Theme V
Replacement and Reduction in Basic Research

Session V-1: Novel methodologies and their potential *in vitro* application for drug development and safety assessment

Session V-1: Oral presentations

V-1-353

A novel platform for automated production and screening of scaffold-free, organotypic microtissues

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Physiological tissue-like culture systems have shown to improve the relevance of *in vitro* testing of substances. Although the advantages of organotypic 3D-cell-culture models to increase the performance of *in vitro* compound assessment have been known for years, complex production and readout processes impeded its industrial implementation. Here, we present a novel automation-compatible 96-well platform to produce organotypic microtissues and its implementation in an automated screening process. This technology allows the production of tumor and primary microtissues in a scaffold-free hanging-drop culture by liquid top-loading similar to standard 96-well monolayer plates. This is achieved by a microfluidic channel connecting an inlet funnel at the top and an outlet funnel at the bottom of the plate, where a hanging drop is formed by a combination of capillary and surface-tension forces in which the tissue is formed. We present an implementation on a robotic liquid-handling device with a 96-multichannel pipette head, showing the same volumetric precision as in standard multi-well plates. This results in an excellent size uniformity of <10% for the microtissues. Finally, a whole screening process using four reference compounds was performed in comparison to classical monolayer cultures, underlining the different behavior of both cell-culture models. Based on the high flexibility of the microtissue production technology using either animal or human primary cells and cell lines, an efficient and automation-compatible technology will further accelerate the use of *in vitro* models in drug development.

V-1-400

The application of pattern recognition data mining and knowledge discovery for systems to replace rodent models in fundamental research

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In spite of the greater awareness of the 3Rs, reliance on rodent models in fundamental biomedical research has increased, largely due to technologies that allow the precise genetic engineering of inbred strains. In competitive research environments, many researchers accept that control of whole organism genotype and broader experimental conditions (e.g. virus or compound dose, diet) are reasons to rely upon laboratory rodents. Through these genetic technologies and control, there is a rodent "system" in place that serves the researcher's needs for whole organism experimentation.

Another system that has developed rapidly is bioinformatics. Within this field the sub-discipline of pattern recognition (or knowledge discovery) has emerged as an efficient strategy with which to analyse enormous databases. This strategy includes advanced statistical analysis, as well as machine learning algorithms developed by computer scientists, and has been successfully applied to a variety of knowledge domains. Recursive partitioning and Support Vector Machines (SVMs) are two examples of machine learning techniques currently being applied to biomedical data as a component of fundamental research in infectious disease and immunology. These and other bioinformatics techniques allow the modelling of multi-dimensional data and the identification of "patterns" associated with a disease outcome. Such patterns, or profiles, can then be summarised as a set of "rules" that allow the clustering of human data associations with an outcome. With these rules, human genetic information can be exploited to assist with the identification of disease genes and pathways after appropriate biological validation of *in silico* models.

V-1-430

Plants as animal alternatives in the production of antibodies and other therapeutic agents

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Antibodies recognize and bind to target antigens with great specificity, a property which allows them to be used for a wide variety of application. Traumatized animals and tissue culture have been used for the production of some of these immunoglobulins. However, fermentation and tissue culture systems are not ideal for synthesizing mammalian therapeutic proteins and injecting an animal with hybridoma in order to induce ascite production in its abdomen is very painful and traumatic. Recently, molecular farming has opened up the opportunity of using plants for the production of antibodies and other proteins in plants. Plants are safe, extremely cost-effective and can carry out post translational modification. New plant expression systems, strategies in the control of post-translational maturation and purification of these recombinant proteins are currently under development to improve the yield and quality of plant-made proteins. Therefore, plants could and should serve as excellent replacement for animals in the preparation of diagnostic and therapeutic proteins thereby avoiding unnecessary suffering experienced by animals.

V-1-584

Organotypic *in vitro* human epithelial models (EpiAirway, EpiDerm-FT) with engineered toxicological reporter functions

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In vitro human 3D (NHu-3D) epithelial models including skin (e.g. EpiDerm, EpiDerm-FT) and airway (e.g. EpiAirway, EpiAirway-FT) are important advances over traditional monolayer cell culture models. For toxicology applications, these animal alternative models provide more realistic, *in vivo*-like structure, barrier properties, metabolic functions and dosing capabilities. Here we describe NHu-3D epithelial models with the added feature of engineered toxicological reporter functions. Early passage normal human epidermal keratinocytes, dermal fibroblasts, tracheobronchial epithelial cells and fibroblasts were transduced with lentiviral vectors containing NFκB reporters linked to either GFP or luciferase. Stably transduced cells were selected by puromycin resistance, expanded several passages and cryopreserved to produce large pools of reporter-expressing cells. Reporter-expressing cells were then utilized to produce NHu-3D skin and airway epithelial models. Organotypic structure and barrier properties of models produced from reporter-expressing cells were found to be similar to models produced from untransduced cells, as determined by histological assessment, barrier assessment by transepithelial electrical resistance and/or resistance to TX-100 penetration. NFκB reporters linked to either GFP or luciferase were found to be activated about 5-fold above background by treatment of the organotypic models with TNFα. GFP was detected in formalin fixed paraffin sections by epifluorescence microscopy. Luciferase activity in tissue extracts was quantified with a microplate luminometer. Production of models containing other reporters of toxicological significance (e.g. for DNA damage, oxidative stress, heavy metal stress, ER stress, etc.) by the same process will provide a suite of human epithelial reporter models that can be utilized to provide mechanistic toxicity screening assays.

V-1-617

Optimized 1- and 3-dimensional isolation and expansion of multipotent human adipose tissue-derived stem cells: evaluation of their multipotency

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In the present study, a modified isolation and expansion procedure for human adipose tissue-derived stem cells (hADSC) in terms of differentiation efficacy and up-scalability is described. The trilineage differentiation potential was investigated upon exposure to various (non)commercially available differentiation media. hADSC, differently isolated, displayed a distinct predestined cell fate. Indeed, cells isolated by means of (i) Ficoll gradient centrifugation and subsequent cultivation as monolayers were identified as having a preference for neuro-ectodermal differentiation. On the contrary, cells with hepatic potency were isolated by (ii) red blood cell lysis treatment and subsequent cultivation as spheres, followed by monolayer culture. Another critical factor affecting differentiation efficacy was the use of selective culture media. Commercially available "cocktail" media, claiming induction of keratinocyte differentiation, mainly led to heterogeneous cell populations with mixed phenotypes. Unidirectional hepatic differentiation, however, was obtained upon exposure to different sequential media, reflecting hepatogenesis *in vivo*. Exposure to the commercially available NPMM®/NPDM® media resulted in the formation of neural structures, whereas oligodendrocyte-like cells were produced using NeuroCult®/NeuroCult Diff® media. In summary, our data show that hADSC-subpopulations harbour as well neuro-ectodermal as hepatic predestined cells. The isolation/purification methods and differentiation culture media used are essential factors for successful unidirectional differentiation.

V-1-372

A mechanistic rationale for the prediction of skin irritancy effects implemented in a workflow process

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An automated workflow algorithm was developed to predict a molecule's potential for skin irritancy (SI) based on *in vivo* legacy data. A key requirement from safety evaluators was the possibility to distinguish irritants from non-irritants but also to predict the severity of effects. The algorithm was developed for diverse chemical classes using a set of chemicals representative of cosmetics. It adopts the combination of rule-based and regression modeling on structural features and physicochemical descriptors relevant to the underlying mechanisms of action (e.g. bioavailability, reactivity, interactions with cell membranes).

Due to their importance in cosmetic formulations, special emphasis on the measurement and calculation of properties associated with surfactants is drawn in this work. Nonionic surfactants containing polyethoxylated chains are of interest due to their potential to induce SI via interactions with the cell membrane's phospholipid components. One representative structural rule is based on the ratio of EO-chains to alkyl chain length, i.e. HLB. The extent to which these molecules partition into lipid membranes is quantitatively taken into account by descriptors such as the packing indicator (total volume/volume defined by head group), and molecular shape. New surfactant properties related to the packing indicator and HLB values have also been developed. Experiments with model phospholipid membranes were devised to study mechanisms and derive new descriptors. Preliminary results indicate that the extent to which the surfactants penetrate into a lipid membrane correlates with a higher potential to induce SI.

Overall this predictive scheme improves the reliability of SI estimations. A pipeline workflow process has been developed to provide a user-friendly implementation for toxicologists.

Session V-1: Poster presentations

V-1-044

Abattoir-sourced isolated ileum from *Gallus gallus domesticus* as an experimental tool

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A study was undertaken to determine the longevity of active muscarinic receptors on abattoir-sourced isolated ileum preparations from *Gallus gallus domesticus*, with a view to using the tissue as an experimental tool for functional response assays in laboratory experiments. A concentration-response curve for acetylcholine (1-256 μM) was plotted, in the presence and absence of 1, 3 and 6 nM atropine. In a second experiment, unknown concentrations of acetylcholine samples were determined by using an interpolation method. In this experiment, four sample concentrations were used and the calculated values were found to be almost equal to the actual values. Finally, an experiment was carried out to elucidate the effects of post-sacrifice time on the contractile response of the tissue. The results showed that the tissue maintained considerable contractile response at the 6-hour post-sacrifice time-point. Competitive antagonistic activity was observed between acetylcholine and atropine on the chicken ileum, and the pA2 value was calculated to be 9.21 by using an Arunlakshana-Schild plot. The results suggest that isolated ileum preparations of *Gallus gallus domesticus*, obtained from a meat abattoir, can be used as a basic experimental tool for bioassays in routine laboratory experiments. However, its potential as a research tool still needs to be confirmed.

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V-1-065

Connexin43 signalling contributes to spontaneous apoptosis in cultures of primary hepatocytes

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Primary hepatocyte cultures are still considered the "gold standard" in the field of liver-based *in vitro* modelling. However, these experimental tools undergo progressive dedifferentiation and the subsequent onset of spontaneous cell death. The current study was set up to investigate dedifferentiation in cultures of adult primary hepatocytes at the level of gap junctions, which consist of two hemichannels of adjacent cells that are composed of connexin32. For this purpose, freshly isolated adult rat hepatocytes were cultivated under conventional conditions for four days with daily monitoring of connexin expression, connexin localization and gap junction functionality. The latter was low after hepatocyte isolation and increased to reach a plateau during cultivation. This was accompanied by gradually decreasing connexin32 protein amounts and the concomitant appearance of the fetal hepatocellular connexin43 species, as observed both at the transcriptional and translational level. Connexin32 was hereby mainly located at the plasma membrane surface, while connexin43 was preferentially associated with intracellular membrane structures. To elucidate the relevance of these findings, three connexin43 inhibitor strategies were applied, all of which resulted in downregulated connexin43 hemichannel functionality and overall gap junction activity. This was paralleled by decreased expression and activity of caspase 3 as well as by reduced production of Bid. Collectively, these data show that connexin43 signalling actively contributes to the occurrence of hepatocellular dedifferentiation *in vitro* by facilitating spontaneous apoptosis. In turn, this finding suggests that counteracting connexin43 channel functionality might be a potential strategy to increase cell survival in cultures of primary hepatocytes.

V-1-079

Reducing animal use: Validating the ovine psoas muscle from the abattoir as a replacement model for testing in extirpated human uteri using the Gynecare VersaPoint II Electrosurgical System

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Gynecare VersaPoint™ II Electrosurgical System is indicated for the removal of benign intrauterine lesions. A 4 mm Bipolar Resecting Loop Electrode was developed for this system to enable the resection of larger tissue volumes than the 2.5 mm Bipolar Resecting Loop Electrode.

The objective of this study was to compare the 4 mm and 2.5 mm bipolar loop electrode performance in ovine psoas muscle from an abattoir to that in freshly extirpated human uteri. The following characteristics were evaluated: (1) maximum depth of thermal injury, (2) gas volume produced, and (3) tissue removal rate. This study was performed to develop a preclinical tissue model that provides clinically predictive data that is similar to that generated in human uteri and to eliminate the need for dedicated research animals.

