformation assessment using a tissue regenerating cell line from fish with GFP-labelled histones

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Regeneration capacities of teleost fish are thought to be based on highly motile, livelong persistent pluripotent stem cells [1]. A cell "community", which proliferates pluripotent cells (KCB-GFP, DSM ACC3285) has been isolated from the carp brain. These cells show a gene expression pattern very similar to embryonic stem cells of vertebrates [2] and can be integrated into different tissues after transplantation. A variant derived from this "cell line" transgenic for H2B-GFP was established. The stable fluorescence signal related to the nuclear structures of these cells reflects impacts on the integrity of the genome in a self-signaling manner. These cells are perfectly suited for detecting cytogenetic abnormalities, particularly those related with genotoxic noxa. Micronuclei (MNi) can be observed and quantified without fixation and nuclear staining. A dose-dependent induction of MNi has been observed for both clastogens and aneugens. Cytotoxic effects can be clearly distinguished from genotoxicity on the basis of apoptotic / necrotic nuclear patterns such as pyknotic or fragmented nuclei (FN). The corresponding frequencies can be used as additional endpoints for ensuring sublethal exposure concentrations. KCB cells are still susceptible to carcinogenic transformation since they are not immortalized or tumor derived, as in the case of most mammalian cells employed for *in vitro* toxicology. Changes in the nuclear fluorescence pattern can be monitored in post-exposure follow up-, or continuous exposure cultures. Preliminary results indicate that transformation to cancer cells – in terms of enhanced proliferation rates and/or alterations of nuclear patterns – can be monitored in the same experiment as MN evaluation. The approach enables the application of cytological- and molecular markers from cancer research, tumor diagnostic and stem cell biology in *in vitro* toxicology and vice versa. The synopsis of data from such a broad spectrum of endpoints enhances the forecasting power of *in vitro* toxicology, so that animal testing can be avoided in many (cancer) risk assessment scenarios.

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From the guideline to the bench – The role of science based policy advice in implementing EU Dir 63/2010 in Germany

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Political decisions and legislation are results of long and complicated negotiation processes in which a number of parties are involved. As protection of animals is highly relevant for scientific research the German National Academy of Sciences Leopoldina accompanied the whole process from the generation of EU Directive 63/2010 at EU level until the implementation into German legislation.

As one of the oldest Science Academies in the world Leopoldina is a society of scholars that is dedicated to the advancement of science. It speaks out on social and political questions, providing a nonpartisan, factual framework for discussion. Interdisciplinary groups of experts publish policy-guiding statements on issues of current interest, such as animal protection. The Leopoldina does never act as research institution, nor does it have any function of a funding agency. During the whole process of policy advice the Academy therefore does not act as a stakeholder but rather as a broker of scientific evidence. Although representing also scientists from institutions that are in fact stakeholders, the Leopoldina does balance its decisions to ensure nonpartisan statements and cooperates also with other Academies of Sciences. In regards to the discussion on the protection of animals used for scientific purposes the discussions and activities of the Academy resulted in two statements: i) one in 2010 [1] on the discussion of EU Directive 63/2010 and ii) one in 2012 [2] on the legislation process in Germany. Even after the legislation process is finished in Germany the Academy tries incentivizing open discussion processes of stakeholders and decision makers. Here the Leopoldina cooperates with other independent institutions to provide a platform for open dialogue of all sides, including scientists, local authorities, federal agencies, NGOs and policy makers. The aim of this dialogue is the identification of current problems and a joint discussion of possible solutions.

The talk will give an overview on the role of the German National Academy of Sciences Leopoldina in the legislation process regarding EU Dir 63/2010 and beyond. It will give an insight into internal processes of policy advice and current development of the topic in academic context.

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Development and establishment of an interdisciplinary international master program for laboratory animal science (MLAS): a contribution to the 3R principles

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With the implementation of the EU Directive 2010/63 on the protection of laboratory animals the principles of the 3Rs proposed by Russel and Burch (Refinement, Reduction, Replacement) are for the first time incorporated into the animal welfare law and a declared goal. Therefore, the impartment of knowledge on 3Rs and alternatives to animal experiments has to be part of the qualification of personnel planning, performing and evaluating animal experiments. The implementation of the directive leads to an increased demand for education and training opportunities in laboratory animal science. The most common teaching format in animal sciences is a FELASA B course. This format mediates the basics but lacks relevant content for persons responsible for planning, evaluating and directing animal experiments. These subjects are part of FELASA C/D or comparable courses, which are only offered rarely or simply do not exist. Therefore, the RWTH Aachen started in 2015 an executive master program in Laboratory Animal Science (MLAS; http://msc-lab-animal.com/). This program is unique in Germany and qualifies students as "Laboratory Animal Specialist" (FELASA D). The MLAS is designed as an international, English-language part-time course. The blended learning concept incorporates e-learning complemented with attendance blocks for practical skills training. For the mediation of learning content in laboratory animal science a BMBF-funded media-supported education concept (emsl VTK) was established. This concept contains modular designed visualizations of knowledge integrated in the MLAS curriculum and are made available on the online learning platform emedia vtk. The curriculum contains modules addressing ethics and legislation in relation to the use of laboratory animals; biometry, statistics, experimental design and facility management; alternatives to animal experiments and laboratory animal science (incl. genetics, breeding, anatomy, physiology, pathology, hematology). For the deeper knowledge courses in animal models, anesthesia and experimental surgery as well as *in vivo* pharmacology, applied toxicology, microsurgery and imaging are offered as compulsory and elective modules. In all the modules 3R principles are addressed and applied.

Within the attendance block practical skills are taught by using toys, videos and training models like the silicon ear in order to shorten the learning curve when finally trained on animals. In addition, *in vitro* and *ex vivo* techniques are taught during practical skill courses. Altogether, this should facilitate the qualification of persons responsible for directing animal experiments and should train the persons to use and advice other researchers in using alternative strategies. An important part of the MLAS program is the developed e-learning modules provided by the emedia vtk platform. The cooperation with the established online platform LAS-interactive (http://www.las-interactive.de) ensures the sustained use and dissemination of the content.

The goal of the MLAS program is to qualify personnel planning, performing and evaluating animal experiments and to ensure an effective distribution and a better knowledge of alternatives to animal experiments. Overall, it is expected, that the master program will significantly contribute to the 3Rs, incorporated in the animal welfare law, and that profound knowledge of 3Rs and alternatives to animal experiments will lead directly and sustainably to reduction and replacement of animal experiments.

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PRIMTRAIN: A COST Action for training and animal behavioral management in non-human primates and other large laboratory animals

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Positive Reinforcement Training and Animal Behaviour Management are essential when working with large animals in biomedical research (in particular with non-human primates). The COST Action "PRIMTRAIN" provides a network for personnel working with non-human primates and other large laboratory animals to facilitate the competence and skills needed to successfully apply Positive Reinforcement Training and Animal Behaviour Management.

Positive Reinforcement Training (PRT) and Animal Behavioural Management (ABM) of non-human primates (NHP) and other large animals used in biomedical experimentation reduce the stress level for the animals, promote more reliable experimental results, facilitate the refinement of methods and procedures and lead to increased safety, both for animals and personnel. However, implementing high quality PRT and ABM, which have substantial and long-term benefits for animal welfare and research, poses a challenge as it requires excellently trained staff. Since research using large animals (including NHPs) is a small field, opportunities for training on PRT and ABM are rare. Therefore it is important to create opportunities for laboratory staff from different facilities to network and exchange knowledge. This talk will outline the planned activities of the COST Action PRIMTRAIN, recently funded by the EU. The network will facilitate regular meetings and allow relevant personnel to get educated about ABM and PRT and to exchange the experience, knowledge and ideas necessary to implement the best possible practice in PRT and ABM in their own facility. The central aim of PRIMTRAIN is to create a network in which knowledge and experience are openly shared and state-of-the-art education and training is promoted. PRIMTRAIN already consists of animal care takers, animal trainers, ABM specialists, ethologists, veterinarians, neuroscientists, and other biomedical researchers using NHP and large laboratory animals from 14 different European countries. The network is still open for interested parties. The Action will offer Workshops, Training Schools, and short-term scientific missions for animal laboratory staff. Moreover a catalogue of relevant literature will be compiled and a recommendation of a minimum European standard for all primate and large animal laboratories with regard to animal training will be developed.

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Supporting evidence in Read-Across approaches: potential and limitations of new approaches and methodologies

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Grouping of substances and read-across is one of the most commonly used alternative approaches for filling data gaps in registrations submitted under REACH. This approach uses relevant information from analogous substances to predict the properties of structurally similar substances.

Registrants for REACH need to make sure that their approach falls within the conditions for using grouping and read-across approaches set out in Annex XI, section 1.5 of the REACH Regulation. As part of its dossier evaluation duties, ECHA has to evaluate whether the provisions in this Annex are met for the adaptation under consideration. Whilst registrants are faced with the challenge of developing and supporting their read-across adaptation, ECHA is faced with the regulatory challenge of scientifically assessing such adaptations. In response to this challenge, ECHA has developed and published in May 2015 the ECHA Read-Across Assessment Framework (RAAF). The RAAF allows for a consistent and structured assessment of the strengths and weaknesses of a read-across approach and identifies possible shortcomings in the documentation, scientific reasoning and/or supporting evidence.

In the context of a grouping and read-across under REACH, supporting evidence substantiates scientific claims or hypotheses made by registrants to establish the basis for predicting properties of a substance from data on another substance. All types of supporting evidence provided are considered when conducting an assessment according to the RAAF. Supporting evidence may range from theoretical considerations or expert systems, to results from *in vivo* or *in vitro* studies. Information on tests constitutes invaluable evidence to support claims of similar toxicological properties among the substances involved in a read-across approach. Information on toxicokinetic properties of substances and mechanistic information (toxicodynamics) also particularly contribute to increasing the robustness of read-across hypothesis.