Both electrodes were tested in human uteri and fresh ovine psoas muscle. The tissues were placed in a saline treatment tank. The electrodes were mounted on a pivot and controlled by a linear actuator that provided a consistent rate of speed across the tissue. The electrodes were operated in the cut and coagulate modes. The tissue volumes resected, gas generated, and tissue necrosis depths (TTC and NBT viability staining) were measured.

The maximum thermal injury depth was similar for both electrodes in ovine muscle and extirpated human uteri. The 4 mm

electrode provided a greater rate of tissue resection and generated a lower volume of gas than the 2.5 mm electrode.

Thus, freshly harvested ovine tissue from the abattoir provides a predictive preclinical model to assess uterine thermal injury and reduces the need to use dedicated research animals. The tissue removal rate for the 4 mm loop electrode was greater than the resection rate of the 2.5 mm loop electrode without an additional safety risk to the patient.

V-1-080

Development of an *in vitro* assay for the assessment of photosensitizers

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Photoactivation and binding of photoactive chemicals to proteins is a known prerequisite for the formation of immunogenic photoantigens and the induction of photoallergy. Up to now, no adequate *in vitro* alternatives for photoallergenic hazard identification were available. Dendritic cells play a pivotal role in the induction of photoallergic dermatitis. Human peripheral blood monocyte derived dendritic cells (PBMDC) were thus perceived as an obvious choice for the development of a novel *in vitro* photosensitization assay using the modulation of cell surface protein expression in response to photosensitizing agents.

CD1a-/CD14+ monocytes are positively selected from human peripheral blood and differentiated by IL-4 and GM-CSF supplementation for 5 days. Known chemicals with photosensitizing, allergenic or non-allergenic potential were pre-incubated with PBMDCs prior to UVA irradiation (1 J/cm^2). Following 48 h incubation, the expression of CD86, HLA-DR and CD83 were measured by FACS.

All tested photosensitizers induced a significant and dose-dependent increase of CD86 expression after irradiation compared to non-irradiated controls. Moreover, the phototoxicity of the chemicals could also be determined. In contrast, (i) CD86 expression was not affected by the chosen irradiation conditions, (ii) increased CD86 expression induced by allergens was independent of irradiation and (iii) no PBMDC activation was observed with the non-allergenic control. The assay proposed here for the evaluation of the photoallergenic potential of chemicals includes the assessment of their allergenic, phototoxic and toxic potential in a single and robust test system and is filling a gap in the *in vitro* photoallergenicity test battery.

V-1-081

In vitro detection of contact allergens: Development of an optimized protocol and performance of an international ring study using human peripheral blood monocyte-derived dendritic cells

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Allergic contact dermatitis is a delayed T-cell mediated allergic response associated with relevant social and economic impacts. Until now animal experiments (e.g. the local lymph node assay) were supplying most of the data used to assess the sensitization potential of new chemicals. However, the 7th Amendment to the EU Cosmetic Directive will introduce a testing ban for cosmetic ingredients after 2013. *In vitro* alternative methods are thus being actively developed. Although promising results have been obtained with cell lines, their reduced functionality and inherent genomic instability led us to reinvestigate the use of peripheral blood monocyte derived dendritic cells (PBMDCs) for the establishment of a reliable *in vitro* sensitization test. To solve the issues associated with the use of primary cells, the culture and exposure conditions (cytokine concentrations, incubation time, readout, pooled vs. single donors and cytotoxicity) were re-assessed and optimized. Here we propose a stable and reproducible protocol based on PBMDCs. Wider acceptance and feasibility of PBMDCs for the reliable detection of human skin sensitizers were tested in a international ring study. First results will be presented.

V-1-085

Evaluation of multiple drug interactions

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In an attempt to reduce the number of mammals used in drug research, we have been examining the use of chick embryos and found that they may be superior for predicting the effects of drugs. There are not very many reports of multiple drug interactions. Almost all of the studies involve just two drugs. At the present study, we investigated the multiple drug interaction in chick embryos.

Fertilized eggs of White Leghorns were incubated and amitriptyline, fluconazole and disopyramide were injected into the fertilized eggs. Electrocardiograms were recorded after the injection, and heart rate was determined.

In this study, the heart rate was significantly decreased by a combination of amitriptyline, fluconazole and disopyramide. In addition, arrhythmia was produced by a combination of amitriptyline, fluconazole and disopyramide.

We have reported that toxic interactions involving just two drugs were demonstrated in chick embryos. The combination with disopyramide modified the pharmacological effects of amitriptyline or fluconazole in chick embryos and led to an arrhythmia detected in the ECGs. These findings indicate that the multiple drug interaction of amitriptyline, fluconazole and disopyramide has a marked influence on the heart rate in chick embryos. Although the exact mechanism underlying the influence of the multiple drug interaction on the pharmacological effects of the drug remains to be clarified, the multiple drug interaction seems to enhance the toxicity of the drug in chick embryos.

In conclusion, our recording system for ECG of chick embryos may be useful for investigating multiple drug interactions.

V-1-092

The Slug Mucosal Irritation (SMI) assay: A tool to predict ocular and nasal stinging, itching and burning sensations

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The aim of this study was to investigate whether the Slug Mucosal Irritation (SMI) test could demonstrate a relation between an increased mucus production (MP) in slugs and an elevated incidence of clinical ocular and nasal stinging, itching and burning sensations.

The stinging potency of an artificial tear (ArtTear), 5 shampoos (A-E), and 6 nasal formulations (F-K) was evaluated with the SMI-test. Additionally, a human eye and nose irritation test (HEIT and HNIT) was set up (24 participants in each study). Evaluation of the test items was performed by participants and an ophthalmologist (only HEIT) at several time points.

For the study with the shampoos, analyses reveal that (1) a significant positive association existed between immediate stinging reaction reported by the participants and the mean total mucus produced by the slugs (MTMP) (Spearman's Rank correlation = 0.986, $p < 0.001$); (2) ArtTear was best tolerated in both tests; (3) moreover, all shampoos induced higher reactions than ArtTear and water; (4) Shampoo B was the best tolerated shampoo in both tests, while C, D and E resulted in more pronounced reactions; (4) Shampoo A induced the highest MTMP and received higher scores for immediate discomfort; (6) lacrimation might not be a valuable parameter to evaluate the general tolerance of a product. The clinical trial with the nasal formulations is ongoing.

The SMI assay shows promise as an evaluation method for discomfort in the human eye and nose. Screening prototype formulations allows formula optimization prior to investment in a clinical trial.

V-1-093

The cytotoxic and inflammatory response of bronchial epithelial cells exposed to cigarette whole smoke and vapour at the air-liquid interface

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Cigarette smoke contains >5000 chemicals distributed in the particulate and vapour phases (VP). The VP contributes over 90% of whole cigarette smoke (WS), therefore, traditional submerged cultures exposed to cigarette smoke lack a vast proportion of WS as they utilise only elements of the particulate phase. We have developed a whole smoke system enabling exposure of cell cultures to WS at the air-liquid interface (ALI).

Bronchial epithelial cells (NCI-H292) were grown in submerged culture to confluence on semi-permeable Transwell® membranes and raised to the ALI immediately prior to exposure. The cells were exposed to 3R4F reference cigarette WS at a range of dilutions (1:400 to 1:5 smoke:air, v:v) for 30 min. In parallel, cells were exposed to the VP (particulate fraction was removed using an inline Cambridge filter pad). Following 24 h recovery, cytotoxicity and secreted mediators of inflammation were measured using the neutral red assay, ELISA and Meso Scale Discovery platform. Spectrofluorometry was used to quantify particulate depositing onto Transwell® membranes during the whole smoke exposure.

Doses of particulate were calculated, using a standard curve of 3R4F total particulate matter and ranged from 0.67 to 58.53 $\mu g/cm^2$ for a 1:400 to 1:5 smoke dilution. Results demonstrate that the VP is significantly cytotoxic in our system and constitutes 82% of the total cytotoxicity derived from WS exposures. Subtoxic dilutions of WS (>1:250) increased the concentrations of inflammatory mediators compared to cells exposed to equivalently diluted VP. Further studies are underway to determine in-line real-time dosimetry analysis of particle deposition during smoke exposure.

V-1-094

The development of an anchorage-independent growth assay for the assessment of the carcinogenicity of whole mainstream cigarette smoke

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Exposure to tobacco smoke has been shown to induce malignant transformation in cells. In general, previous studies employed cigarette smoke in the form of total particulate matter (TPM) or aqueous extract solution (CSEaq). Whilst this has provided a wealth of information, TPM and CSEaq do not include all the components of cigarette smoke.

Recent technical advances have enabled the exposure of cells to whole mainstream smoke (WS) at the air-liquid interface (ALI), allowing for a more physiologically relevant route of exposure. Here we describe the use of this approach, using an exposure chamber developed in BAT (Patent publication No. WO 03/100417 A1) in conjunction with the anchorage-independent growth (AIG) assay to assess the carcinogenic potential of WS.

BEAS-2B cells, a human bronchial epithelial cell line, were seeded into Transwell® inserts and left to incubate for 24 h. The inserts were transferred to the exposure chamber and exposed to varying dilutions of WS generated by a Borgwaldt RM20S smoking machine under ISO conditions (35 ml puff drawn over 2 s every min) for 15 min. Following a 48 h recovery period, cytotoxicity was assessed using the CellTiter-Glo® assay. In parallel, exposed cells were plated in Noble agar and incubated at 37°C. Colonies were scored after 21 days using the GelCount™ colony counter.

Initial results demonstrate that WS exposure induces colony formation in BEAS-2B cells, with colony sizes directly proportional to the smoke concentration. This model may provide a valuable tool in the assessment of the carcinogenicity of WS and other aerosols.

V-1-096

Involvement of acetylcholine and response to reduction in phosphorylated connexin43 in drug development research for ischemic heart disease

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Pathological sympathetic overactivation and vagal withdrawal are thought to reduce the survival rate after acute myocardial infarction (AMI). Previously, we tested if aortic depressor nerve stimulation improved the survival rate after AMI in rats. AMI was induced by ligating the left coronary artery. After the ligation, 10 Hz stimulation of the intact ADN was started and continued for 30 min in the treatment group, whereas no stimulation was performed in the control group. The survival rate at 60 min after AMI was only 6.6% in the control group, whereas it increased to 76.5% in the treatment group. With regard to life-threatening arrhythmias in acute ischemia, the effect of vagal nerve stimulation has been reported to prevent ventricular fibrillation in animals. Therefore, we investigated the effect of acetylcholine (ACh), a parasympathetic nerve system neurotransmitter, on the gap junction component Cx43 using H9c2 cells.

H9c2 cells, which are spontaneously immortalized ventricular myoblasts from rat embryos, were used due to their conserved electrical and signal transduction characteristics. The cells were cultured in DMEM supplemented with 10% FBS and antibiotics. H9c2 cells were pretreated with 1 mM ACh for 8 h, followed by 1 h of hypoxia. When cells were subjected to hypoxia, the total Cx43 protein level was decreased. The hypoxia group showed a marked reduction in the amount of phosphorylated Cx43, whereas the hypoxia-ACh group showed only a slight reduction compared with the hypoxia group. These results suggest that ACh is responsible for restoring the decrease in the Cx43 protein level, resulting in functional activation of gap junctions.

V-1-105

Novel culture configuration accelerating and enhancing hepatocyte polarization

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We present a novel approach to accelerate and enhance hepatocyte polarization by spatially patterning hepatocytes in a collagen gel on an oxygen-permeable membrane. We fabricated circular microcavities made of collagen gel having 100 μm depth on an oxygen-permeable membrane, and aligned primary hepatocytes within the microcavities. Aligned hepatocytes were overlayed with collagen gel. As a result, the hepatocyte clusters were constantly made in the microcavities ranging from 40-120 μm in diameter. We show that pattern size induced the embedding of some hepatocytes, thus allowing control of the number of hepatocytes used to induce hepatocyte polarity. These hepatocytes in clusters were more rapidly polarized compared with conventional non-patterned hepatocyte collagen sandwich culture and formed functional bile canaliculi at 2 days of seeding. Furthermore, these hepatocyte clusters had wider areas of bile canaliculi compared with those of fully polarized hepatocytes in conventional sandwich culture. We also demonstrated that width of Mrp2-positive bile canaliculi and the cell layer of each patterned hepatocyte cluster were thicker compared with those of conventional non-patterned hepatocyte collagen sandwich culture. Especially, the formation of bile pools at the center of the cluster could be controlled by varying diameter of circular cavity. The culture configuration of our approach would reduce time and culture size for the estimation of drug metabolism including biliary excretion without animals.

V-1-119

Endocrine disruption and steroidogenesis: integrated evaluation from gonadal steroidogenic enzymes *in vitro* and *in vivo* to hormonal balance and fertility assessment *in vivo*

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In the present study, we propose an integrated evaluation of the effects of some endocrine disruptors *in vivo* in male and female adult rats, and *in vitro* in several models. We focused our work on the last step of steroidogenesis and the aim was to determine to what extent *in vitro* models could be predictive of *in vivo* alterations.

We have evaluated the selected chemicals for their ability to (i) modulate the expression and activity of steroidogenic enzymes *in vitro* and *in vivo*, to (ii) disrupt hormonal balance, and to (iii) disrupt certain fertility parameters, during a sub-acute exposure. Gene expression profiles were assessed by RT-qPCR, *in vitro* and *ex vivo* aromatase activity was assessed by the method of tritiated water, blood and gonadal steroid concentrations were evaluated with LC-MS/MS and ELISA.

Our results showed that all treatments induced disturbance of hormonal balance, which was related to gene expression disorders in some cases. For example, rats treated with atrazine presented high levels of estradiol and low levels of testosterone. This observation may be related to aromatase induction observed in testes and ovaries as well as in gonadal primary cultures and cell lines. A decrease of testosterone level is also observed in male rats treated with methoxychlor, which can be linked not only to aromatase induction, but also to HSD17B3 down-regulation.

Taken together, these results contribute to identify toxic endpoints as well as relevant biomarkers of endocrine disruption. Further studies should determine if, on the basis of these data, a new predictive tool could be developed for endocrine disrupting chemicals assessment.

V-1-122

The Doerenkamp-Zbinden Foundation's Chairs on alternatives to animal experimentation: Projects

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The DZF is a Swiss foundation that has dedicated its activities and support to the development and promotion of alternatives to animal experimentation in biomedical research and education according to the well-known 3R principle (replacement, reduction and refinement) in the field of biomedicine. Lately the foundation's strategy has been focused on establishment and support of university chairs on alternatives to animal testing worldwide.

Up to now six endowed chairs have been established worldwide:

1 The Doerenkamp Professorship for Innovations in Animal and Consumer Protection, University of Erlangen, Germany
2 The Doerenkamp-Zbinden Chair of *in vitro* toxicology and biomedicine, University of Konstanz, Germany
3 The Doerenkamp-Zbinden Professorship for Alternative Methods in Toxicology, Utrecht University, The Netherlands
4 The Doerenkamp-Naef-Zbinden Professorship on Alternative Methods to Animal Experimentation, University of Geneva, Switzerland
5 The Doerenkamp-Zbinden Endowed Chair for Evidence-based Toxicology, Johns Hopkins University, Baltimore, USA
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V-1-124

Development of human T cell priming assay using PBLs depleted in regulatory cells for *in vitro* screening of weak contact allergens

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Allergic contact dermatitis (ACD) is mediated by IFNγ-producing hapten-specific $CD8^+$ T cells and down-regulated by different subsets of suppressive cells, i.e. $CD4^+25^+$ Tregs and $CD56^+$ NK cells (Vocanson et al., 2009; 2010). Based on these observations we have developed an *in vitro* human T cell priming assay (hTCPA) based on a classical autologous mixed DC-lymphocyte reaction (MDLR) in the presence of contact sensitizers. Priming of T cells is measured at day 6 by cell proliferation and IFNγ production after *in vitro* restimulation with submitogenic doses of PHA. We have shown that the sensitivity of hTCPA to detect the sensitizing properties of haptens is directly and negatively correlated to the presence of regulatory cells in the assay: i) hTCPA using whole PBLs can only detect strong haptens; ii) when PBLs are depleted in $CD25^+$ cells (depletion of $CD4^+$Tregs), hTCPA can detect strong and moderate haptens; iii) when both $CD25^+$ (Tregs) and $CD56^+$ (NK) cells are depleted, hTCPA can detect strong, moderate and weak haptens. Positive results were obtained with TNBS, pPD, TMD, MGN, Oxazolone, Eugenol, Isoeugenol. The specificity of the hTCPA is excellent, since no significant priming was obtained using non-sensitizer compounds (Glycerol, Salicylic Acid or Lactic Acid). Collectively our results provide strong evidence that hTCPA is a sensitive method to test for the immunogenic properties of contact allergens, especially those endowed with weak sensitizing properties.

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V-1-132

Modification of an existing mouse model of human skin infection to reduce animal use

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There are many murine models of human skin and soft tissue infections in the literature. The majority of these models (90%) involve a single infection lesion per animal. We investigated the impact on bacterial load, lesion size, and body weight of a second local skin infection in the same animal.

Mice were infected subcutaneously in one or both flanks of the animal with *Pseudomonas aeruginosa* suspended in dextrin beads. Animals were weighed and treated twice-daily for two days with doripenem (DOR), a parenteral carbapenem antibiotic, starting one hour after infection. Forty-eight hours post-infection, the animals were euthanized, weighed, skin lesions measured, collected and the number of bacteria in the skin was determined.

Untreated control animals had bacterial loads of 8.7 and 8.8 log10 CFU/g skin tissue present in animals with single- or double-sided infections, respectively, 48 h after infection. Treatment with DOR (1.6-100 mg/kg/day) resulted in similar, dose-dependent reductions in bacterial load and lesion size in animals having either single- or double-sided infections. No significant statistical difference in bacterial load, lesion volume, or % body weight loss was observed in animals with single- vs. double-sided infections. This improved method resulted in reducing animal usage by 50%, while increasing the throughput of compounds tested in this model.

V-1-133

pH cycling models for evaluating the efficacy of fluoridated mouth rinses for caries control

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New anticaries fluoride formulations in the USA are required to meet animal caries testing requirements as defined by the US FDA anticaries final monograph (21 CFR 355.70). The final monograph allows for the use of alternate models of "equivalent accuracy" to the animal caries model. pH cycling models are *in vitro* protocols that involve exposure of dental substrate (either enamel or dentin) to a series of demineralizing and remineralizing conditions to mimic the dynamics of mineral loss and gain involved in caries formation. pH cycling models have been shown to demonstrate fluoride dose responses equivalent to those observed in the animal caries model. Therefore, several pH cycling studies with varied protocols were carried out to study enamel fluoride uptake and remineralization efficacy of fluoridated mouth rinses to provide additional support for the anticaries potential of marketed products: Listerine® Total Care® (LTC) and Listerine® Smart Rinse™ (LSR), marketed by Johnson and Johnson Consumer Companies Inc.

Using pH cycling studies and combinations of response variables, such as surface micro hardness (SMH), cross-sectional micro hardness (CSH) and fluoride content (from various enamel depths), we evaluated different mouth rinse formulations with varying fluoride concentration as well as different treatment regimens. These studies provided an understanding of various factors and processes that determine fluoride efficacy in mouth rinses and also allowed us to successfully demonstrate the remineralization efficacy of LTC and LSR. Controlled pH cycling models that have been optimized with respect to the substrate used, lesion depth, and measurement and analysis tools are reproducible and cost effective in evaluating anticaries potential of fluoridated mouth rinses. These models have effectively mitigated the need for animal caries testing or human clinical studies to support efficacy claims. Further improvements to the pH cycling protocols are being implemented to improve their predictive value by including environments that mimic complex salivary components and plaque fluid, ionic concentration, etc.

V-1-147

Alginate based 3D hydrogels as an *in vitro* co-culture model platform for the toxicity screening of new chemical entities

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Accurate prediction of human response to potential therapeutic drugs and vaccines is done by conventional methods of *in vitro* cell culture assays and expensive *in vivo* animal testing. Alternatives to expensive animal testing require sophisticated *in vitro* model systems that replicate *in vivo*-like function for reliable testing applications. Advancements in biomaterials have enabled the development of three-dimensional (3D) cell encapsulated hydrogels as systems to mimic *in vivo*-like function for *in vitro* models used in drug screening. In this study, we have developed a platform to enable 3D high density (~10^7 cells/ml) culture of liver cells combined with a monolayer growth of two-dimensional (2D) target breast cancer cell line (MCF-7) in static culture environments for *in vitro* drug toxicity testing. A test platform that incorporates a porous poly-carbonate disc is integrated within standard cell culture plates to enable the co-culture of multiple cell types. Alginate hydrogels encapsulated with serial cell densities of HepG2 cells (10^6-10^8 cells/ml) were supported by the disc platform and co-cultured with MCF-7 breast cancer cells during a 3 day study period. The clearance rates of drug transformation by HepG2 cells were measured using the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) metabolized to HFC (7-hydroxy-4-trifluoromethyl coumarin). The platform was used to test for HepG2 toxicity using commercially available known drugs such as acetaminophen, diclofenac, rifampin and quinidine. The cytotoxicity 50% value of candidate drugs derived from dose-dependent testing using our platform correlate well with the reported *in vivo* LD_{50} values. The developed test platform allowed us to evaluate drug dose concentrations to predict hepatotoxicity and its effect on the breast cancer cell line. The *in vitro* 3D co-culture platform provides a scalable, reusable and flexible approach to test multiple-cell types in a hybrid setting within standard cell culture plates and therefore opens up novel, relatively inexpensive techniques to screen NCE compounds.

V-1-154

An image processing analysis of corneal alteration induced by chemicals

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Sodium fluorescein is widely used for the evaluation of ocular surface integrity. The fluorescent property of this molecule allows the corneal defects to be directly examined and visualized by the intensity of the ocular surface staining. For ophthalmological practitioners, slit lamp biomicroscope examination and the assessment of the type, depth and area of corneal injury are performed after sodium fluorescein instillation and observation using a cobalt blue filter. This routine and useful exam permits the discrimination and classification of an ocular irritant based on the extent of the corneal defects. Several *in vitro* ocular models currently exist to predict moderate and severe/corrosive ocular irritation; nevertheless these models lack accuracy to discriminate between mild/slight/very slight ocular irritants. Even if these methodologies are highly informative, they do not offer at the same time a rapid and direct assessment of the area and the depth of the corneal injury. The aim of this study was to propose and investigate the reliability and validity of a method based on the Principal Component Analysis of injured cornea images for objective and quantitative measurement of surface and depth of corneal injury induced by chemicals. Such methodology could be a useful tool for cosmetic industries for the safety assessment of ingredients and finished cosmetic products.

V-1-162

Evaluation of the individual and synergistic value of the HET-CAMVT and the dynamic solubility model in order to predict the potential of new IV formulations to cause injection site reactions

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Prior to administering an intravenous (IV) drug formulation in humans, it is necessary to evaluate its vascular irritation potential so that clinical injection site reactions (ISRs) and, as a consequence, a delay in the clinical development of a compound, can be prevented.

Two *in vitro* models that have shown promise for improving the screening process and reducing the need to evaluate formulations *in vivo* are a dynamic solubility model developed by Yalkowski et al. (2003) and the HET-CAMVT (Vascular Toxicity Hen's Egg Test-Chorioallantoic Membrane). While in the dynamic solubility model, the precipitation of the solution infused in a tube is monitored, in the HET-CAMVT the irritation potential of the formulation is evaluated after topical application on to the vasculature (coagulation, hemorrhage and lysis).