A considerable effort in developing new approaches and methodologies for investigating properties of substances has been made over the past years. Significant results have been obtained in the development of *in vitro* test batteries as our understanding of adverse outcome pathways expanded. The emergence of high throughput screening methods and omics data opened avenues in gathering a vast amount of information on the interaction between substances and the organism. The potential of these techniques as supporting evidence in read-across approaches has been a main theme of a recent ECHA workshop on New Approach Methodologies in Regulatory Science (http://echa.europa.eu/view-article/-/journal_content/title/topical-scientific-workshop-new-approach-methodologies-in-regulatory-science). Nevertheless, the associated limitations and challenges relating to performance standards of these methods (to demonstrate robustness and reproducibility) and reporting standards are emerging.

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Automation-compatible EST assay

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

EBs were formed for 5 days in in a 96-well hanging drop plate to achieve EB formation and induce cardiomyocyte differentiation. At day 5 EBs were directly transferred into an adhesive 96-well plate by placing the HD-plate on top of a receiver. Adhered EB's were monitored for cardiomyocyte differentiation at day 10. ESCs aggregated in the hanging drop and formed round shaped EBs of uniform size within 5 days. Size analysis of EBs resulted in diameters of 319 µm ±3.0% at day 3 and 466 µm ±5.2% at day 5, respectively with a contraction efficiency of 88% ±13% at day 10. Compounds selected from the ECVAM test-validation panel resulted in similar classifications as with the original EST protocol.

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

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3Rs in surgical skill trainings

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To develop the medical students' and residents' surgical skill is a crucial point in their education. To get a stable knowledge can be an important basic help in their later clinical practices.

Our Department has a Semester course titled Basic Surgical Techniques for 3rd year medical students. It involves the rules of asepsis, the behavior in the operating room, the knotting and sewing techniques and the basis of laparoscopic techniques. The program would not be complete without usage of a living organism. In planning the program we took into consideration the 3Rs.

For residents, who will study a surgical specialty e.g. surgery, gynecology, traumatology etc., have a 4-week-long course to acquire some basic and advanced surgical techniques. For repeating and acquiring new wound closing techniques we use *ex vivo* tissue (pigs' skin, vessels, bowels, stomach for "macro" surgery, silicone tubes, chickens' legs for microsurgery, pelvitrainers and tissues for laparoscopy). The residents practice in these models before they work on an animal. For developing their surgical skill we use rats for microsurgery. Vessel and nerve anastomosis is performed in living animals. For surgical operation pigs are used.

Reduction: Rats as re-used animals should not be used in experiments any more. (The severity of the earlier experiments on these rats is mild. They are mainly used in behavioral pharmacological research.) Medical students operate rats only in one practice from seven practices. Two or three student use one animal and more procedures are performed in one animal under the same anaesthesia. In resident courses rats are used only in the last day of the microsurgery week and all these animals are re-used (see above). Two residents use one animal and more vessel and nerve anastomosis is performed in one animal under the same anaesthesia. For "normal" surgery pigs are used. 5 students work on an animal and they do as many operations as they can in one pig.

Refinement: Professional assistants and veterinarian take care of the animals and they are responsible for the anaesthesia. The students are allowed to work on the animals in the last practice when they have enough experience in the surgical techniques. The residents are allowed to work on the animals after practicing in *ex vivo* models.

Replacement: This is the most effective part of 3Rs in our education. Students learn knots in simple thin ropes, using skin skill model, *ex vivo* animal tissue for sewing and special boxes, so called pelvitrainers, for laparoscopy. Most of the cases e.g. *ex vivo* tissues or skill models can replace the animals. For learning handling laparoscopic instruments, working in special situations and tying knots intracorporally pelvitrainers are used.

Apart from earlier routine the practices of surgical techniques underwent major changes. The number of used animals is radically reduced but surgery cannot be taught without living tissue and "blood". A well-planned animal consumption can make balance between the aims of skill training and complying with the 3Rs.

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Applying the harm benefit assessment under the EU Directive – what does it mean in practice for animals?

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The utilitarian approach of weighing the harms done to the animals against the benefits to humans of animal experiments now lies formally at the heart of Directive 2010/63. A positive harm benefit assessment (HBA) is now a prerequisite for approval of a project involving harmful use of animals. For some countries in Europe the HBA is a new process, for others it has been a requirement for many years. However, even in those countries the HBA appears to be being done on a very rudimentary basis, by those directly involved in animal research and can be dominated by consideration of the 3Rs which is not the same as ethical evaluation.

In the presentation I review the conduct of the HBA across Europe and provide evidence of, and solutions for, a number of practical and theoretical issues with its current performance. Some of these issues are surmountable and could be overcome by greater transparency, the production of clearer criteria, and insistence on rigorous and more impartial, wider evaluation involving the views of general society. However, others are inherent and unavoidable. For example, it is unlikely that the process can ever be entirely unbiased, because it is dominated by the opinions of those set to benefit and not by those set to be harmed. There are also fundamental problems with the inability to assess harms and benefits at the same point in time and failure to consider the likelihood that any single animal experiment is likely to translate to a human medical benefit.

With the review of the Directive, however, we can start to address some of the improvements with the HBA process that can be made to ensure that there is better balance with respect to public opinion and animal welfare.

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Application of serum-free cultured human TK6 cells in the *in vitro* micronucleus test

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The effort to develop new or refine established *in vitro* test systems rises due to animal welfare, scientific and/or regulatory reasons (e.g. the animal testing ban concerning the risk assessment of cosmetic product ingredients in March 2013). This progress, among others, leads to an increased performance of cell-based assays. The majority of model cell lines are routinely cultured using medium supplemented with fetal bovine serum (FBS) in amounts between 5-10%. The application of serum-substitutes will provide a reduction of the animal number needed, which corresponds to the guiding principles of the three R's (3R), described by Russel and Burch in 1959. In addition, chemically defined serum-substitutes have the potential to reduce the inter-experimental variability of test conditions caused by the inherent differences in chemical composition across FBS batches [1], resulting in a refinement of *in vitro* testing.

In this study, human TK6 cells were gradually adapted to serum-free conditions, where they show comparable growth gradients at the exponential phase. For cells under serum-free conditions a mean doubling time of 19.3 (± 1.7) h was observed while FBS supplemented cells showed a doubling time of 13.5 (± 0.8) h. The *in vitro* micronucleus test protocol included a 4 h treatment with 0.63, 1.25, 2.50, 5.00, 10.00 and 20.00 µg/mL methyl methanesulfonate, followed by a 20 h recovery period. Both conditions showed a significant increase of the micronucleus frequency. Considering the prolonged doubling time of the serum-free cultures, an extension of the recovery time from 20 h to 26 h would provide a preferable adjustment of the method.

The results indicate that serum-free cultured cells can be used in the *in vitro* micronucleus test. However, further testing of reference substances have to be done to confirm the suitability of this application.

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Advanced training course for Veterinarians to qualify for Animal Welfare Officer

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Going beyond the Directive 2010/63/EU on the protection of animals used for scientific purposes, the latest amendment of the German Animal Welfare Act rules that facilities and establishments where vertebrates or cephalopods intended for use in animal experimentation or whose organs or tissue are intended to be used for scientific purposes are kept or used shall have animal welfare officers [1]. The animal welfare officer may not also be the person responsible for the breeding or holding of the animals pursuant to § 11 section 1 paragraph 1 number 1 [2]. Only persons who have graduated with a university degree in veterinary medicine may be appointed as animal welfare officers [2]. They shall possess the knowledge and abilities required and the necessary trustworthiness for the performance of their duties [2]. Therefore, together with the Academy for Veterinary Continuing Education of Federal Chamber of Veterinary Surgeons an advanced training course for veterinarians to qualify animal welfare officer was established in 2015. The first part of the course covers the following session's animal law, statistics and ethics, further more ethology, management and applications as well as laboratory animal sciences and transgenic animals. The second part includes anesthesia, analgesia, pathology and hygienic aspects as well as communication skills, and the function of the national committee, animal models and alternative methods. Experienced experts in the field, the competent authority and members of the national committee gave lectures, which were evaluated as very good by the participants. With this advanced training course, we guarantee the provisions, conditions, and requirements concerning knowledge and qualification of animal welfare officers to ensure highest standards in animal welfare and consulting of scientists within the institutions.

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Hepatocyte-like cells derived from human adipose stem cells: a new model for genotoxicity testing

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EU and international legislation aimed at the protection of human health requires genotoxicity assessment for nearly all types of substances on the market. Genotoxicity assessment follows a step-wise approach, beginning with a basic battery of *in vitro* tests and if positive results are obtained followed by *in vivo* testing. However, it has been estimated that, at present, around 80% of the *in vivo* genotoxicity tests in the EU are performed due to false *in vitro* positives [1]. An important reason for the false results are the mammalian cell lines used in current *in vitro* tests and their inadequate expression of metabolic enzymes that in the organism catalyze the activation and detoxification of genotoxins. In that respect, new cell models with metabolic capacities similar to primary hepatocytes are being developed. A promising strategy is generation of hepatocyte-like cells (Hep-LCs) trough differentiation of mesenchymal stem cells, which have the potential to differentiate into various types of tissue cells, including liver cells [2]. The aim of our study was to develop new Hep-LCs from adipose stem cells (ASCs). To gain optimal conditions for their cultivation and effective differentiation to hepatic cells, we examined the influence of starvation, serum supplement and different coatings of culture plates (matrigel, collagen I and laminin) and evaluated several three-step (endoderm induction, hepatic induction and hepatic maturation) protocols in which concentrations of key hepatogenic growth factors (Activin, FGFs, EGF, BMPs, HGF, OncostatinM and Dexamethasone), their combinations and order of addition were varied. The characteristics and specific hepatic functionality of differentiated cells were confirmed through morphology, immunocytochemistry and periodic acid-Schiff staining along with the analysis of mRNA expression of distinct hepatocyte markers, such as *ALB* and *GATA4*. The metabolic activity of differentiated cells was assessed by determining DNA damage induced by a model genotoxic compound benzo(a)pyrene (BaP) that needs metabolic activation. Successful differentiation of ASCs to Hep-LCs was obtained by culturing the cells on collagen I coated plates without serum with all tested growth factor combinations sequentially added at appropriate concentrations. The generated cells displayed typical features of hepatocytes, including polygonal morphology, expression of *ALB* and *GATA4*, and ability to store glycogen and secrete albumin. The comet assay revealed that exposure of the generated Hep-LCs to BaP (30 µM; 24 h) induced significant increase in the amount of DNA strand breaks, confirming their metabolic activity. On the contrary, exposure of ASCs to B(a)P did not induce DNA damage. These data point out that the differentiated Hep-LCs have the potential to be used as a new model for the prediction of genotoxicity of chemicals *in vitro*; however, further validation of these cells is needed. They will be evaluated in detail for their metabolic capacity with various classes of genotoxic carcinogens, which are activated through different metabolic enzymes and characterized regarding their karyotype stability, p53 response pathways and DNA repair mechanisms, which are all very important in obtaining the reliable results in genotoxicity testing.