The aim of this study was to evaluate the individual and synergistic value of both models in order to predict ISRs. Different IV formulations with known *in vivo* ISR were tested in a dose dependent manner both in the HET-CAMVT and the dynamic injection model.

It was shown that the results of the HET-CAMVT model correspond well with the observed *in vivo* ISR. In the dynamic solubility model a clear dose response was observed, although at other concentrations compared to HET-CAMVT. By testing more compounds that cause irritation by different mechanisms and by evaluating the importance of different parameters in the dynamic solubility model, both models can help us to guide whether a compound structural change or a formulation change is needed to avoid clinical ISRs.

V-1-193

Development of an integrative approach for the prediction of systemic toxicity: Combination of cell toxicity, pharmacological and physical chemical properties

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Ethical, scientific and economic constraints have motivated the scientific community to develop alternatives to animal testing. Developing alternatives for acute/chronic systemic toxicity testing represents a challenge because of the complex biological processes implied. A realistic approach could rely on the combination of data generated for multiple endpoints. The Ctox panel®, which is a multiparameter cell-based *in vitro* system for predicting rat acute systemic toxicity, is a typical example. Preliminary studies conducted in a blinded manner showed a good sensitivity and specificity (91% and 78%), while defining a LD_{50} threshold at 2000 mg/kg. However, the model failed to accurately predict very toxic chemicals displaying LD_{50} below 300 mg/kg. Further to an in-depth analysis of the misclassified chemicals, we concluded that both pharmacological data (for the reduction of false negatives) and physical-chemical properties (for the reduction of false positives) had to be considered. The modified approach was applied to 76 non-proprietary compounds previously tested with the standard method. A significant improvement in the prediction of the GHS categories was observed. Indeed, 75% of the chemicals pertaining to GHS 1, 2 and 3 were correctly classified, compared to 50% with the standard model. In addition, at an arbitrarily defined LD_{50} threshold of 500 mg/kg, the sensitivity and specificity were 85% and 89% with the new model against 71% and 83% with the standard model. Future directions will consist of challenging the newly built model with a new set of chemicals and foreseeing the application of such a strategy for repeated dose-toxicity.

V-1-199

Development of an integrative approach for the prediction of systemic toxicity: Combination of cell toxicity and metabolism data

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Increasing societal concerns for animal welfare and current legislation constraints have made the industry enter a new phase in its innovation and R&D processes. For acute and chronic toxicity testing, which consumes a large number of laboratory animals, no alternative is available, one of the reasons being the complexity of the biological processes involved. As such, many research programs have been launched with the aim of developing integrative approaches that would accurately predict such an endpoint. The purpose of the study presented herein was to develop an integrated testing strategy on the basis of the data previously generated with The Ctox panel®, a multiparameter, cell-based *in vitro* system and the Solidus® DataChip/MetaChip platform, developed to assess metabolism-mediated toxicity. The set used consisted of 63 proprietary chemicals, categorized as Toxic (25 compounds) and Non Toxic (38 compounds) on the basis of a LD_{50} threshold of 500 mg/kg in rat following oral administration. A statistical analysis of the data led to the construction of several integrative models. The most predictive model (discriminant analysis) required the consideration of a total of 22 parameters. On the basis of a LD_{50} threshold of 500 mg/kg, the sensitivity and specificity of the prediction was 92% and 87%. The next steps will consist of challenging the model with a set of diverse chemical classes. It is noteworthy that the number of parameters considered for the model correlates well with the complexity of the endpoint mentioned above.

V-1-211

Cardiovascular model for cardiac toxicity testing

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The heart has been shown to be particularly prone to toxic effects of cardiac and noncardiac drugs. Adverse cardiac drug reactions are typically serious and even fatal. The aim of this study was to develop a cardiovascular model for *in vitro* testing of pharmacological and toxicological potential of compounds. The model was constructed by plating neonatal rat cardiomyocytes on the top of the co-culture of human fibroblasts and endothelial cells forming a network of tubular structures. As controls, cardiomyocyte monoculture and tubular network co-culture were established. The specific goal was to investigate whether the presence of tubular structures has an influence on survival and functionality of primary cardiomyocytes.

The results showed that by culturing cardiomyocytes with tubular structures the survival and functionality of neonatal rat cardiomyocytes was improved. Cardiac cells contracted synchronously for at least 14 days a cardiomyocyte monoculture maintained contractile function only for 7 days. The number of cardiomyocytes and the composition of stimulation media were found to be critical. Immunocytochemical staining demonstrated that the cardiomyocytes were oriented in line with the formed tubular structures, and the typical morphology of mature cardiomyocyte was observed.

In conclusion, interaction between tubular structures and cardiomyocytes seems to be important in promoting neonatal cardiomyocyte cell survival and their functionality. The developed model has great potential to be further developed to a routine test for pharmaceutical and toxicological industry. Further studies aim at accomplishing a completely human cell based cardiovascular model by using human pluripotent stem cell derived cardiomyocytes.

V-1-216

An organotypic microliver platform for high throughput drug testing

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Predictive toxicology testing is a major challenge in regulatory toxicology and drug discovery. Traditionally, many toxicity tests are performed in animals. However, the need for testing thousands of compounds in a high-throughput manner would require millions of animals for toxicity testing. Therefore, novel models for predictive toxicity testing *in vitro* are urgently required. So far, mainly monolayer cultures of primary hepatocytes were used to study drug metabolism, enzyme induction and compound hepatotoxicity *in vitro*. While 2-dimensional sandwich cultures improve maintenance of hepatocyte function, these systems are technically challenging, of little use for chronic toxicity testing and only fairly suited for higher throughput. In order to preserve liver-specific functions of hepatocytes over extended time periods and to provide a versatile platform for toxicology testing, we developed a novel scaffold-free, organotypic production technology for a 3-dimensional culture of rat liver microtissues in a standard 96-well microtiter plate format. The GravityPLUS system allows the production of regular-sized microtissues with a morphology and architecture close to native liver tissue when co-cultured with non-parenchymal liver cells. Stainings of paraffin-sections indicate tight cell-cell contacts, extensive glycogen storage and formation of bile canaliculi between hepatocytes. The rat liver microtissues show stable cell survival and CYP3A induction over 5 weeks in culture. Our results demonstrate the validity and suitability of the GravityPLUS hanging drop platform for liver microtissue culture, offering a highly functional and reliable *in vitro* toxicology testing system which can easily be implemented in standard lab automation processes.

V-1-221

A pertinent screening tool to measure permeability coefficient: Episkin® reconstructed human skin model

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According to their similarities to native human tissue in terms of morphology, lipid composition and biochemical markers, reconstructed human epidermis (RhE) has been identified as a useful tool for the *in vitro* testing of phototoxicity, corrosivity and irritancy. These last years, some papers claim that RhE is an appropriate alternative to human skin for the assessment of skin permeation and penetration *in vitro*.

Among all RhE models commercially available, Episkin® from SkinEthic is particularly adapted for testing. Indeed, its design allows to measure penetration directly in the insert without mounting the tissue in a diffusion cell. These results lead to the development of reliable protocols for the upstream ranking assessment of the skin penetration of cosmetic ingredients under their conditions of cosmetic use.

Permeability coefficient (Kp) measurement requires sampling as a function of time. It could be done using a flow through diffusion cell (as PermeGear cell) or using the insert directly with total or partial replacement of a given volume of receptor fluid at given time gaps. Both approaches have been tested with caffeine as reference compound. With sink condition and infinite dose, flux as a function of time should reach a constant value corresponding to the steady state. Results show that steady state is not reached with the PermeGear cell contrary to the insert.

Comparison with human skin data reinforces previous studies' conclusions on RhE model as a relevant alternative to human skin for *in vitro* penetration studies.

V-1-225

Alternative approach to maximum flux for TTC applied to safety evaluation of cosmetic ingredients

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REACH legislation will increase the toxicology evaluation drastically. To meet this challenge, relevant and accurate prediction of dermal uptake/exposure of topically applied chemicals is essential for risk assessment. This could be obtained without the need for an experimental measurement, avoiding any problems with ethical issues, recruiting volunteers or housing animals, using QSAR models.

Typically, QSAR model predicting permeability coefficients (i.e. kp) are used. Many models were developed; all of them led to the same conclusion: small lipophilic chemicals have the greatest skin permeability. This analysis often causes confusion. The dataset used to build up this relation considered percutaneous transport from aqueous solution. Whereas, kp increases with log P, aqueous solubility decreases with lipophilicity. The resulting flux and effective absorbed amount of chemical are then balanced between two competitive factors (permeability and solubility).

The concept of maximum flux means that a chemical cannot cross the skin faster than the flux measured at steady state with a chemical applied on the surface in saturated solution (or in neat chemical form). It allows assessing the maximum absorbed dose. This concept was recently used in a TTC approach for cosmetic ingredients. A classification of potential of cutaneous chemical absorption was proposed on the basis of the substances' physicochemical properties. Unfortunately, the proposed classification does not cover the full range of molecular weight and log P. Moreover, some physicochemical properties known to affect cutaneous absorption (i.e. ionisation, volatility) are not considered. At least, the default proposed values greatly overestimate the absorption obtained experimentally.

An alternative approach was developed by L'Oréal to overcome these limitations.

V-1-227

Adaptation of the validated SkinEthic RHE skin corrosion test method to 0.5 cm^2 tissue samples

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In vitro human reconstructed epidermal models have been used to develop protocols able to discriminate corrosives from non-corrosives. The SkinEthic RHE test method, using 0.63 cm^2 inserts, was validated in 2006. Due to manufacturing constraints, the model is now produced in 0.5 cm^2 instead of 0.63 cm^2 sample sizes.

This study demonstrates that the RHE skin corrosion protocol could be adapted from 0.63 cm^2 to 0.5 cm^2 RHE samples. For such purpose, the protocol size adaptation was performed using 25 chemicals including the 12 OECD TG431 reference chemicals. To test the robustness and relevance of the test method, particular attention was given to choosing chemicals that are correctly classified (non-corrosive, corrosive) but also chemicals known to be misclassified (false positives/negatives). Results obtained with the 0.5 cm^2 skin corrosion test method showed that all corrosives were correctly classified, and 11 out of 13 chemicals were identified as non-corrosives. The overall accuracy over the 25 chemicals was 92%. The specificity and the sensitivity of the OECD chemicals were 100%.

In addition, the quality of RHE tissues was not only maintained but also improved. The quality control, performed on 136 (0.63 cm^2) and 262 (0.5 cm^2) RHE batches, showed a mean of 0.99 and 1.17, respectively. This similarity over years demonstrates the high quality production of the tissues using both viability and morphology parameters.

In conclusion, the quality of 0.5 cm^2 SkinEthic RHE tissues was thoroughly maintained over 9 years and the performance of the skin corrosion test method fully met OECD and ECVAM requirements.

V-1-248

Development of an alternative to the oral mucosa irritation test by modified STE test

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In recent years, the economy of urban areas in China has been greatly developed, and many oral care products are imported from overseas. However, the classification of oral care products used to be unclear in China. Since 2008 they have been classified as cosmetics, although oral mucosa irritation has rarely been evaluated. The Shanghai CIQ is responsible for safety management of domestic and import cosmetics, and Japan and China collaborated to develop an alternative method for evaluation of oral mucosa irritation. An oral mucosa irritation study was performed in Hartley guinea pigs with reference to CTFA Guidelines and obtained the irritation score. The alternative method employed was a modified STE method (mSTE), where the cell viability was evaluated after exposing the study article (concentrations: 5.0%, 0.5%, 0.05%) for a short time to SIRC cells or 3T3 cells. Eighteen raw materials for oral care products and fifteen products (eleven marketed products) were tested, and the results were compared with those of animal studies. CPC and SLS showed potent irritation of the oral mucosa, although glycerin and sorbitol did not show any irritation. In addition, all of marketed products showed slight irritation. These results corresponded well with those obtained from the mSTE test, and the high concordance rate was seen when SIRC cells were used and the exposure concentration was 0.5%. Based on these results, it was suggested that the mSTE could possibly be used as an alternative method for an irritation study in the oral mucosa.

V-1-249

Improving human vascularized mucosa/intestine models to study substance adsorption phenomena

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Robust and reliable dynamic bioreactors for long-term maintenance of various tissues at ml-scale within a vascularized matrix, i.e. BioVaSc®, have been developed at the Fraunhofer IGB in Stuttgart, Germany. Once seeded with Caco-2 cells, such vascularized matrix bioreactors support self-assembly of an intestinal microenvironment with the typical histological appearance of villi structure and morphology (Schanz et al., 2010). This indicates that in dynamic bioreactors microcapillary vasculature and proper architecture support organoid self-assembly. To further improve the equivalence of the system to human *in vivo* performance we have replaced the Caco-2 cell seeding by seeding of human primary intestinal tissue. An improved bioreactor hardware operating at least four identical dynamic bioreactors simultaneously has been engineered. The hardware supports long-term bioreactor operation over weeks and months. Process parameters, such as nutrient perfusion rate, medium composition in the inner vascular space and in the inner gut lumen, as well as culture time/duration, have been optimized to qualify the system for repeated dose testing of orally administered drug candidates on adsorption properties. Daily intravascular and inner lumen samples have been analyzed to monitor metabolic activity of the tissue culture. Histo- and immunostaining of cryopreserved or paraffin-embedded tissue slices have been analyzed to compare self-assembled organoid tissue structures with their corresponding *in vivo* counterparts. Evidence is provided for the use of the system for reliable evaluation of adsorption properties of drugs at different dosages over long periods.

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V-1-268

Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes

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Technological developments are driving *in vitro* methods towards integrated "omic" strategies. However, there is still an over reliance on classical viability assays for dose range finding. Such assays require termination of the experiment, which makes it difficult to monitor temporal alterations. To this end, we investigated the use of lactate production as a marker of cell stress in long term repeat dose *in vitro* experiments.

We conducted daily exposures to 8 compounds at 5 concentrations for 14 days on human renal proximal tubular cells (RPTEC/TERT1), human hepatoma cells (HepaRG) and mouse fibroblast (BALB-3T3) cells. Compounds were chosen from a training set used in the 7th EU Framework project Predict-IV. At days 1, 3, 7 and 14, lactate was measured in the supernatant medium. At day 14, cells were assayed for resazurin reduction capability and subsequently lysed in methanol for ATP determination.

Compound-induced loss of viability was comparable across all cell lines. In all cell types, lactate production was induced prior to a decrease in viability. In some situations, lactate also fell below control values, indicating cell death. Thus, temporal alterations in supernatant lactate alone gave information on the time and concentration of stress induction and the time and concentration of loss in viability.

Supernatant lactate production is a simple, cheap and non-invasive parameter. Since many molecular pathways converge on the glycolytic pathway, enhanced lactate production may be considered as a global marker of sub-lethal injury and thus an ideal marker for investigating temporal alterations in long-term repeat dose testing *in vitro* regimes.

V-1-285

Correlation of *in vivo* and *in vitro* degradation profiles for bio-absorbable polymer implants

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A key goal in characterization of bio- absorbable polymer is a good understanding of its *in vitro* and *in vivo* degradation behavior. One of the challenges of absorbable polymer research is to develop a tool that correlates the *in vitro* degradation strength profile from several formulations (different buffer and temperature settings) to the *in vivo* degradation strength profile. Developing a predictable *in vitro* tool not only saves animals used for preclinical studies but also shortens the product development cycle. Our poster showcases examples of correlation studies conducted for bio-absorbable polymer implants that can be used as guidance for future correlation work.

In vivo degradation is performed by implantation in Long Evan rats' posterior dorsal subcutis (between the skin and skeletal muscle). Animals are sacrificed at specific time points and test materials are explanted. *In vitro* degradation is conducted by submerging the samples in phosphate buffered solution of pH between 7.27 and 8.8 and the temperatures of the bath can vary between 37-55°C depending on the polymer. The samples are tested using a material testing machine. Testing method is specific to each material (tensile, shear, etc.).

Example 1: Poly(p-dioxanone) (PDS) anchor attached to PDS suture. *In vitro* conditions: pH 7.27 maintained at 37°C temperature. Both *in vitro* and *in vivo* testing were conducted on a weekly basis for 10 weeks. Results from the *in vitro* and *in vivo* studies indicate that the strength profiles have similar trends with a drop in strength after 7 weeks. Statistical analysis was conducted using Minitab software to correlate the *in vitro* and *in vivo* degradation profiles.

In vitro strength (lbs) = bo+ co* *in vivo* implant strength (lbs), where bo & co are constants.

Example 2: PALG anchor. *In vitro* conditions: pH 7.27 maintained at 37°C temperature. Tests were conducted on up to 91 days; *in vitro* and *in vivo* curves for shear and tensile tests.

Shear strength *in vivo* = b1 * *in vitro*, where b1 is a constant.

Tensile strength *in vivo* = c1* *in vitro*, where c1 is a constant.

These studies indicate that good *in vitro* models can be developed to predict *in vitro* polymer behavior.

V-1-303

Development of cell-based endocrine disruptor screens using automated image analysis to quantitate receptor binding and dynamics

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Endocrine disruptors are compounds that alter steroid activity, thereby perturbing endocrine system functionality. Environmental endocrine disruptors have been linked to numerous adverse health effects and reproductive problems in both humans and wildlife. The advent of complex chemical library production in addition to a large catalog of existing compounds necessitates an automated procedure to assess a chemical's *in vitro* endocrine activity prior to investigating potential organismal and environmental impact. Utilization of *in vitro* screens for potential endocrine active chemicals (EACs) reduces animal testing by categorizing and prioritizing chemicals based on their ability to alter endocrine receptor activity.

We have designed a cell-based workflow to efficiently test chemicals for their ability to activate redistribution GFP-tagged steroid receptors estrogen receptor alpha (ERα) and androgen receptor (AR). These GFP-tagged receptors form nuclear foci in response to stimulation that can be easily imaged and quantitated by high content analysis, thereby establishing an automated *in vitro* assay for EACs. Redistribution ERα and AR cell lines are assayed under similar conditions, simplifying EAC screen-

ing. The dose response for each test chemical is compared to a vehicle control, a weak response control, and a positive control dose response via user-defined thresholds to visualize both the potency and magnitude of the EAC response. Additional outputs provide insight into the dynamics of receptor activation while simultaneously monitoring cell cycle perturbations and cytotoxicity. Together these assays provide a detailed *in vitro* mechanism of endocrine receptor activation resulting in a more thorough assessment of the chemical's potential *in vivo* endocrine disruption.

V-1-305

Replacing the use of animals in antibody production

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Monoclonal antibodies (mAbs) are ubiquitous in research and medicine. They have emerged as effective therapeutic treatments for cancer, various auto-immune disorders, and other illnesses. In molecular biological research, mAbs are reagents often used to detect and measure protein and drug levels in biological fluids and to register changes in cellular proteins after exposure to a chemical agent.

The mouse ascites method of mAb production is now widely discouraged due to the pain and distress it causes the animals used and the wide availability of *in vitro* alternatives. However, the misnamed "*in vitro*" method continues to rely on mice for initial generation of the antibody and presents serious animal welfare concerns as well as methodological limitations.

Fortunately, there are non-animal alternatives available for the generation of antibodies. Aptamers and recombinant antibodies (rAbs) can be created without the use animals or animal tissues and can be used in the same ways as traditional monoclonal antibodies and can be engineered to recognize a wider range of epitopes than traditional mAbs. We explore the proven applications of both rAbs and aptamers while illustrating the efficiency and efficacy introduced when these fully *in vitro* methods are employed.

V-1-317

Ex vivo tape striped human skin model: an alternative method to animal testing for skin pharmacology studies and the pharmacological evaluation of cosmetic ingredients and finished products

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Topically applied products need to be evaluated with regard to safety and clinical efficacy. Mostly the efficacy of cosmetic products is delivered by active ingredients in the formulation. Previously the efficacy of the ingredients or formulated product was determined by testing in animal models before conducting human clinical studies. For pharmacological efficacy, few non-animal models existed which were as physiologically similar to human skin. We therefore established a model involving skin obtained from non-invasive tape-striping, studying effects on skin cells attached to the tape and quantifying skin physiological responses. The tape striped human skin model is an effective way to quantify biomarkers from the skin of subjects in clinical studies of various skin conditions, such as diaper rash, aging, and the measurement of antimicrobial peptides present on facial skin as related to acne. This quantification has been validated in freshly acquired clinical samples as well as after prolonged storage at subzero temperatures. Since these noninvasive methods can also be used to evaluate skin *in vivo* as a function of age, regional site variations, and external challenges, this technique is ideal for clinical assessment and as an investigative tool for skin biomarker studies as it is simple, non- invasive and versatile. To summarize, this model is a sensitive, non-invasive technique to assess skin inflammation, oxidative stress, skin differentiation and biomarker expression in clinically acquired samples in lieu of animal testing.

V-1-331

In vitro BBB modeling: From research to high-throughput screening

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Since the early 90's, our goal is to focus on the development of relevant *in vitro* Blood-Brain Barrier (BBB) models adapted for all stages of R&D and for all users. The BBB is a physical and metabolic barrier located at the level of brain capillaries, which regulates exchanges between blood and brain, maintains brain homeostasis and provides defense against blood-borne toxins or infective agents.

Our most famous BBB model consisting of a co-culture of bovine brain capillary endothelial cells (BBCE) together with rat glial cells has been successfully used for medium-throughput screening and mechanistic purposes in a number of major pharmaceutical companies for more than a decade. By modifying this highly predictive model, a procedure has been developed to obtain a differentiated endothelial cell monolayer after only 4 days and without using primary glial cells, which substantially reduces the use of animals. This model represents the first *in vitro* BBB model suitable for High-Throughput Screening (HTS) of compounds (drugs, chemicals, cosmetics and consumer products). Furthermore, a new generation BBB *in vitro* model that is easy to use, quick and suitable for HTS, is now available in frozen ready-to-use format and consists of BBCE frozen onto collagen-coated 24-well cell culture insert plates. This model considerably reduces the technical needs to obtain after only 4 days a functional *in vitro* BBB model.

These models answer growing needs to identify compounds that have the lowest risk for toxicity and highest probability of success in accordance with the concept of the 3Rs.

V-1-336

New tools for discovery: stem cells, adipose tissue engineering and small animal imaging

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Tissue engineering represents a key technology that will ultimately enable the replacement and reduction of animal use. Our tissue engineering strategy is based on adipose-derived stem cells (ASCs). These accessible and abundant cells can differentiate into a variety of cell types, including adipocytes. We have used human ASCs to recreate *in vitro* reconstructed tissues very similar to their native counterparts. Using the self-assembly approach of tissue engineering, tridimensional adipose tissues, devoid of exogenous materials but featuring functional adipocytes, were reconstructed. They secreted a variety of bioactive molecules (leptin, VEGF, Ang-1) in addition to their capacity to mediate lipolysis following stimulation by various agonists. When endothelial cells were incorporated, they assembled into a newly formed network of capillaries throughout the adipose construct. The structure of this network was modulated by use of TNFα. Therefore, reconstructed adipose tissues represent innovative tools to test cosmetic/pharmacological products and study their impact on metabolic functions of the adipocyte/vasculature, which play central roles in obesity and related complications. Finally, translational magnetic resonance imaging (MRI) was performed in order to image and quantify the volume of the reconstructed adipose tissues after implantation onto nude mice. MRI allowed the non-invasive assessment of the grafted tissues on the same animal over weeks, reducing by at least 80% the number of animals required. In summary, the joint evolution of stem cell and tissue engineering research, coupled with advanced imaging modalities, will contribute to the reduction of animal use in both basic and applied research.