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Characterization of stably transfected HEK-293 cells expressing uptake transporters using fluorescent 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), Na^+-taurocholate co-transporting polypeptide (NTCP, gene family SLC10A1) and Organic cation transporters (OCT, gene family SLC22) belong to the uptake transporters and mediate the uptake of a broad range of substrates including several widely prescribed drugs into 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 in assays that can be performed in standard laboratories.

Transporter activities of OATP1A2, -1B1, -1B3, -2B1 and NTCP as well as OCT1, -2, -3 were analyzed with five fluorescent substances (Fluorescein methotrexate (FMTX), Fluorescein, Rhodamine 123, Dibromofluorescein (DBF), Cholyl-Lysyl-Fluorescein (CLF)).

FMTX was specifically transported by OATP1B1 and -1B3. Fluorescein is a substrate only for OATP1B1, while Rhodamine 123 is one for HEK-OATP1A2 and OCT1, -2, -3. CLF was characterized as substrate for a wide range of transporters: NTCP, OATP1B1 and -1B3. The compound DBF is transported by HEK-OATP2B1. Rifampicin functions as inhibitor for all investigated OATPs. The transport activity of all 3 OCTs could be inhibited by Quinidine. Cholate efficiently inhibited the uptake of CLF mediated by NTCP.

In summary, the transport function of stably transfected HEK-293 cells expressing the above mentioned transporters could be analysed with fluorescent substances. This platform can be used for identification of specific transporters involved in drug uptake and also for the evaluation of drug efficiency to inhibit transporter activity.

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Non-animal testing for skin sensitization: Replacement or mere supplement?

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Under REACH, information on skin sensitization potential is requested for all substances and regularly assessed using the murine local lymph node assay (LLNA). To replace animal testing, a number of non-animal methods have been developed [1] and recently two of these methods (DPRA and ARE-Nrf2 Luciferase Test Method) were adopted as OECD test guidelines, while endorsement of a third method (h-CLAT) is currently pending.

To cover the complexity of skin sensitization, single non-animal methods are combined into integrated testing strategies (ITS). In contrast to the single methods, ITS are not formally validated, which leaves uncertainty about their adequacy, completeness and overall evaluation. Hence, animal studies will still have to be performed and full replacement will only be achieved if (i) all necessary single methods and (ii) ITS are validated and accepted.

An ITS combining three non-animal methods is the "2 out of 3" weight of evidence approach, which provided a good predictivity of the skin sensitization potential in humans exceeding that of the LLNA [2]. For this approach, applicability domains, uncertainties [3,4] and feasibility to detect pre- and pro-haptens [5,6] were analyzed and a modified approach using kinetic measurements of peptide reactivity was shown to predict GHS potency classes [7].

Hence, full replacement of animal tests to assess skin sensitization potential is attainable. The scientific validity of the single methods and their combination in an ITS has been demonstrated. Alas, the regulatory acceptance is lacking behind and remains incomplete in stopping at single method validation and neglecting validation and formal approval of integrated approaches.

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Preclinical evaluation of CD30 as a novel molecular target in systemic mastocytosis

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Systemic mastocytosis (SM) is a hematologic neoplasm characterized by increased expansion and pathologic accumulation of neoplastic mast cells (MC) in various organs. In most patients, neoplastic cells exhibit transforming KIT mutations. While several KIT-targeting drugs have been developed and found to induce clinical responses in SM patients, responses are often short lived. The Ki-1 antigen (CD30) is an established therapeutic target in Hodgkin´s lymphoma and anaplastic large cell lymphoma. Recent data suggest that neoplastic MC in human patients with aggressive SM (ASM) and mast cell leukemia (MCL) aberrantly express CD30. We therefore hypothesized that the CD30 targeting drug brentuximab vedotin might represent a promising treatment option for advanced SM patients. We examined the expression of CD30 in various human and canine MC lines (HMC-1, MCPV, NI-1, C2) and in primary neoplastic MC obtained from SM patients. As assessed by flow cytometry, neoplastic MC expressed cell surface CD30 in most patients, namely in 10/11 with ISM and 4/7 with ASM/MCL. The immature, RAS-transformed MCL line MCPV and the canine MC line NI-1 expressed substantial amounts of cell surface CD30, whereas HMC-1 and C2 cells expressed only little or no CD30. We next applied brentuximab vedotin (Millennium: Takeda Oncology Company, Cambridge, MA, USA), an antibody-drug conjugate consisting of a chimeric anti-CD30 antibody conjugated to the microtubule inhibitor monomethyl auristatin E. Brentuximab vedotin has recently been described to induce dose-dependent growth arrest in CD30+ lymphoblastic cell lines. In the present study, brentuximab vedotin was found to inhibit the proliferation of MCPV, HMC-1, NI-1, and C2 cells in a dose-dependent manner, with lower IC50 values found in CD30+ MCPV, HMC-1.1, and C2 cells (5-10 µg/ml) than in CD30− HMC-1.2 and NI-1 cells (10-30 µg/ml). As assessed by AnnexinV/PI staining and staining for active-caspase-3, brentuximab vedotin also induced dose-dependent apoptosis in MCPV, HMC-1.1, NI-1, and C2 cells. Furthermore, brentuximab vedotin induced dose-dependent apoptosis in primary neoplastic MC in patients with CD30+ SM, but did not induce apoptosis in MC in patients with CD30− SM. Next, we examined the effects of a drug combination consisting of brentuximab vedotin and the KIT D816V-targeting drug midostaurin. We found that both drugs synergize with each other in inhibiting the *in vitro* proliferation of MCPV and NI-1 cells. To confirm our *in vitro* data we conducted *in vivo* experiments using NOD-SCID-IL-2Rγ-/- (NSG) mice. In these experiments, pre-incubation with brentuximab vedotin was found to inhibit engraftment of MCPV cells in NSG mice. In conclusion, our data generated in an interdisciplinary comparative oncology setting provide evidence that CD30 is expressed on the surface of neoplastic MC in most patients with advanced SM as well as in canine mastocytoma cells. Our data also show that the CD30-targeting antibody-drug conjugate brentuximab vedotin induces growth arrest and apoptosis in neoplastic human and canine MC and synergizes with midostaurin in inhibiting the growth of CD30+ neoplastic MC. First clinical data from a small case series support our preclinical data, yet the clinical impact of this targeted drug in patients with advanced SM remains to be determined.

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Serum-free media and serum alternatives

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The use of Foetal Calf Serum (FCS) in the biosciences has been regarded critically for decades. From a scientific point of view, the use of undefined media supplements such as FCS is problematic for a range of applications, e.g. when ingredients mask the toxic effect of substances which bind to them. From an ethical perspective, the production of FCS is connected to various serious animal welfare problems because it involves the heart puncture of live foetuses. Nevertheless, to date FCS is used at large scale, particularly for cell cultures.

This presentation reports on the outcome of a 3rd workshop on this topic, held in June 2016, which brought together key players in the field, to connect to previous activities and investigate solutions for the future.

The 1st workshop, held in Utrecht, The Netherlands, in 2003 [1] was initiated after publication of a comprehensive report on fetal pain and distress during blood harvesting for FBS production [2] to create awareness and to discuss possibilities to reduce or replace the use of FBS in cell culture media. A follow-up workshop was organized in Copenhagen, Denmark, in 2009 to discuss current *in vitro* methods devoid of FBS or other animal components [3].

Recent years showed tremendous efforts in the establishment of human platelet lysates as one of the most valuable alternatives to FBS as cell culture supplement. This promising development, together with successful serum-free applications in microphysiological systems and organ-on-chips technologies, prompted us to organize a 3rd workshop on FBS, serum alternatives and serum-free media. In light of these developments, three main topics were identified to discuss at this workshop: (1) the serum controversy, (2) alternatives to FBS, with special emphasis on human platelet lysates, databases on serum-free media, commercialization of chemically defined media, and (3) serum-free *in vitro* applications.

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Education and Training Platform for Laboratory Animal Science (ETPLAS)

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It is mandatory that staff carrying out procedures, designing procedures and projects, taking care of animals or killing animals should have received instruction in a scientific discipline relevant to the work being undertaken and have species-specific knowledge. Education and training (E&T) are important contributors to the 3Rs and are aimed at increasing the quality of both animal welfare and science. With this in mind, the EU Commission proposed that a portal (ETPLAS) should be developed for exchanging information on laboratory animal science E&T for all stakeholder groups. In this context, the initial focus of ETPLAS is to build a database of training providers, with a point of contact for trainees across the EU, who can offer courses meeting the requirements of Directive 2010/63/EU.

ETPLAS has been established to enable information sharing and communication between EU Member States' authorities, accrediting/approval bodies, training providers and trainees involved with all aspects of E&T in laboratory animal science (LAS). The Steering committee of ETPLAS is composed of representatives from all these groups and has developed a database of Contacts to aid communication. A Reference Group of contacts provides additional review and input to the work of the Steering Committee. ETPLAS has Observer status at the meeting of National Contact Points of the EU competent authorities where its work is shared and discussed.