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V-1-337

Utility of the *in vitro* mutagenicity assay in Muta™Mouse FE1 cells for regulatory assessment of genotoxicity

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In vitro assays for mutagenicity assessment provide a rapid and effective means for evaluating various types of chemical products. However, mammalian cell assays that are commonly used for regulatory decision-making show high sensitivity, but low specificity for *in vivo* genotoxicity and carcinogenicity. In other words, these assays elicit a high frequency of "false" or "irrelevant" positives for *in vivo* effect. The *in vitro* assay based on pulmonary epithelial cells (i.e., FE1 cells) from Muta™Mouse has proven useful for mutagenicity assessment of a variety of chemical agents including PAHs, aromatic amines, nitroarenes, extracts of complex environmental matrices, and nanoparticulate materials. The performance of the FE1 mutagenicity assay was evaluated by examining the response (i.e., frequency of lacZ mutations) to 9 non-DNA-reactive (i.e., Ames test-negative) chemicals that have been reported to elicit irrelevant positives in regulatory *in vitro* assays (i.e., chromosomal aberrations and micronuclei in various cell lines, and/or tk mutations in mouse lymphoma cells). When tested up to the 10 mM/5 mg/plate limit, 8 compounds were negative in the FE1 Muta™Mouse assay, and one compound (eugenol) was positive at the highest sub-toxic doses. Analysis of true positives and true negatives confirmed satisfactory assay performance (i.e., ~20-fold greater than control and no significant response, respectively). All compounds are currently being re-tested in the presence of an exogenous S9 metabolic activation mixture to confirm the negative findings. In addition, detailed cytotoxicity assessment is underway. Collectively, these results will contribute to the continuing evaluation of the *in vitro* mutagenicity assessment system based on Muta™Mouse FE1 cells.

V-1-350

Development of a multicolor luciferase assay system for *in vitro* chemical risk analysis

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The reporter assay system using luciferase is widely used as a conventional and powerful tool for quantitative monitoring of gene expression. Recent advances in luciferase technology, involving improvements in both the luciferase and the detection system and a newly cloned luciferase gene, allow us to monitor the expression of multiple genes simultaneously when luciferase are used that induce differently colored emission spectra in the catalysis of a common substrate. Recently, we have developed a multicolor luciferase assay system in which three gene expressions can be simultaneously monitored using green- (SLG, λ_{max}=550 nm), orange- (SLO, λ_{max}=580 nm), and red-(SLR, λ_{max}=630 nm) emitting luciferases. To develop a rapid- and high reliance-chemical *in vitro* risk analysis assay system, we applied the multicolor luciferase assay system to immunotoxicity tests. We generated a multicolor stable cell line by introducing three reporter vectors (IL-2-SLG, IFNγ-SLO and G3PDH-SLR) into Jurkat cells. To verify the accuracy of the measurement system, the stable cell lines were seeded on a 96-well plate and respective luciferase activities were measured. We successfully measured expression of two marker genes and one internal control gene (total 288 gene expressions) within 20 min, with a coefficient of variation of less than 15%. In addition, we confirmed that induction of IL-2 and IFNγ expression stimulated by PMA/ionomycin were stably maintained during eight weeks after start of culture. These results suggest that combined use of the measurement system and the stable cell line established in this study are useful tools for chemical risk analysis.

V-1-357

Quantitative proteomic profiling for drug toxicity prediction in human organ model systems

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The objective of the European Union funded project Predict-IV is to implement an effective drug test system using specific markers to predict toxicity in an early development stage and reduce costs and time. Therefore, primary cells and cell model systems for kidney, liver and CNS are treated with organ specific reference toxins and different omic approaches are used for biomarker identification.

Human renal proximal tubular epithelial cells (RPTEC/TERT1) for nephrotoxicity profiling were treated with reference toxins. Processed cells and controls were harvested after 1, 3 and 14 days in 3 biological replicates. After tryptic digestion and isobaric labeling using iTRAQ, peptides were separated by capillary high performance liquid chromatography (HPLC) followed by LTQ-Orbitrap mass spectrometry (MS). We used the open source software OpenMS which allows the combination of different search engines to increase the number of identifications and the quantification of labeled peptides over several iTRAQ-experiments.

HPLC-MS analysis of 27 samples comparing untreated cells with cells that have been exposed to the immunosuppressant drug Cyclosporin A led to the identification of more than 2500 proteins. Differential quantification is based on the observation of iTRAQ reporter ions representing the changes in protein abundances induced by drug stimulation. The obtained dataset serves as a basis for systematic pathway analysis using bioinformatic tools in order to reveal proteins that are indicative of toxic effects in target tissues. Putative biomarkers will be further corroborated by analyzing the effects of other reference compounds and comparison with transcriptomic and metabolomic data.

V-1-358

Development of a new reconstructed human epidermis (RhE)-based screening assay for contact allergens

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Recent changes in regulatory restrictions and social views against animal testing called for the development of reliable *in vitro* tests for predicting skin sensitizing potential of a broad range of cosmetic raw materials. Also, many of these raw materials include lipophilic compounds and complex mixtures, which could be out of the applicability domain for known cell-based assays and peptide reactivity assays. To address both the political and technical issues, we employed commercially available reconstructed human epidermis (EpiDerm), on which test samples can be applied at high concentrations in the same manner as in animal testing. We first determined gene expression profiles in EpiDerm after application of the known allergens DNFB and oxazolone (OXA). A unique set of genes encoding redox regulatory enzymes was upregulated significantly by DNFB and/or OXA, but not by a non-allergen, benzalkonium chloride. Real-time PCR analysis demonstrated that expression of the selected target gene(s) was robustly (>3-fold) induced by hexyl cinnamic aldehyde, abietic acid, and ethyleneglycol dimethacrylate, which are categorized as weak allergens in LLNA, but not by non-allergens, methylsalicylate and lactic acid. Furthermore, lipophilic sensitizing compounds, such as beta-propiolactone and palmitoyl chloride, induced marked expression of target genes without affecting cell viability, but not so a lipophilic non-allergen, hexane. Not only do our results demonstrate the predictivity of our new assay platform, which we called Epidermal Sensitization Assay (EpiSensA), they also highlight the much broader applicability domain against raw materials including highly-lipophilic compounds and complex mixtures.

V-1-377

Development of a non-injuring cell test to measure acetylcholinesterase activity for neurotoxicological high-throughput alternative methods

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Acetylcholinesterase (AChE) is a membrane-bound enzyme with its active site on the cell outside. It is an important enzyme studied in neurotoxicology and neuropathology, since it is involved in neuron system development and neurophysiological processes.

The aim of this study was to develop a modified Ellman's method for AChE activity assay without cell damage, allowing its use on *in vitro* neurotoxicological test batteries, reducing costs, experimental times, procedures, and contributing to future replacement of animal testing in neurotoxicology.

150 μl of phosphate buffered saline (PBS) or Dulbecco's modified Eagle's medium (DMEM) high glucose, with or without 10% fetal bovine serum (FBS), were added with 1 mM acetylthiocholine (AceScol) and either 0.150 mM or 0.050 mM of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) on a 96 well microplate, following OD_{412} for 50 minutes. The reaction medium with lower interference (background) was subsequently tested in the presence of undifferentiated neuroblastoma (SH-SY5Y) cells, seeded 24 h before ($4x10^4$ cells/well).

FBS induced a fast degradation of AceScol, possibly due to butyrylcholinesterase. PBS media presented the lowest background of AceScol degradation (0.012 OD_{412}/h). In the presence of cells, it was possible to detect enzyme activities using 1 mM of AceScol and 0.05 of DTNB in PBS, allowing up to 0.6 OD_{412}/h of activity.

In summary, changes in the pattern of DTNB reaction on microplate wells with and without neuroblastoma cells indicate a possible method to measure AChE activity of these cells.

V-1-378

A performance evaluation of Simcyp Dog – a fully mechanistic physiologically-based pharmacokinetic dog model – based upon a variety of theophylline i.v. and oral formulations

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The purpose of this study was to evaluate the performance of Simcyp Dog – a physiologically-based pharmacokinetic (PB-PK) dog model – to predict the pharmacokinetics of theophylline (THP) following intravenous (IV) and oral (immediate release (IR) and sustained release (SR) formulations) dosing.

Simcyp Dog is a 10 kg virtual beagle dog, which uniquely includes both inter-individual variability of major oral absorption-related parameters and a fully mechanistic gut wall permeability model. Simulations were performed to predict THP plasma concentration-time (Cp-t) profiles following the administration of 4 IV, 3 IR and 3 SR formulations (fed vs. fasted); the latter characterised by *in vitro* dissolution profiles. Simulation results were compared against published results (Tse and Szeto, 1982; Shiu et al., 1989; Mongozzi et al., 1998; Ochoa et al., 2010) to evaluate model performance.

Simulated THP Cp-t profiles for 3 of 4 IV doses were in good agreement with published literature. Preliminary simulations with IR formulations indicated a slight under-prediction of Cp-t profiles for 2 of 3 formulations tested whereas SR formulation results were in good agreement with the published data.

Thus, the Simcyp virtual Beagle model is reasonably successful in predicting THP Cp-t profiles after IV and SR formulations. The slight under-prediction of Cp for IR formulations may be attributed to factors like gastric emptying time and *in vitro* and *in vivo* dissolution rate differences. This study demonstrates promising potential for virtual veterinary drug development.

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V-1-396

Evaluation of neurotoxicity using automated image analysis

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There is increasing belief that environmental exposure to industrial chemicals, certain food additives, or substances used as therapeutic agents can contribute to development of a variety of neurodegenerative and pathophysiological conditions, such as Alzheimer's and Parkinson's disease. It is within these abnormalities that changes in neuronal cell morphology, overall cell death, neurotransmitter activity, and outgrowth, elongation, and branching of neurites are observed. Automating an *in vitro* process to screen chemicals for neurotoxic effects would allow further insight to mechanisms at the cellular level to help prioritize further selection for animal studies, while offering ease of manipulation, and increased scalability.

For this study, a select panel of neurotoxins (including retinoic acid, mercuric chloride, K252a, and okadaic acid) were tested on Neuroscreen™-1 (NS-1) cells, an established *in vitro* model system for neuronal physiology and toxicity. Neuronal viability and general morphology were evaluated in conjunction with neurite outgrowth following NGF treatment with a dose-response of various neurotoxins over 96 hours. Cells were then fixed and fluorescently stained to identify the nucleus, cell body, and neurites. Samples were analyzed using automated image analysis tools to evaluate which compounds showed general decreases in viability, as well as those that affected neurite elongation and/or branching. Multiplexing these features with automated image analysis can aid in pre-screening potential neurotoxicants and help decrease the overall need for animal testing.

V-1-411

Prevalidation of the Cultex® system assessing the inhalation toxicity of nanoparticles by direct exposure of cells at the air-liquid interface

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Over the last decade nanochemistry has developed with a tremendous pace, not without realising that the safety and risk assessment of nanoparticles will challenge current toxicological approaches. As for many nanoparticles the respiratory tract is a main portal to the human body, thus inhalative exposure route requires special attention. The Cultex® RFS system has specifically been designed to model inhalation *in vitro*, also addressing issues such as atmosphere generation, particle distribution and deposition, in order to optimise and standardise the *in vitro* exposure of cells at the air-liquid interface.

The usefulness of this system for the assessment of the acute inhalative toxicity of nanoparticles is currently explored in a prevalidation study. In this A579 cells are exposed to several well-defined nanoparticles, which have been selected primarily based on the availability of toxicological data and substances, measuring cytotoxicity as the main endpoint. In a classical approach with three laboratories, the method including all test protocols and the technical equipment was successfully transferred by the lead laboratory to the two "naïve" laboratories. Within- and between-laboratory reproducibility are being investigated by testing up to twelve different nanoparticles in independent experiments. Data are reported and analysed by the fourth project partner, who will also develop a preliminary algorithm that relates the *in vitro* results to *in vivo* reference results.

V-1-437

Need and perspectives for the implementation of relevant *in vitro* methods in the field of inhalation toxicology

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The EU REACH legislative for chemicals of 2006 represents one of the largest challenges for toxicological testing, because 68,000 to 101,000 chemicals have to be investigated according to the newest estimates. In the field of acute toxicology, internationally accepted methods are available with regard to oral toxicity. Comparable validated approaches for inhalation toxicology are not available at the moment, probably due to the difficulties in exposing cells of the respiratory tract directly to inhalable substances in a way comparable to the *in vivo* situation. In the last ten years, the optimization of the biphasic cell culture and exposure techniques as well as the availability of human cell lines for such studies offer promising possibilities to integrate this type of *in vitro* study into the research strategies for inhalable chemical compounds. Particularly, prevalidation studies are under investigation for analysing the biological activity of gaseous and particulate matter using special *in vitro* exposure systems like the CULTEX® RFS module for exposing cultivated cells at the air-liquid interface (ALI). Special attention is placed on issues like controlled generation, distribution and deposition of the test atmosphere in order to optimise and standardise the *in vitro* exposure of cells at the air-liquid interface.

V-1-474

The Bionas Discovery™ 2500 system – applications for *in vitro* alternative tests to identify eye irritants

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Advanced *in vitro* methods help to identify pharmacodynamic properties of substances and the prediction of compound toxicity. Recently, the cytosensor microphysiometer, measuring the extracellular acidification rate of cells by using a pH sensor, was recommended for the use as part of a tiered testing strategy for regulatory classification in a Bottom-up/Top-down approach to identify ocular corrosives/severe irritants. So far, this device is the only validated *in vitro* test method recommended to identify chemicals not classified as eye irritants.

The Bionas Discovery™ 2500 system measures extracellular pH in combination with two additional parameters in a very similar way. Here the suitability of this similar instrument was shown in experiments performed according to the Test Guidelines of the ECVAM, using the INVITTOX protocol 102 as a standard. L929 fibroblasts were grown on silicon sensor chips hosting the sensors for pH, oxygen and impedance measurement. The cells were exposed to increasing concentrations of test chemicals and the metabolic rates i.e. the change in pH, were determined. In addition, a potential regeneration from the toxic insults was investigated. Toxic effects induced by the test chemicals were indicated as MRD50, a value which is calculated from the concentration response curve to provide a measure of the ocular irritancy potential.

The use of the Bionas Discovery™ 2500 system serves as an alternative method for animal experiments. Regarding the similar principle of measurement, the system provides a high potential to investigate the toxicity of chemicals for eye corrosion.

V-1-544

A strategy combining high-content screening and zebrafish larvae to predict human drug-induced hepatotoxicity

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Drug-induced liver injury (DILI) represents a major challenge for pharmaceutical preclinical development as it signifies a major reason for pre-and clinical compound attrition and market withdrawal. High-content cytotoxicity screening (HCS) on human HepG2 cells has been proposed as a valuable early screening model for the prediction of DILI in humans (O'Brien et al., 2004). With a specificity of 100%, the sensitivity of detection for HCS is 67% for the prediction of strongly hepatotoxic compounds and 53% when all hepatotoxicants are included. To further improve the prediction of DILI, we propose a combined strategy including HCS and a whole-liver system. Although several 3D-liver models are currently under development, at this time no *in vitro* model is capable of mimicking the complexity of a whole liver system; zebrafish larvae nonetheless represent an attractive *in vivo* alternative. While the simplicity of HepG2-based HCS allows high-throughput screening, the liver of zebrafish larvae enables detection of more complex mechanisms of hepatotoxicity at a later stage of the discovery phase. Our evaluation of zebrafish larvae as an additional model to predict human hepatotoxicity shows that higher sensitivity indices are achieved, indicating that the whole-liver system of the zebrafish is capable of detecting more hepatotoxicants in comparison with HCS. However, the new complex model system of zebrafish larvae also requires thorough investigation of uptake and metabolism, and evidence of similarity of the most important mechanisms of drug-induced hepatotoxicity between zebrafish and humans needs to be investigated before zebrafish results are applied in decision-making processes in the drug discovery phase.

Reference

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V-1-547

Evaluation of an integrated testing strategy: comparison of *in silico* predictions with *in vivo* toxicity

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This research was conducted within a larger program to investigate possibilities and limitations of the use of non-animal data (*in silico* and mammalian *in vitro* data on kinetics, modeling and toxicity) for predicting human risk. To this aim a set of 17 widely varying chemicals was selected based on the availability of *in vivo* toxicity data. In the absence of animal data, a first step could be the use of *in silico* predictions to select *in vitro* test systems. Here we compare the output of QSAR and statistically based software with the observed *in vivo* toxicity.

Structural alerts were investigated using the knowledge-based systems DEREK (Lhasa Ltd, UK), the OECD Toolbox, and the quantitative structure-activity relation (QSAR)-based TOPKAT®. *In vivo* toxicity data for rodents after oral exposure were obtained from North American and European risk assessment agencies.

Several, but not all, *in vivo* effects are predicted *in silico*. Critical effects were generally targeted in the predictions, although this information was in several cases included in the software. For carcinogenicity, skin toxicity and hepatotoxicity false positives were predicted (respectively 7, 7, and 2 out of 17 chemicals). More problematic for human chemical safety, false negatives were also observed, in particular in the predictions for developmental toxicity, hepatotoxicity, nephrotoxicity, and neurotoxicity (respectively 4, 4, 3, and 8 out of 17 chemicals).

Formation of metabolites is not yet included in the *in silico* predictions, while this is in several cases critical for *in vivo* toxicity. This emphasizes the importance of the inclusion of metabolite formation *in silico* (followed by classifying the metabolites by structural similarities and feeding into toxicity prediction software).

V-1-560

Ex vivo tumour sphere approach as a potential alternative method for tumour xenografts in preclinical drug efficacy assays

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We previously demonstrated that 3D colospheres, directly derived from mechanically dissociated colorectal cancer tissue, are a short term culture model formed exclusively by cancer cells and associated with tumour aggressiveness (Weiswald et al., 2009). Similar approach gives successfully mastospheres from breast cancer xenografts. Cancer preclinical assays involve both *in vitro* and *in vivo* testing. Consequently, spheres as potential alternative method to reduce mouse numbers in drug screening assays was investigated.

We used human breast and colon cancer xenografts established in *Nude* mice directly from patient samples. Their mechanical dissociation gave rise to numerous spheres in only 3 days. Spheres and xenografts were analysed for gene expression profiles and anticancer drug responses.

Clustering analysis of gene expression clearly showed that spheres matched with their parent xenografts, demonstrating 1) the lack of *ex vivo* culture artifacts, and 2) the confirmed relevance of spheres for mimicking *in vitro* tumour cells.

Design of colosphere collecting method, culture conditions and cell viability/toxicity assays allowed us to obtaining reproducible results. Besides, preliminary results show that the *in vivo* chemosensitivity responses of xenografts are reproduced in derived spheres.

As one xenograft is able to give rise to abundant spheres which closely reproduce parental tumours, spheres deserve further investigation to determine if they are relevant alternative method for drug screening, leading to decreased number of mice used in anticancer drug pipeline.

Reference

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V-1-571

Application of Upcyte® technology to primary cells for developing alternatives to current *in vitro* ADME-Tox models

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We have developed a new technology to allow the proliferation of differentiated primary cells without inducing permanent immortalization, uncontrolled cell growth, or loss of phenotype. The Upcyte® ("upregulated") technology involves a viral gene transfer system to introduce a unique combination of genes that induce and maintain cell proliferation until the cells reach confluence. This allows the primary cells to be passaged many times and the generation of billions of cells. Upcyte® technology has been applied to different cell types, including keratinocytes, endothelial cells, proximal tubular kidney cells and hepatocytes. We summarise some of the comparisons we have made between primary cells and their Upcyte® equivalents. The flexibility of the application of Upcyte® cells to different cellular-based assays, together with their abundant availability from different donors for routine testing, means models are now available with sustained quality and in sufficient quantities to allow for reproducible and reliable *in vitro* ADMET studies.

V-1-578

Micro electrode chip assay (MEA) as method to detect neurotoxicity *in vitro*

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Detection and characterization of chemical-induced toxic effects in the nervous system represent a major challenge for registration and assessment of chemicals. So far, no *in vitro* method for evaluating the neurotoxic hazard has yet been validated and accepted for regulatory purposes. *In vivo*, neurotoxicological assessments exploit the fact that activity of neurons in the central and peripheral nervous system has functional consequences.

The microelectrode array (MEA) assay consists of a culture chamber with an integrated array of microelectrodes, capable of measuring electrophysiology (spikes and bursts) from electroactive tissues, such as primary cultures of nervous system tissue. Recordings from such an *in vitro* cultured system are non-invasive, label-free evaluations and provide a higher throughput than conventional electrophysiological techniques.

In this first study 21 blinded substances (12 known to be neurotoxic, 2 non-neuroactive and non-toxic and 7 non-neuroactive but toxic) were tested in a dose-response curve on embryonic rat cortical neuronal networks on a MEA for their toxicity. The experimental procedure consisted in the evaluation of the firing activity (spiking rate) and modification/reduction in response to the chemical administration. Native/reference activity, 30 minutes of activity recording per dilution, plus the reversibility/recovery points (after 24 h) were recorded. The IC_{50} values were calculated using Hill Equation Fitting tool of the averaged data. The preliminary data show a good predictivity (sensitivity: 0.93; specificity: 0.57; predictivity: 0.81). Thus, the MEA with a neuronal network could potentially become a powerful tool to evaluate neurotoxicity *in vitro*.

V-1-602

Predicting dermal toxicity using the OECD TG 404 integrated testing strategy: an evaluation of the SkinEthic RHE test methods

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The use of testing strategies based on alternative methods was adopted by the OECD test guidelines TG404 for predicting dermal toxicity. To date the test methods using the SkinEthic Reconstructed Human Epidermis (RHE) were independently validated for dermal skin corrosion and skin irritation.

The aim of the present study was to develop a testing strategy combining both SkinEthic RHE skin corrosion and skin irritation test methods to support the ongoing revision of OECD TG404 and TG431.

For this purpose, about 40 chemicals (from the ECVAM validation studies) were evaluated in both skin corrosion and skin irritation. The results showed that 15 *in vivo* irritant chemicals were identified *in vitro* as non-corrosives but correctly classified as irritants using the SkinEthic RHE skin irritation test method. In addition 15 *in vivo* non-irritants and non-corrosives were correctly predicted *in vitro* using both skin corrosion and irritation test methods. Finally 12 corrosive chemicals identified by NICEATM/ICCVAM as incorrectly predicted *in vitro* were evaluated. The results showed that corrosive chemicals misclassified in the *in vitro* corrosion test were identified as irritants – with the exception of one substance – using the SkinEthic skin irritation test method. So, when applying the OECD TG404 testing strategy on the substances identified by NICEATM/ICCVAM as potentially false negative corrosives, all these substances were correctly identified.

In conclusion, when the determination of corrosivity or irritation cannot be made using a weight-of-the-evidence analysis, a preferred sequential testing strategy (skin irritation/corrosion), which includes the performance of accepted *in vitro* SkinEthic RHE tests should be considered.