The aims of ETPLAS are to provide:

- a forum for exchanging information on LAS education and training for all stakeholders
- training providers with the necessary information to establish additional education and training courses
- the user community with information on available education and training courses including continuing education (CPD)
- authorities/employers with the necessary information to facilitate the process of mutual recognition of education & training to promote free movement of personnel involved in LAS

ETPLAS has developed a website http://www.etplas.eu which is intended to be a one-stop shop for all involved in LAS.

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The EU-ToxRisk project: A European flagship program for mechanism-based safety sciences and risk assessment

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On January 1, 2016 the EU-ToxRisk project started with support form the European Commission Horizon2020 program. The vision of EU-ToxRisk is to drive a paradigm shift in toxicology towards an animal-free, mechanism-based integrated approach to chemical safety assessment. The project will unite all relevant disciplines and stakeholders to establish: i) pragmatic, solid read-across procedures incorporating mechanistic and toxicokinetic knowledge; and ii) ab initio hazard and risk assessment strategies of chemicals with little background information. The project will focus on repeated dose systemic toxicity (liver, kidney, lung and nervous system) as well as developmental/reproduction toxicity. Different human tiered test systems are integrated to balance speed, cost and biological complexity. EU-ToxRisk extensively integrates the adverse outcome pathway (AOP)-based toxicity testing concept. Therefore, advanced technologies, including high throughput transcriptomics, RNA interference, and high throughput microscopy, will provide quantitative and mechanistic underpinning of AOPs and key events (KE). The project combines *in silico* tools and *in vitro* assays by computational modelling approaches to provide quantitative data on the activation of KE of AOP. This information, together with detailed toxicokinetics data, and *in vitro-in vivo* extrapolation algorithms forms the basis for improved hazard and risk assessment. The EU-ToxRisk work plan is structured along a broad spectrum of case studies, driven by the cosmetics, (agro)-chemical, pharma industry together with regulators. The approach involves iterative training, testing, optimization and validation phases to establish fit-for-purpose integrated approaches to testing and assessment with key EU-ToxRisk methodologies. The test systems will be combined to a flexible service package for exploitation and continued impact across industry sectors and regulatory application. The presentation will highlight the main concepts of the EU-ToxRisk project which will further be illustrated by more detailed presentation in the rest of the symposium.

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Improving transparency on quality and translatability of animal studies using new science driven approach

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The 3Rs have undoubtedly raised awareness to the way we work with animals in biomedical research. However, implementation of the 3R principles in animal-based research appears to be very challenging [1,2]. Additionally many shortcomings have been identified such as poor experimental design, inadequate reporting and publication bias [1]. To boost improvements in animal-based research, we support a new science driven approach in laboratory animal science, namely the conduct of systematic reviews of animal studies.

Systematic reviews (SRs) are a transparent and thorough method to summarize and synthesize available research evidence. SRs are already standard practice in clinical research and considered the highest level of evidence in the chain of evidence-based research. Even though a substantial number of animal studies are conducted as part of preclinical research, SRs of animal studies are not yet widely conducted.

SRs of animal studies have already proven to be of great value, e.g. to provide evidence-base input for the design of new animal studies (including reduction and refinement strategies) and clinical trials. Additionally, SRs provide insights into the translational value of animal research, which should, from both an ethical and scientific point of view, be one of the leading principles in justification of animal use in biomedical research.

The methodology used in current reviews of animal studies differs substantially [4]. Only 52.7% of the assessed SRs performed some type of risk of bias analyses. Because only 24.6% of the primary studies applied randomisation en only 14.6% blinding, the risk of bias in the primary studies in these reviews appears to be high. This illustrates the need for better design, reporting and evaluation of animal studies, and additionally also clear guidelines (and education) for the conduct of SRs of animal studies, in order to achieve valid scientific conclusions on quality and translation.

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Isolation of astrocyte cell culture from human brain

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Objective

To study mechanisms of neurotrauma and neurodegeneration, *in vitro* organ culture systems with live neural cells are highly appealing. Astrocytes are especially a focus in research. Mostly, these cells have been isolated from animal tissue and may not be representative enough. We established a relatively quick and easy protocol for isolation of astrocytes from the brain biopsies with a high yield and low risk for contamination.

Methods

Human astrocytes can be obtained following cranial operations, especially in neurotrauma patients after brain necrectomy. In sterile conditions, fragments of viable tissue that was removed during the operation were collected. The tissue was cut, grinded and seeded through mesh system. After sequential centrifugation and separation, sediment was harvested and cells seeded in suspension, supplemented with special media (DMEM Advanced) containing high nutrient level (FBS) and antibiotics (streptomycin, penicillin). Characterization was made and subisolation cells followed.

Results

In appropriate environment, isolated cells retained viability and proliferated quickly. Attachment was observed after 8 to 10 hours and proliferation after 5 days. Time to confluence was 21 days. Cell proliferation, apoptosis and cell senescence were examined after 21 days in culture. The cells were stable. Under standard culture conditions, cell proliferation and cluster formation was observed. Cell viability was 90%. GFAP and DAPI immunohistology was made for characterisation and the cells were highly positive, confirming the astrocyte markers.

Conclusions

The demonstrated isolation process is simple, quick and economical, allowing viable long-term primary cell culture of human origin. The availability of such system will permit the study of cell properties, biochemical aspects and the potential of therapeutic candidates for traumatic and neurodegenerative disorders in a well-controlled environment on human instead on animal astrocyte cell culture.

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Effects of association of lipoperoxide nanoemulsion and cisplatinum for a cancer targeted therapy in DLD-1 human colon adenocarcinoma cells

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Several clinical studies evidence that redox status of patients could influence tumor growth and subsequently overall survival. The oxidation therapy, an unique antitumor strategy, is based on delivering excess oxidative stress or disrupting the antioxidative defense systems into tumor cells. Lipid-hydroperoxides (LOOHs) derived from polyunsaturated fatty acids, are stable intermediates of oxidative process, and at non-toxic concentrations can act as signalling transductors of ROS, modulating gene expression and cell proliferation. In our previous studies, LOOHs, derived by a patented extra-virgin olive oil having a defined peroxides titre (600mEq/kg O2, K600), after a controlled peroxidative process, evidenced selectively cytostatic effects on several cancer cells, by a ROS mediated mechanism.

As known, nanoscale delivery systems, as oil-in-water nanoemulsions, show a great potential for enhancing the bioavailability of lipophilic drugs. Therefore, we verified if K600-nanoemulsions (NK600) could exert stronger effects than K600, at 0.05 and 0.1% doses. In human adenocarcinoma cells (DLD-1), NK600 resulted in a marked cytostatic effect, just after 24h, compared to that observed in K600-treated cultures. MTT index evidenced that mitochondrial compartment was more compromised by NK600 than K600.

After 48h exposure to 0.1% NK600, DLD-1 cells revealed a dramatic increase of extracellular ATP levels, as marker of bioenergetic impairment, and of TBARs, as end products of lipid peroxidation.

Many conventional anticancer drugs exhibit antitumor activity via ROS generation if not totally, so DLD-1 were treated with cisplatinum (CIS), a chemotherapeutic drug, alone and in association with NK600. After 48 hours exposure to 15 and 30 μM CIS, DLD-1 MTT viability index was reduced to 60 and 20%, respectively, vs untreated cultures, while the membrane integrity results to be less affected (NRU index). The simultaneous exposure to CIS with both tested doses of NK600 dropped viability to almost undetectable values.

Analysis carried out by Compusyn software revealed a synergic effect of NK600 and CIS.

To better simulate the *in vivo* microenvironments, 3D cultures of DLD-1 were performed in commercial hydrogel, and in this model it was observed a more chemosensitivity to experimental treatments.

In order to evaluate some mechanisms underlying the growth inhibitory of CIS and NK600, we analysed poly(ADP-ribose) polymerase (PARP) cleavage, and sulforedoxin expression (Srx), an antioxidant protein recently related to promotion of colorectal cancer cell invasion and metastasis. The obtained results confirmed the strengthened of association of CIS with NK600. Just after 24 hours exposure, a marked increase in PARP cleavage and a dramatic reduction of Srx protein confirmed the trigger of apoptotic pathway and the inhibition of proliferation, respectively.

Taken together these preliminary data, DLD1 has proved to be a useful *in vitro* model to study the effectiveness of oxidation therapy as adjuvant of conventional antineoplastic regimen.

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High-throughput microfluidic platform for culture of 3D-kidney tissue models

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Drug toxicity remains a major issue in drug discovery and stresses the need for better predictive models. Here, we describe the development of a perfused renal proximal tubule cell (RPTC) model in Mimetas' OrganoPlate® [1] to predict kidney toxicity. The OrganoPlate® is a microfluidic platform, which enables high-throughput culture of boundary tissues in miniaturized organ models. In OrganoPlate®, extracellular matrix (ECM) gels can be freely patterned in microchambers through the use of PhaseGuide technology. PhaseGuides (capillary pressure barriers) define channels within microchambers that can be used for ECM deposition or medium perfusion. The microfluidic channel dimensions not only allow solid tissue and barrier formation, but also perfused tubular epithelial vessel structures can be grown. The goal of developing a perfused RPTC model is to reconstruct viable and leak-tight boundaries for performing cytotoxicity, as well as transport and efficacy studies.

Human RPTC (SA7K clone, Sigma) were grown against an ECM in a 3channel OrganoPlate®, yielding access to both the apical and basal side. Confocal imaging revealed that the cells formed a tubular structure. Staining showed tight junction formations (ZO-1), cilia pointing into the lumen (acetylated tubulin) and correct polarization with microvilli on the apical side of the tubule (ezrin). Tightness of the boundary over several days was shown by diffusion of a dextran dye added to the lumen of the tubule. Addition of toxic compounds resulted in disruption of the barrier which could be monitored in time. The time point of loss of integrity corresponds with the concentration and the toxic effect of the compound. Furthermore, fluorescent transport assays showed functional transport activity of in- and efflux transporters.

The 3D proximal tubules cultured in the OrganoPlate® are suitable for high-throughput toxicity screening, trans-epithelial transport studies, and complex co-culture models to recreate an *in vivo*-like microenvironment.

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Stability of human skin explants: usability and effect of cultivation media

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Evaluation of beneficial as well as side effects of ingredients used in pharmacological, dermatological and/or cosmetic industry is important for safety of users. Each new compound requires the assessment of effectiveness and safety for humans. Skin of human volunteers and animals represents models that allow studies closer to reality. However, their use is restricted due to the ethical reasons. On this purpose various models have been developed. Human skin organ culture (skin explant) presents 3D organisation and combination of major skin cells such as keratinocytes, melanocytes, Langerhans cells and dermal fibroblasts and extracellular matrix components e.g. collagen and elastin. Compared to artificial skin, explants are cheap and can be easily and quickly prepared from superfluous skin from plastic surgery operations. However, skin explant use has its limitations such as a period of usability due to irreversible changes. The aim of this study was to determine changes in selected antioxidant parameters during short-term cultivation of skin explants. Effect of different media on skin explant stability was also evaluated.

The use of skin tissue complied with the Ethics Committee of the University Hospital in Olomouc and Faculty of Medicine and Dentistry, Palacký University, Olomouc (reference number 41/09) and all patients had signed written informed consent. Skin tissue was washed with phosphate buffered saline (PBS), cut into pieces (~1×1 cm) and cultured in petri dishes in a humidified atmosphere with CO2 (5%; v/v) at 37°C. To study stability and effect of culture media, explants were cultured in i) complete skin medium (CSM; the mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 Nutrient Mixture (1:3) supplemented with foetal bovine serum (FBS), antibiotics and growth factors); ii) DMEM with L-glutamine supplemented with FBS, penicillin, streptomycin and amphotericin B (DMEM). Explants were harvested immediately and after 1-7, 14 or 21 days in culture. Samples were fixed in formalin for histological analysis or frozen for Western Blot and antioxidant parameters determination.

Histological examination (hematoxylin and eosin staining) showed minimal changes in explant structure during cultivation. Western blot analysis showed time-dependent modulation of inflammatory markers (cyclooxygenase-2, interleukin-6) and catalase, decrease in fibulin, increase in caspase-3 and superoxide dismutase-2 and minimal changes in glutathione peroxidase and superoxide dismutase-1. Further modulation in activity of catalase and superoxide dismutase, increased activity of glutathione transferase and amount of glutathione and malondialdehyde and minimal changes in activity of glutathione peroxidase and glutathione reductase were found. Concerning effect of media, minimal changes in antioxidant parameters were found between CSM and DMEM during first four days of cultivation. Minimal changes in skin structure were found during three-week cultivation in CSM and DMEM.

In conclusion, in experiments on skin explants modulation of various marker have to be taken into account. There were found no differences in structure between explants incubated in CSM and DMEM. However, due to changes in other parameters cheaper DMEM-based medium should be used for short-term cultivation of skin explants.

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Use of human derived liver cell lines for the detection of genotoxic carcinogens

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Chemicals are routinely tested *in vitro* for their DNA-damaging properties; when positive results are obtained in these experiments, they are further investigated in animal trials in regard to their genotoxic and carcinogenic activities. At present, a large number of *in vivo* experiments are conducted since false-positive results are obtained in many *in vitro* trials [1]. One of the main reasons for their low reliability is the inadequate representation of drug metabolizing enzymes in currently used cell lines. Recent findings indicate that certain human derived liver cell lines have retained the activities of phase I and phase II enzymes, which catalyze the activation and detoxification of genotoxins. Some studies indicate that these cell lines can be used instead of currently employed lines, which require the addition of enzyme mixtures from rodent livers with active phase I (but inactive phase II) enzymes. We tested 11 different liver cell lines in regard to their sensitivity towards representatives of different classes of DNA-reactive carcinogens, which are of human relevance, namely AFB1, a mycotoxin, IQ and PhIP, which are heterocyclic aromatic amines, NDMA, which belongs to the nitrosamines and B(a)P, a polycyclic aromatic hydrocarbon. These chemicals require activation via different enzymes. Furthermore, we characterized the cell lines in regard to their similarity to primary human hepatocytes (morphology, karyotype, growth kinetics, p53 status). We obtained positive results with all diagnostic mutagens in single cell gel electrophoresis (SCGE or comet) assays, which are based on the detection of DNA-damage by use of electrophoresis, with two cell lines (HepG2 and Huh6). The former line was used in earlier studies, but is relatively unstable, while Huh6 was never used in genetic toxicology so far. HepaRG is a line, which is currently marketed commercially, and was postulated to be suitable for *in vitro* toxicological studies as a surrogate of human primary hepatocytes. However, this line failed to detect the amine IQ and has an extremely long generation time. Other lines (i.e. Hep3B, Huh7, JHH6, PLC/PRF, SK-Hep1, SNU-398, SNU-449 and WRL68) were not suitable for the detection of the different genotoxins. Taken together, our findings suggest that Huh6 is a highly promising line, which can be used for *in vitro* genotoxicity assays. The obtained positive results show that drug metabolizing enzymes, which are required for the activation/detoxification of the different pro-carcinogens, are represented. Further experiments concerning the validation and standardization of genotoxicity experiments with this cell line are currently in progress.

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Assessment of pre- and pro-haptens using non-animal test methods for skin sensitization

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Due to ethical and regulatory reasons, several non-animal test to assess skin sensitization potential of chemicals have been developed and validated. In contrast to animal tests, they lack or provide limited metabolic capacity. For this reason, identification of pro-haptens but also pre-haptens, which require molecular transformations to gain peptide reactivity, is a challenge for these methods.

In this study, 27 pre- and pro-haptens were tested using non-animal tests. Of these, 18 provided true positive results in the DPRA, although lacking structural alerts for direct peptide reactivity. The reaction mechanisms leading to peptide depletions were therefore elucidated using mass spectrometry. Hapten-peptide adducts were identified for 13 of the 18 chemicals indicating that these pre-haptens were activated and that peptide binding occurred. Positive results for five of the 18 chemicals can be explained by dipeptide formations or the oxidation of the sulfhydryl group of the peptide.

Nine of the 27 chemicals were tested negative in the DPRA. Of these, four yielded true positive results in cell-based assays. Likewise, 16 of the 18 chemicals tested positive in the DPRA were also positive in either one or both of the cell-based assays. A combination of DPRA, KeratinoSens™ and h-CLAT used in a "2 out of 3" WoE approach identified 22 of the 27 pre- and pro-haptens correctly (sensitivity of 81%), exhibiting a similar sensitivity as for directly acting haptens.

This analysis shows that the combination of *in chemico* and *in vitro* test methods is suitable to identify pre-haptens and the majority of pro-haptens.

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In vitro demonstration of surfactant antagonism in the Open Source Reconstructed Epidermis (OS-REp) model

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Surfactants are main constituents of different consumer products, e.g. detergents or cosmetic cleansing products. Since surfactants show an intrinsic skin irritation potential, dilutions are used in the final products to avoid adverse effects like irritant contact dermatitis from product use. Moreover, a surfactant is often formulated in combination with other surfactants, as it is a long-standing experience that those mixtures exhibit much lower acute irritation potential than expected from the mere summation of their individual irritation potential, an effect coined surfactant antagonism.

Only few studies were performed to gain a more fundamental understanding of the effect, and it's mechanistic basis remains unclear. However, a thorough understanding of the surfactant antagonism is not only of value for the formulation of products that are considered "mild to the skin". It is also important for the classification of products according to the CLP Regulation in cases when data of the mixtures is missing, because simply adding the irritating effects of all ingredients usually results in over-classification as skin irritant.

Due to the progress in the development of alternatives to animal testing, different *in vitro* methods have become available to determine the skin corrosive and irritating properties of substances, respectively (OECD TG 431 and 439). These methods, which are based on the viability assessment of topically treated epidermal equivalents, aim at deriving a classification for skin irritation/corrosion effects according to the CLP regulation. However, even though these *in vitro* methods are currently the preferred assays for acute skin damage testing, to our knowledge hitherto isolated investigations on the surfactant antagonism were only performed either by human patch test studies or by non-standard *in vitro* assays [1].

In this study, the irritation potential of binary mixtures of sodium dodecylsulfate (SDS), linear alkylbenzene sulfate (LAS), cocamidopropyl betaine (CABP) and alkylpolyglucosid (APG) was compared with the irritation potential of the single compounds by using Open Source Reconstructed Epidermis (OS-REp) models. Combinations of SDS or LAS with CABP and APG, respectively, clearly decreased the irritation potential compared to the irritation exerted by the single surfactants, even though the total surfactant concentration was higher in the mixtures. This kind of surfactant antagonism was also observed with a mixture of CABP and APG.

To our knowledge this is the first time that the effects of surfactant antagonism have been demonstrated with a 3D epidermal equivalent. The reduced irritation potential of mixed surfactants came along with both reduced skin penetration of fluorescein and reduced LDH release. Since no surfactant antagonism was observed in monolayer cultures of keratinocytes, it is assumed that the barrier function of the skin model protects the underlying viable keratinocytes from damage as long as the surfactants do not penetrate the barrier. Hence, surfactant antagonism appears to be primarily driven by the mixture's lower ability to damage the skin model's barrier.

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The current status of nonanimal alternatives in the life sciences in China

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Background
A brief introduction on the concept, classification, process and abroad development of nonanimal alternatives in life science were reported.

Objective
To understand the current status of nonanimal alternatives in life science in China.

Method
Key words and titles searched with terms non-animal alternatives, animal testing alternatives or animal use alternatives on Chinese databases VIP, CNKI and WANFANG, search date from database first issue till July 20, 2015. Data were collected on institute, law, education, methods, etc. and results were described as follows.