V-1-613

Comparative sensitivity of tumor and non-tumor cell lines to cytotoxicity of anionic lysine-based surfactants

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Surfactants are applied as additives in topical pharmaceuticals, cosmetic products, and recently in intracellular drug delivery systems. In this context, amino acid-based surfactants deserve particular attention and could be a promising application in these fields. Here we have compared the toxicological effects of five anionic lysine-based surfactants with heavy (tris and lysine salts) and light (sodium, lithium and potassium salts) counter-ions in their structure by *in vitro* cytotoxicity assays (MTT and NRU endpoints) using two tumor and four non-tumor cell lines.

Cytotoxicity was assessed in mouse fibroblasts (3T3 and 3T6), human keratinocytes (HaCaT and NCTC 2544), human breast cancer (MCF-7) and human cervical cancer (HeLa) cell lines. Uptake of vital dye neutral red (NRU) and MTT reduction assays was used as an endpoint to evaluate cell viability. Cytotoxicity of each surfactant was expressed in terms of IC_{50} (50% inhibitory concentration) and individual values were analysed by ANOVA followed by Tukey *posthoc* test.

Following 24 h exposure, surfactants showed varied levels of cytotoxicity to cell lines. IC_{50} values revealed that the cytotoxicity of surfactants with light counter-ions was in general more pronounced than that of surfactants with heavy counterions. Moreover, cytotoxic effects were greater in the MTT assay than in the NRU assay. Comparative analysis of cell lines showed that the NCTC 2544 and 3T6 cells were the most sensitive, while tumor cells were markedly less sensitive to the surfactant with lithium counter-ion. These differences between cells may be due to specific mechanisms underlying toxic response, and highlight the choice of cell type as very important in toxicity studies.

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V-1-635

Validation of a 3D skin model for cosmetic, chemical and medical device phototoxicity testing (EPARS)

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We have enhanced our validated *in vitro* phototoxicity test using human skin models by exploring inflammatory mediator and gene expression endpoints. The Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS) is based upon a 3D skin model that closely parallels human skin morphology. Major advantages of this test system are that test substances can be applied topically, avoiding the problems of (1) difficulty in solubilizing test materials, and (2) indirect application of test materials to cell monolayers via culture media. In addition, the tissues are composed of differentiated layers of primary human keratinocytes, a more relevant model than mouse tumor fibroblasts. Phototoxic effects are determined by measuring the viability of UV irradiated vs. non-irradiated exposed tissues. In order to increase the sensitivity and specificity of the test, we have measured the release of cytokines into the culture media via ELISA. The release of the inflammatory factor PGE2 was shown to be an early predictor of the toxic effects demonstrated in the viability assay. When compared to human phototoxicity test results and the 3T3 NRU PT validation test material set, EPARS had 100% accuracy, sensitivity and specificity. Microarray analysis of gene expression showed that chlorpromazine treatment with UVA irradiation caused changes in gene expression over time that were not observed without UVA irradiation. These genes include those for keratins, collagens and fibronectins. EPARS is an accurate and sensitive test for detecting phototoxic substances at doses representative of those that cause actual human skin reactions. Thus, EPARS is a highly predictive phototoxicity assay, with endpoints of inflammatory mediator and gene expression that allow for investigation into the mechanisms of photosensitivity in a wide variety of consumer products.

V-1-636

In vitro phototoxicity test methods compared: 3T3 NRU PT vs. phototoxicity assay in reconstituted skin

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Currently, only the 3T3 Neutral Red Uptake Phototoxicity Test (3T3-NRU PT) has been approved as a non-animal phototoxicity test by governmental regulatory agencies. However, the 3T3-NRU PT has serious drawbacks as a model for human skin, specifically: it uses a monolayer cell culture consisting of mouse tumor cells, it is overly sensitive resulting in many false-positives, and test substances must be soluble in tissue culture media. Thus, we have developed a more relevant *in vitro* system based on three-dimensional, differentiated human keratinocyte cultures, which can accommodate a wide range of vehicles and allow direct topical application of test substances, a "Phototoxicity Assay in Reconstructed Skin (PARS)". We present a side-by-side comparison between the 3T3-NRU PT and the PARS test systems using the same Solar Simulated Light (SSL) source and the 8 reference standard chemicals used for validation of the 3T3-NRU PT. The PARS test correctly predicts with 100% accuracy the phototoxic potential of all reference test substances. The concentrations of test agents needed to induce cytotoxicity in reconstituted skin, when compared to the Neutral Red assay in 3T3 fibroblasts, is one to two orders of magnitude higher, reflecting the thickness and complexity of a three-dimensional tissue structure. This better approximates the exposure levels of chemicals needed to induce a phototoxic effect in animal tests and actual human skin. In addition, the most important practical advantage gained over the 3T3 NRU PT is that test substances can be applied topically, overcoming both pH and solubility problems encountered when dosing via the culture media.

V-1-637

Gene expression profiling of an *in vitro* human skin model after psoralen plus ultraviolet light-induced phototoxicity

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To reduce the number of animals for safety screening of potential irritating chemicals and phototoxins, efforts have been made to develop more predictive *in vitro* assays. One model in more common use is reconstituted human epidermis (MatTek Epiderm) resembling *in vivo* human skin. In this investigation, these human skin models were exposed to a known skin irritant, 8-methoxypsoralen (8MOP), at a dose comparable to the EC10. The samples were also either kept in the dark or exposed to solar simulated light (SSL). Gene activity was analyzed with mRNA microarrays at 1, 6, and 20 h to determine potential cellular and molecular mechanisms of action. Two levels of biological control samples were used: a) samples not treated with 8MOP and kept in the dark or exposed to UV light and b) samples treated with sodium dodecyl sulfate (SDS) [positive control]. Purified, labeled, and fractionated cRNA isolated from each of the biological samples were hybridized onto whole human genome mRNA expression microarrays, each containing 41,000 unique probes. Data analysis was done by a tiered approach. Coefficients of variation (CV) from all the probes passing quality measures or a total of 11,335 probes for each biological sample within the treatment groups ranged from 18.5-33.1%. The least variability was observed with the principal components analysis (PCA) for the negative control samples (those not exposed to 8MOP) and the samples exposed to 8MOP under dark conditions. The most activity was seen with 8MOP and SSL exposures at 6 and 20 h as well as exposures to SDS, the positive control. Several genes in common between treatments with SDS and 8MOP were CXCL14, fibrillin2, tropomyosin alpha 1, CYP26B1, HSP70B and VEGF-A.

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V-1-638

Oxidative stress and hypoxia as factors in phototoxic damage to a reconstituted human skin model: Gene expression profiling evidence

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Currently there is worldwide emphasis on more predictive *in vitro* assays for phototoxicity, especially in screening dermatological and pharmaceutical products. Reconstituted human skin tissues modeling human skin *in vivo* were exposed to a known phototoxin, chlorpromazine (CPZ) with and without ultraviolet light, at a dose correlating with the EC10. The goal was to understand what cellular and genetic mechanism(s) contribute to the phototoxic response. The skin models were treated with CPZ in the dark as well as exposed to solar simulated light (SSL) and gene activity was measured with mRNA expression microarrays at 1, 6, and 20 h post-exposure. For comparison, biological control tissues were concurrently treated with vehicle alone or left untreated, and kept either in the dark or exposed to UV light. Purified, labeled, and fractionated cRNA isolated from each of the tissue samples were hybridized onto whole human genome mRNA expression microarrays. The microarrays contained 41,000 unique probes corresponding to the full complement of sequenced human genes. The data was analyzed using a tiered approach. Coefficients of variation (CV) from all the probes passing quality measures or a total of 10,299 probes for each tissue sample within the treatment groups ranged from 5.3 to 19.2%. The least variability was observed with the principal components analysis (PCA) for the skin model samples not treated with CPZ under either dark or light conditions. The profiles for each treatment group were more similar by time point rather than by light/dark exposure. The numbers of down-regulated genes ranged from 10 to 52 and the numbers of up-regulated genes ranged from 96 to 236. Noteworthy genes which were down-regulated included genes involved in differentiation and normally expressed in epithelial tissue, such as CXCL14 and DAPL1. Several genes (e.g. HSPA6, ERO1L and ANGPTL4) related to oxidative stress and hypoxias were up-regulated.

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V-1-640

Decellularized liver matrix, a remarkable tool in the bio-engineering of three-dimensional *in vitro* liver systems

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Simplified liver-based models are gaining ground as *in vitro* tools to evaluate drug metabolism, efficacy and safety. Yet, a major drawback is the progressive loss of hepatic functionality, limiting their applicability over time. As cell-extracellular matrix and cell-cell contacts partially overcome this problem, the development of a three-dimensional *in vitro* model, closely resembling the *in vivo* liver architecture, seems of major importance. Here we describe the development of a three-dimensional, naturally derived liver scaffold with an intact microvascular system and capable of withstanding fluid flows in the three hepatic circular systems. Whole rat livers were subsequently perfused with a selection of mild detergents to specifically remove cellular components, while preserving all major extracellular matrix components, including laminin, collagen I, collagen IV and fibronectin and extracellular matrix bound growth factor islets. This unique scaffold is available within 60 minutes and represents a remarkable tool in the bio-engineering of complex three-dimensional *in vitro* systems for pharmaco-toxicological purposes.

V-1-641

Acceptance criteria: the challenge in the development of stem cell based toxicity tests

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Currently, a big effort is directed towards the optimization of *in vitro* cell models for reliable and human-based toxicity testing. However, current human cell models are either based on primary tissues that are difficult to standardize or rely on cell cultures with carcinogenic origin which have often unwanted/unknown characteristics that might impact the cellular response to xenobiotics. The availability of human pluripotent stem cells and their derivatives opens up a new avenue to overcome these shortcomings and offers the opportunity to convert stem cell based models into test systems that can support new ways of safety assessments based on a mode of action paradigm. However, stably culturing pluripotent stem cell lines and obtaining homogenous differentiated cell cultures are still challenging. The quality of the initial undifferentiated stem cell culture can affect the differentiation process, the phenotype or functionality of the differentiated cells. Thus, it is pivotal to optimize current cell culturing methods for appropriately growing undifferentiated stem cells and to establish efficient differentiation protocols in order to get toxicologically relevant cell derivatives. To do so, it is mandatory to standardise the quality control assays serving as acceptance criteria to judge the suitability of a stem cell line and their derivatives for toxicity testing. The poster describes the establishment of a set of quality criteria standards for undifferentiated cells and their derivatives which are needed for pluripotent stem cells-based toxicology studies as well as the endeavour to encourage a consensus with the use of pluripotent stem cells based *in vitro* systems.

V-1-646

Stem cells for relevant, efficient, extended and normalized toxicology

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In the development of products for use by humans it is vital to identify compounds with toxic properties at an early stage of their development, to avoid spending time and resources on unsuitable and potentially unsafe candidate products. Human pluripotent stem cell lines offer a unique opportunity to develop a wide variety of human cell-based test systems because they may be expanded indefinitely and triggered to differentiate into any cell type. SCR&Tox aims at making use of these two attributes to provide *in vitro* assays for predicting toxicity of pharmaceutical compounds and cosmetic ingredients. In order to demonstrate the value of pluripotent stem cells for toxicology, the consortium will focus on four complementary aspects:

– Relevance, i.e. establishing and maintaining discrete cell phenotypes over long-term cultures; providing large versatility to adapt to assays of specific pathways.
– Efficiency, i.e. i) automated cell production and differentiation, ii) cell engineering for differentiation and selection, and iii) multi-parametric toxicology using functional genomic, proteomic and bioelectronics.
– Extension, i.e. i) scalability through production of cells and technologies for industrial-scale assays and ii) diversity of phenotypes (5 different tissues) and of genotypes (over 30 different donors).
– Normalization, i.e. validation and demonstration of reproducibility and robustness of cell-based assays on industrial-scale platforms to allow for secondary development in the pharmaceutical and cosmetic industry.

SCR&Tox is part of a Research Initiative of the European Commission and the European Cosmetic Association in which 7 projects are aiming to contribute towards the replacement of *in vivo* repeated-dose systemic toxicity testing.