Results
There were 219 records on nonanimal alternative published in Chinese from 1999 to 2014, mainly descriptions on systemic toxicity testing, alternative animal model, etc. There are some studies on experiment education, but limited on alternative system, law, policy and validated methods, etc.

Conclusion
There were limited system policy and methods studies on nonanimal alternatives available in China. It is necessary to develop the nonanimal alternative system in life science focus on policy, education and methods, etc.

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Defining the optimal animal model for translational research using gene set enrichment analysis

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The mouse is the main model organism used to study the functions of human genes because most biological processes in the mouse are highly conserved in humans. Recent reports that compared identical transcriptomic datasets of human inflammatory diseases with datasets from mouse models using traditional gene-to-gene comparison techniques resulted in contradictory conclusions regarding the relevance of animal models for translational research. To reduce susceptibility to biased interpretation, all genes of interest for the biological question under investigation should be considered. Thus, standardized approaches for systematic data analysis are needed. We analyzed the same datasets using gene set enrichment analysis focusing on pathways assigned to inflammatory processes in either humans or mice. The analyses revealed a moderate overlap between all human and mouse datasets, with average positive and negative predictive values of 48% and 57% significant correlations. Subgroups of the septic mouse models (i.e., *Staphylococcus aureus* injection) correlated very well with most human studies. These findings support the applicability of targeted strategies to identify the optimal animal model and protocol to improve the success of translational research.

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A modular cell sheet-based approach towards 3D tissue models

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Cell sheet engineering as an alternative approach to classical scaffold-based tissue engineering relies on thermoresponsive surfaces which allow for the detachment of confluent cell sheets with their extracellular matrix simply by temperature reduction without enzymatic or mechanical cell treatment [1]. The surfaces are coated with polymers that switch between a rather hydrophobic state allowing for cell adhesion and a hydrophilic state enabling cell detachment. We envision producing 3D *in vitro* models for biomedical research and drug development as an alternative to animal testing by combining polyglycidyl ether based thermoresponsive surfaces, a biocompatible cell adhesive and optimized cell culture conditions.

A proof of concept for temperature triggered cell adhesion and cell sheet detachment on/from these thermoresponsive coatings was accomplished with coated gold substrates [2]. Currently, we are in the process of transferring these functional coatings to common cell culture materials. In addition to the polymer and surface properties of the correspondingly coated substrates, we found the cell culture and detachment conditions to vastly influence the detachment efficiency and quality of the fabricated cell sheets. Detachment of NIH/3T3 cells was more reliable and faster when cells were cultured with low glucose (1.0 g/l) rather than high glucose (4.5 g/l) medium; further reduction of the detachment time of confluent cell sheets was achieved with a second washing step after the initial triggering of the cell sheet detachment with PBS (room temperature). This technique was further proven to be applicable to the fabrication of sheets of primary human dermal fibroblasts which are more relevant for 3D tissue models. Initial experiments with polybetaine-based biocompatible cell adhesives revealed the concentration-dependent aggregation strength of these adhesives on human erythrocytes and dermal fibroblasts in a microscopic agglutination assay without lysing the cell membrane.

Cells and confluent cell sheets can be detached via a thermal trigger from polyglycidyl ether-based surfaces. However, in addition to the polymeric surface characteristics, also the culture and detachment conditions have a strong impact on cell sheet detachment. Based on these results we are currently developing methods and engineering tools to arrange cell sheets into 3D tissue structures with the help of a synthetic, biocompatible cell adhesive.

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EPAA contributions in the area of ADME and carcinogenicity testing

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EPAA is a voluntary collaboration between the European Commission, companies and European trade associations from seven industry sectors. The partners are committed to pooling knowledge and resources to accelerate the development, validation and regulatory acceptance of alternative approaches to animal testing. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing.

The presentation will describe two recently completed projects that were supported by EPAA and relate to the areas of ADME (Absorption, Distribution, Metabolism and Excretion) and Carcinogenicity.

The ADME project was conducted by scientists at the Health and Safety Laboratory in UK; it developed further MEGen, an existing web-based tool, to be able to simulate and visualize toxicokinetics (PBTK) based on input from ADME properties (absorption, distribution, metabolism and excretion). The tool is a user-friendly and free to use downloadable application that allows *in vitro* and *in vivo* exposure prediction from non-animal test methods [1]. Output examples include tissue concentration versus time profiles (forward dosimetry) or external exposure versus time profiles (reverse dosimetry). Following this "proof of concept" project, the prototype will be further improved and validated in a next project that is planned to be funded by ECETOC and Cefic LRI. This is a concrete example showing the cross sector synergies built between EPAA and its member organisations in order to promote novel alternative approaches.

The Carcinogenicity project was carried out by the University of Wageningen in collaboration with the Medicines Evaluation Board (The Netherlands).

The overall objective was to identify opportunities for improving the science supporting the regulatory testing of medicines and chemicals and to achieve Reduction if it proves possible to waive long carcinogenicity studies at an early stage during clinical development of a pharmaceutical, e.g. at the end of Phase 2. Pharmacological data and data from 6-month subchronic toxicity studies from rats are available in that stage. Data from other studies (e.g. 9-months data from non-rodents), may contribute to a weight-of-evidence approach. For this purpose, a dataset similar to that of Sistare et al. [2] from previously submitted sub-chronic toxicity and carcinogenicity studies of pharmaceutical compounds was extended with pharmacology data in line with Van der Laan et al [3]. A peer-reviewed publication with the conclusions from the extended database analysis is expected to provide the scientific evidence needed for developing an ICH concept paper that EPAA can discuss further with regulators to achieve waiving of the 2-year carcinogenicity study on rats.

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3D networks of iPSC-derived neurons and glia for high-throughput neurotoxicity screening

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The assessment of neurotoxicity remains a major scientific challenge due to the complexity of the central nervous system. Current strategies to evaluate toxicity of drugs and chemicals are predominantly based on *ex vivo* or *in vivo* animal studies. These models have limited predictability for neurotoxicity in humans and are not amenable to high-throughput testing. In order to overcome these limitations we are developing a neurotoxicity model based on iPSC-derived neurons in OrganoPlates™ [1,2]. This microfluidic platform enables high-throughput screening of miniaturized organ models. A mixed population of human iPSC-derived neurons consisting of GABAergic and glutamatergic neurons with supporting astrocytes was cultured in 3D, closely representing the physiology of the human brain. As a part of the validation, proper network formation was observed by neuron-specific immunostainings and neuronal electrophysiology was analyzed by a calcium sensitive dye indicating spontaneous neuronal firing. Additionally, we investigated the dose-response neurotoxic effects of methylmercury and endosulfan on neuronal viability. The OrganoPlate™ platform enables real time analysis of neurotoxic effects of compounds in high-throughput. This iPSC-derived neuronal model can be used to refine animal experiments and has the potential to better predict adverse effect in humans and hence to improve clinical development success.

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Reducing animal numbers by half: A hybrid preparation technique for the simultaneous investigation of histology and broncho-alveolar lavage parameters of the rat lung

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Lung histopathology and bronchoalveolar lavage (BAL) are both necessary to examine the pulmonary toxicity of inhaled toxicants. While histopathology is indispensable to diagnose complex morphological changes and is the gold standard in international guidelines, BAL data are more easily quantifiable and can be subjected to statistical analyses. To obtain both types of data, established protocols use two separate groups of animals for lavage and histopathology. Here, a preparation technique was established which allows to maintain all necessary scientific information while collecting lavage and histopathological data from only one animal group. Method, limitations and pitfalls of this hybrid-preparation will be described.

Experiments were carried out with sham and quartz particle-subjected rats, to evaluate the applicability of the new procedure in animals with pulmonary inflammatory changes 21 days after instillation. While the lung was left in situ with the trachea being cannulated and the left bronchus clamped, the right lung was manually lavaged. Thereafter, the clamp was removed and the complete lung was fixed with formalin. For both, sham control and quartz-instilled animals, it could be shown that the unilateral lavage had no disadvantage compared to the bilateral approach in terms of quantitative evaluation of the lung structure and also of inflammatory parameters. As the procedure allowed no lung weight determination, a modified protocol was developed: Rats were sacrificed by exsanguination under narcorene®anesthesia. The thorax was opened and the trachea cut distal to the larynx. Lung, trachea, heart, thymus, and lung-associated lymph nodes were resected as a whole and the weight of this organ complex was determined. The left bronchus was clamped about 3 mm distal to the bifurcation and the right lung was lavaged (2 or 4 times to harvest cells, proteins and enzymes) via a stainless steel cannula inserted into the right bronchus. The left bronchus was re-opened and the cannula retracted to a position upstream the bifurcation. Heart, thymus, and lung associated lymph nodes were removed. Their weights were subtracted from that of the organ complex enabling lung weight determination. Finally the lung was filled with 10% neutral-buffered formalin at constant pressure (30 cm H_2O). With both procedures the non-lavaged left lung was maintained in excellent condition for routine histopatholoy. Moreover, the tissue structure of the lavaged right lung was similarly good, enabling full histopathology evaluation of all four lobes.

The described method is in full accord with the 3R principle: It reduces the number of animals for this type of tests by half and a refines the analyses, as more data are gathered from each animal which is always beneficial in case of inter-individual variation. This approach in line with the animal welfare spirit of the current draft renewal of OECD test guidelines 412 and 413.

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A fetal bovine serum free cytotoxicity test following ISO 10993-5

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Tests for cytotoxicity (or biocompatibility) are commonly performed according to ISO 10993-5 [1]. To perform the test in a chemically defined environment and to avoid suffering of animals, a protocol without the use of fetal bovine serum (FBS) was developed. L929 fibroblasts were adapted to FBS-free media as described by van der Valk et al. [2] and a cytotoxicity assay with three replicates, positive control, negative control and blank was developed.

L929 cells were cultivated in a 50:50 (v/v) mixture of Dulbecco's Modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with insulin, transferrin and selenium (DME/F12+ITS) medium for several passages. DME/F12+ITS medium was also used for the assay. As positive control Pattex Multi Alleskleber (Henkel AG, Germany) and as negative control SILASTIC (Dow Corning Europe S.A., Belgium) was used. Cells were cultivated in 6well plates with three replicates for up to 72 h and the condition of all cultures was evaluated according to ISO 10993-5:2009. A showcase experiment with aluminum as test material showed full functionality of the assay. The results show that a test for cytotoxicity/biocompatibility can be easily performed with an FBS-free assay.

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Modeling fungal infections within a three-dimensional perfused respiratory system

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Aspergillus fumigatus, a saprophytic and spore-forming fungus found worldwide, exerts an influence on the respiratory tract in many ways [1,2]. Aspergillus causes respiratory disorders and the clinical syndromes range from colonization to deep-seated infections [1]. Current studies primarily involve the use of animal models and cell lines to understand the fungal invasion process. To reduce animal experimentation and work in a more physiological human system, we aim in unfolding mechanisms underlying the primary interactions and invasion process between the upper and lower respiratory epithelium and the pathogen. Multiple predisposing factors such as role of immune cells or cytokines released at the sites of infection need to be studied additionally in a sophisticated *in vitro* respiratory model.

To simplify and optimize the conditions in the perfused respiratory 3D system, we now employed normal human bronchial epithelial (NHBE) cells in air-liquid interface culture (ALI) instead of alveolar cells and applied these cells in the Kirkstall QV600 System. We studied epithelial differentiation using confocal, scanning electron (SEM) and live cell microscopy and compared perfused NHBE cells with their static counterparts. Additionally, we added dendritic cells (DCs) to the basolateral layer of the epithelial cells to mimic the *in vivo* situation, where DCs are present alongside the upper respiratory tract to sense incoming pathogens, too.

Analyses over time by confocal microscopy showed that NHBE cells differentiated in ALI to form tight junctions, produced mucus and developed cilia and this differentiation process was significantly accelerated by perfusion as assessed by live cell microscopy. SEM studies of respiratory cells infected with Aspergillus demonstrated that in the initial phases the host cells were able to produce factors which inhibited fungal growth as well as hyphae production. DCs also sensed the presence of fungal pathogens and migrated through the epithelia to the sites of conidial infection. After optimization of the perfused conditions using NHBE cells, we will extend our studies by not only using upper but also lower respiratory tract and lung cells in combination with relevant immune cells (neutrophils, alveolar and interstitial macrophages, dendritic cells).

Our preliminary data will have future implications to use differentiated, perfused upper and lower respiratory cells in combination with immune cells in a 3D setting reflecting *in vivo* conditions. This model will support better understanding of pathogenesis, detection, and treatment of co-infections.

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CON4EI: Short Time Exposure (STE) model

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Assessment of ocular irritancy is an international regulatory requirement in the safety evaluation of industrial and consumer products. The objective of the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project is to develop tiered testing strategies for eye irritation assessment for all drivers of classification. For this, a set of 80 reference chemicals (38 liquids and 42 solids) was tested in eight *in vitro* test methods. Here, the results obtained with Short Time Exposure (STE) model are shown. The primary aim of this study was an evaluation of the performance of the test method to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling for inducing serious eye damage (Category 1). In addition, the predictive capacity in terms of *in vivo* driver of classification was investigated. In a second step, it was investigated if STE can be used as part of a tiered-testing strategy for eye irritation assessment when assessing chemicals that fit the applicability domain.

For the STE method, the accuracy in identifying Cat 1 chemicals was 61.3% with 23.7% sensitivity and 95.2% specificity. Excluding non-qualified results did not affect the ability to correctly identify Cat 1 chemicals (accuracy 61.2% with 26.9% sensitivity and 100% specificity). The accuracy of the STE test method to identify No Cat chemicals was 72.5% with 66.2% sensitivity and 100% specificity. Excluding non-qualified results improved the predictivity (accuracy 87.8% with 85.4% sensitivity and 100% specificity).

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Novel perspectives for *in vitro* models of human skin wounds

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Predictable models of human skin wounds are not only needed for basic research, but even to a bigger extent, for preclinical testing of novel dressings and therapeutics for skin wound application.

Even though animals (especially rodents) differ from the human body in terms of anatomy, immune system and wound healing processes, the preclinical phase of developing novel therapeutics is still largely dominated by animal testing. And due to the increasing incidence of chronic wounds and wound infections, the number of preclinical candidates is constantly increasing.

Thus, predictive models which closely mimic the *in vivo* situation of human skin wounds are urgently needed to assess safety, efficacy and absorption of novel wound therapeutics. Cell monolayers based on cultivated keratinocytes serve as simple migration assays for which a dense monolayer is artificially disrupted (wounded) by a blunt specimen. The extent of repopulation of this defected area allows for evaluation of "wound healing" capacity and extent. However, these so called "scratch tests" can solely be used for testing liquid formulations, as cell monolayer destruction upon removal of the system under investigation and its excessive hydration by total immersion limit their applicability. Further, such tests do not reflect influences of interactions of different cell types and three dimensional effects. To address these issues, current progress was focusing on model hydration comparable to the physiological *in vivo* situation as well as on the option to remove the tested system without disturbing the integrity of the cell monolayer. Further, three-dimensional skin cultivates existing of different cell types as well as excised human skin samples can 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. Besides the model itself, valid assessment of readout parameters requires suitable analytical techniques. Standard techniques are destructive and limited regarding in-depth investigation on molecular level.

The talk will present the existing wound models highlighting their individual potential and discussing their limitations as well as introduce novel approaches and developments. Further, analytical techniques for assessment of wound healing will be discussed and their significance and potential to advance current knowledge will be presented.

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Teaching practical applications of animal welfare – a review over 10 years of the FELASA B-Course in Vienna

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In order to maintain animal welfare in science an adequate training of people working in animal experiments is of great ethical importance and legal demand.
Therefore, the Institute of Laboratory Animal Science is offering the University Course "Introduction to Laboratory Animal Science" since 2006 in accordance with the Guidelines for FELASA postgraduate courses category B which is certified by the GV-SOLAS. This course serves to introduce and to train interested people for experimental use of laboratory animals. In addition to the wide range of subjects discussed, the course's main focus is on the safe, responsible handling and care of animals used in scientific experiments.

Animal welfare is not only a theoretical aspect of this course but also a practical application: Implementing the Principals of the "3Rs" into practice, dummies, films and novel training devices are used for teaching in order to protect animals from unnecessary adverse effects and to reduce distress or harm.

The aim of this course is passing on expertise in animal handling (and animal welfare) but also the development of awareness for the laboratory animal.

Since this course has run successfully over the past 10 years, teaching experience and methods as well as developing demands of participants are continuously reviewed and upgraded.

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A medium-throughput method for the analysis of (combinatorial) endocrine effects using the nematode *C. elegans*

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The soil nematode *Caenorhabditis elegans* (*C. elegans*) is a well-characterized biological model organism that has been successfully employed in research in many fields. Several pathways found in *C. elegans* are highly conserved among different species including humans (Corsi, Wightman et al., 2015). This and other advantages like its ability to self-fertilize, its short lifecycle, its high fecundity, and its ease of culture make *C. elegans* ideal as a model in toxicology (Tejeda-Benitez and Olivero-Verbel, 2016). Developmental and reproduction toxicity endpoints in particular might be much easier and faster accessed than in studies using vertebrate animals. These endpoints are very interesting for numerous chemicals including pesticides with potential endocrine disruptive activities. Due to the large number of used pesticides and the unresolved issue of combinatory effects (European Union: European Commission, 2012), a fast and efficient test system to address these endpoints is especially important.

Using five potential endocrine disrupting azole fungicides (epoxiconazole, prochloraz, cyproconazole, propiconazole, tebuconazole), we developed a medium-throughput method with automated analysis of growth inhibition and fecundity reduction in *C. elegans* as a test system for the identification of reprotoxic or endocrine active substances. This was accomplished by employing a simple 96 h toxicity assay with subsequent automated fluorescence microscopy image acquisition using a High Content Screening System (Opera Phenix, Perkin Elmer) combined with automated data analyses employing a newly developed pipeline for the open-source cell image analysis software CellProfiler (Broad Institute).

In accordance with the 3R principle (Replacement, Reduction, Refinement), this test might be used upstream of vertebrate testing (Reduction) as a screening tool to identify high priority substances. Most importantly, this method allows the efficient analysis of combinatory effects that are of particular interest for the assessment of cumulative exposure to active substances used in pesticides.

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Mouse Dried Blood Spot (DBS) analysis to reduce animal testing

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Mice are the most widely used laboratory animals in biomedical research. According to the German Federal Ministry of Food and Agriculture (BMEL) in 2014 approximately 1.2 million lab mice were used in Germany. As (i) mice share 99% of their genes with humans and (ii) the CRISPR/Cas9 technology facilitates the generation of mouse models, it can be expected that both the number and the diversity of mouse models will further increase in the future. Numerous scientific studies investigate the concentration of proteins and biomarkers in murine tissue and body fluids, for instance to monitor or control novel therapeutic approaches. Indeed, regulated proteins could be detected in the plasma of genetically modified mouse models of pancreatic cancer. The human homologues of these proteins allowed the effective distinction between pancreatic cancer patients and healthy controls. Such studies often employ the usage of methods such as Western blot or ELISA that rely on the usage of antibodies and consequently have inherent limitations. For instance, the multiplexing capacity, i.e. the number of proteins that can be analyzed in parallel, is severely limited, whereas the required blood volume (volume/protein) is relatively high. For ELISA approximately 20-200 µL of mouse blood are required for the parallel quantification of only 10 proteins. A laboratory mouse, however, has only ~1.4 mL of blood and repeated blood collection should not exceed 140 µL. Hence, extended studies and time-course experiments as conducted e.g. in toxicology currently demand the usage and sacrifice of numerous mice.

We directly address this issue through the combination of DBS technology and quantitative mass spectrometry (MS): Using dedicated, sensitive and highly specific MS-based assays will allow the parallel (absolute) quantification of up to 250 proteins from only 10 µL of mouse blood. Compared to many ongoing mouse studies this enables the parallel and more accurate quantification of up to 20 times more proteins from up to 20 times less blood. Therefore, our technology leads to a massive reduction of laboratory mice usage (Reduce) and will (ii) reduce suffering (Refine) while concurrently (iii) boosting the gain of knowledge for biomedical research and (iv) substantially reducing costs. Our aim is to share the developed MS-based assays with the research community and to additionally offer them as service. Therefore, we are developing quality control standards, standard operating procedures and analysis pipeline based on instrumentation that is available in any major university or clinics (triple quadrupole MS).

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Combining human cell-based assays to identify biocompatible poly[acrylonitrile-co-(N-vinyl pyrrolidone)] nanoparticles

Nan Zhang [1,], André Said [1], Christian Wischke [2,3], Vivian Kral [1], Robert Brodwolf [3,4], Pierre Volz [4], Alexander Boreham [4], Christian Gerecke [5], Wenzhong Li [2], Axel Neffe [2,3], Burkhard Kleuser [5], Ulrike Alexiev [3,4], Andreas Lendlein [2,3] and Monika Schaefer-Korting [1,3]*

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Objectives
Novel drug delivery systems like nanoparticles (NP) offer exclusive local admission and low side effects, but need to be tested for biocompatibility before their use in medicinal and cosmetic products. Animal testing provides limited information about biocompatibility to humans [1]. In this study, a series of human cell-based methods, replacing animal testing is adopted according to OECD and INVITOX guidelines to study the impact of the size and physicochemical properties of poly[acrylonitrile-co-(N-vinyl pyrrolidone)] NP on cell/tissue compatibility.

Methods
A series of poly[acrylonitrile-co-(N-vinyl pyrrolidone)] NP of varying size and composition were synthesized [2]. The evaluation of biocompatibility was conducted in a size- and composition-dependent manner, including cytotoxicity by MTT assay, cell uptake by fluorescence-lifetime imaging microscopy [3], irritation potential by red blood cell test and bovine cornea opacity and permeability assay, genotoxicity by ROS assay and Comet assay, and at last skin penetration by Franz cell experiment.

Results
According to the cell uptake and cytotoxicity data, NP hydrophilicity largely defined the interaction with normal human keratinocytes and dermal fibroblasts, as NP hydrophilicity improves cell uptake and appears to be associated with lower biocompatibility. Moreover, higher penetration into barrier-damaged skin was demonstrated with small NP and moderate lipophilicity. Neither irritation potential nor induction of ROS or genotoxicity were detected.

Conclusion
Human cell-based assays permit to gain insights into crucial criteria for biocompatibility of nanoparticles. Less efficient and less biocompatible NP can be eliminated for further (non-)clinical studies, guarding against unnecessary animal testing. Specifically, altering NP hydrophilicity by systematic variation of the copolymer composition may allow delivering drugs into the cell, but may in some cases also be associated with increased cytotoxicity.

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Intestinal organoids: Functional studies on nutrient and drug transport, hormone secretion and intracellular signaling

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Studies on intestinal nutrient and drug absorption as well as on sensing and gut hormone secretion are of high relevance for biomedical, pharmacological and nutritional applications. Appropriate *in vitro* models that would allow a replacement or significant reduction of animal experiments have been lacking to date.

Immortalized mammalian cell lines are currently the prime *in vitro* model used to assess intestinal transport processes or the secretion of gut hormones in response to luminal stimuli. Although well-established and easy in handling, these cultures do not reflect the complexity of the intestinal epithelium with multiple cell types and a region-specific architecture. Primary intestinal epithelial cell culture seems a better approach to study intestinal functions *in vitro*. However, primary cell cultures are limited by short-term culture hence their generation requires a large number of animals, and enterocytes are poorly differentiated.

Few years ago, the generation and long-term *in vitro* cultivation of 3-dimensional intestinal organoids (so called "mini-guts") of human and murine origin has been reported. Intestinal organoids are generated from isolated intestinal tissue and can be cultured and multiplied in the lab over months. They contain all cell types of the intestinal epithelium and display the main characteristics of the mammalian intestine.

Research on intestinal nutrient transport and sensing is of high interest for the therapy of metabolic disorders such as obesity and diabetes. Our studies reveal for the first time that murine small-intestinal organoids can be used as a new *in vitro* model system enabling concurrent investigations of nutrient and drug transport, sensing and gut hormone secretion as well as fluorescent live-cell imaging of intracellular signaling processes. By generating organoid cultures from wild type mice and animals lacking certain intestinal transporters, we show that organoids preserve the main phenotypic and functional characteristics of the intestine. We demonstrate (i) functional transport of sugars, peptides and drugs, (ii) secretion of the incretin hormones GIP and GLP-1 in response to different luminal stimuli, and (iii) fluorescent live-cell imaging of intracellular calcium signaling and pH changes.

As an *in vitro* system, intestinal organoids come closest to physiology thus providing a wide range of application areas from gastrointestinal and nutritional research over drug bioavailability studies and sensory science. Even though animal studies cannot be completely replaced by organoid cultures in all cases, this novel model system has the potential to significantly reduce the number of *in vivo* animal experiments, as it gets closer to physiology than any other *in vitro* model before.

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How to determine severity degrees for genetically altered rodents?

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Genetically altered animals are frequently used research models with continuously increasing numbers. Apart from its scientific value, the genetic alteration can compromise animal well-being. However, the large variety of phenotypes is challenging when it comes to severity degree determination. A guide on severity classification will therefore largely contribute to the harmonisation of severity assessments of genetically altered mice and rat lines throughout Europe.

The breeding of genetically altered animals needs to be authorized by the competent authority within the scope of the European Directive 2010/63, if a harmful phenotype is likely to occur. Hence, the severity classification plays an essential role in pro- and retrospective severity assessment. Since 2013, severity assessment and classification of genetically altered laboratory animals has to be established at all European research institutes.

The German Federal Institute for Risk Assessment developed guidelines for a basic welfare assessment [1,2]. According to these guidelines a final assessment form has to be filled in for each established line containing the severity degree, a description of the phenotype and refinement recommendations.

The *Arbeitskreis Berliner Tierschutzbeauftragte* (Working Group of Berlin Animal Welfare Officers) collected and reviewed severity classifications of genetically altered mouse and rat lines. We compiled data from Berlin's principle research institutes whose *in vivo* research uses almost exclusively mice and rats with genetic alterations and who keep some 180.000 rodents in total. Data of the years 2013 through 2015 has been included.

The *Guide on severity assessment and classification of genetically altered mice and rat lines* contains examples of symptoms and syndromes caused by genetic alterations. Examples are assigned to a particular severity category (none, mild, moderate, severe) including recommendations for refinement strategies.

These classifications are based on a consensus reached by experts in veterinary medicine, laboratory animal science, and animal welfare. In addition, recommendations of the British Home office [3], and the European Commission Expert Working Group on severity classification [4], as well as selected scientific publications on phenotypes have been taken into account.

This collection of severity classifications will serve as guidance in the complex process of severity degree determination and assignment. Animal welfare bodies and researchers are encouraged to contribute information on phenotypes which will be incorporated into the collection. The list will be reviewed periodically in order to provide a large collection of different syndromes and clinical symptoms and will be a way to increase the harmonisation of the approach across scientific research institutes.

http://www.ak-tierschutzbeauftragte.berlin
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Modeling severe infection in human lung tissue

Katja Zscheppang [*], Johanna Berg, Diana Fatykhova, Maren Mieth, Mario Tönnies, Torsten Bauer, Paul Schneider, Jens Neudecker, Jens-Carsten Rückert, Stephan Eggeling, Maria Schimek, Achim Gruber, Norbert Suttorp, Stefan Hippenstiel and Andreas Hocke

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Pneumonia is a leading cause of death worldwide and mortality rates are constantly high since decades [1]. Influenza A virus (IAV) and *Streptococcus pneumoniae* (*S. pneumoniae*) especially in subsequent co-infections contribute significantly to the fatal outcome [2]. Since antibiotic resistance increases and causal viral therapies are often lacking, it is pivotal to apprehend the molecular interplay between viruses, bacteria, and pulmonary target cells to enable for innovative immune modulatory and adjunctive therapies next to pathogen-directed clinical approaches. However, the underlying disease determining mechanisms are so far mainly investigated in cell-based assays or animal models, which, due to structural and functional differences, often fail in the transferability of the results from mice to human.

To fill this gap, we established a living *ex vivo* human lung infection model [3,4] to study pathogen host interactions, the cellular regulation of cytokines and innate immune related mechanisms contributing to the susceptibility of viral and bacterial infection.

In previous studies we revealed the *S. pneumoniae* induced cell-type specific regulation of cyclooxygenase-2 and its related metabolites as well as the pneumolysin dependent inflammasome activation [4,5]. We revealed the cellular tropism of IAV and analysed the subtype dependent differential replication, which has been compared to the severe MERS-CoV infection [6]. Moreover, we introduced a new intra-vital imaging method on human lung tissue slices to investigate viral and bacterial cell damage directly in the alveolar compartment down to the mitochondrial level. Based on these data, a current study focuses on the mutual immune interference of viral and bacterial co-infection, demonstrating that IAV induced interferons suppress the pneumococcal inflammasome activation and subsequent GM-CSF production, which might result in impaired bacterial clearance and alveolar repair. The use of human lung tissue to study viral and bacterial (co-)infections displayed distinct differences on the cellular and molecular level compared to mice.

Taken together the human lung tissue model is suitable and necessary for the simulation and characterization of innate immune events determining the regulation of pneumonia. In comparison to mouse models, human lung tissue can be directly infected with viral and bacterial strains isolated from infected patients, which therefore strengthens the relevance of the data and allows for direct testing of new pharmacological substances to treat severe infections.

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