Session I-2: Addressing systems toxicology

Session I-2: Oral presentations

I-2-460

Integrated testing strategies and *in vitro-in vivo* extrapolations: their role in chemical risk assessments

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There is increasing need for efficient approaches to assess the environmental or human health risk for many chemicals. Today's heavy reliance on approaches using animal models increasingly meets criticism for ethical, economical as well as scientific reasons.

A shift in the paradigm on assessing risk focusing more on the mechanisms of toxic action rather than apical endpoints in animal studies is emerging. This approach integrates: 1) *in silico* methods for relating chemical structure and physico-chemical properties to biological activities (QSARs), 2) modern cell biology techniques for *in vitro* measurements of data related to the toxic mechanisms by applying systems biology methods, "omics", imaging techniques, etc., with 3) computer-aided kinetic modelling (PBBK). This allows the use of these data in a quantitative *in vitro-in vivo* extrapolation (QIVIVE) by relating the *in vitro* concentration-effect relations to an *in vivo* dose-effect relationship, which then can be used as a point-of-departure in risk assessments.

A limited number of studies have been performed using this approach. In general these have shown many possibilities and some limitations for risk assessments not requiring animal toxicity studies.

I-2-218

Human multi-organ chips – a possible solution for animal free systemic ADMET testing

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Animal free systemic toxicity and ADME testing predictive of human exposure is currently the major challenge for science, regulatory bodies and the industry involved. On the one hand, modern test system engineering focuses mainly on single organ equivalents, rather than the systemic combination of organs. On the other hand, none of the currently available systems ensures long-term homeostasis of the respective tissues over months. This is primarily caused due to the lack of *in vivo*-like vasculature.

On the basis of three design pillars – device, micro-architecture and culture process – we have designed a "multi-organ-chip" (MOC) platform technology. The MOC platform is

based on a self-contained sensor-controlled dynamic micro-tissue-bioreactor, the shape of a standard microscope slide. A fast and flexible standardized prototyping procedure has been established allowing continuous improvement of the MOC design. The current fourth MOC generation supports the continuous maintenance of either human micro-scale liver tissue or miniaturized human full skin organoids (epidermis-dermis) optionally containing micro hair follicles. A micro-channel circulation system, which can be covered with human endothelial cells, ensures the circulation of nutrients through the micro-organoid growth chambers. The MOC design supports relevant substance exposure routes. Data on organoid self-assembly, indicators for equivalence to human *in vivo* performance and evidence for long-term tissue behaviour will be presented. The next generation of MOC prototypes combining human liver and skin organoids within a common vasculature will be discussed. Finally, the challenges and opportunities of this platform technology in comparison to the existing dynamic bioreactors will be addressed.

I-2-231

Long term Cyclosporin A increases transepithelial electrical resistance in renal proximal tubule monolayers: a possible role of claudin rearrangement

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The immunosuppressive compound Cyclosporine A (CsA) is known to be a chronic nephrotoxin. CsA has been shown to have diverse adverse effects on renal cells in culture including induction of reactive oxygen species, enhanced glycolysis rates and decreased cell proliferation. Here we provide evidence that CsA can also effect proximal tubular epithelial barrier function by inducing alterations in claudin isotypes.

The human renal proximal tubule cell line RPTEC/TERT1 was cultured on microporous growth supports for 2 weeks. Cells were treated with 5 and 15 μM CsA every day for 14 days. Trans epithelial electrical resistance was measured every day using the ENDOHM/EVOM system and cells were harvested on day 1, 3 and 14 for RNA isolation and subsequent transcriptomic analysis.

CsA caused a mild induction of TEER by day 2 at both concentrations. At day 7, 15 μM CsA had induced TEER to 7.8 fold and remained heavily induced until experiment termination. Transcriptomic analysis revealed a CsA induction of claudin 1, 4, 12, 15 and 23 with a decrease in claudin 2, 10 and 16. Additionally, E-Cadherin, cingulin and ZO-3 were decreased by CsA, whereas alpha catenin and ZO-1 were induced. The majority of the claudins induced by CsA are sealing claudins, while those decreased are all pore forming. Interestingly, we have also demonstrated that CsA impacts negatively on dome formation, which may be related to the fact that claudin 2 has recently been demonstrated to be able to form paracellular water channels.

Long term treatment of human renal proximal tubular cells with CsA induces TEER dramatically, which is most likely due to a loss of pore forming claudins in the tight junctions and an increase of sealing claudins. Such a dramatic alteration in proximal tubule function could have serious consequences for homeostasis.

I-2-237

Cardiac safety testing of human pharmaceuticals using fresh human tissue

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Despite the rigid regulatory framework, advances in preclinical modelling and the emergence of *in silico* technologies, approximately 25% of human pharmaceuticals are still failing Phase II trials or being withdrawn from the market due to off target effects on the heart. Using fresh, functional cardiac tissue we have attempted to bridge the gap between the currently utilised cell-based assays, animal models and the clinical situation.

The effects of various compounds on the resting tone and contractile properties of both small (<500 μm) and large (500-1000 μm) isolated functional human coronary arteries were assessed using standard myography methods. Strips of endocardial muscle were isolated from the left ventricle of functional human hearts and mounted in an organ bath. Using electrical field stimulation we assessed the effects of drugs on the contractility of the muscle. In addition, artificial arrhythmias were induced in the tissue by increasing the frequency of the field stimulation. The effects of drugs on the threshold for producing an arrhythmia were investigated.

Using the approaches described above we investigated the use of fresh, functional cardiac tissue in assessing the safety of human pharmaceuticals. Each assay was shown to confirm the observed off-target clinical effects of drugs such as cisapride and sumatriptan as well as highlighting potential mechanisms for issues observed with tegaserod and mephedrone. The data demonstrates that human tissue can be used for translational endpoints which may not be detected in whole animal or cell-based models.

I-2-110

Challenges and solutions associated with transfer of *in vitro* cellular monolayer techniques to 3D tissue models

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Although three dimensional (3D) reconstructed tissue models theoretically can provide numerous advantages to the field of *in vitro* toxicology, it is not always trivial to directly utilize techniques developed for monolayer cell culture with the appropriate 3D models. Even methods as basic as cellular viability assays may prove difficult to apply because of the difficulty in evenly treating the 3D tissue surface, targeting relevant cell types within the construct or extracting colored marker chemicals from the often complex matrix of 3D models. Other difficulties may occur during the exposure phase, e.g. adequately modeling the evaporation of volatile test materials, understanding whether topical doses applied *in vitro* are similar to those obtained *in vivo*, determining penetration of the test material, or assuring that containment of the 3-D model is sufficient to permit chemical exposure only via the intended biological barrier. Examples of solutions to these and similar challenges will be presented.

Session I-2: Poster presentations

I-2-049

Development of *in vitro* panel of assays for rapid toxicological assessment of novel munition compounds

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The U.S. Army Environmental Quality Technology and Ordnance Environmental Program is dedicated to finding replacements for substances causing environmental and/or occupational risks to health. This includes recent efforts to find a less hazardous replacement for the explosive 1,3,5-trinitrohexahydro-1,3,5-triazine (RDX) used since World War II. There are 35 energetic candidates under development. No toxicity data, however, exist for these novel formulas. Fast, high-throughput methods are needed to assess relative toxicity of new compounds proposed for use. Toxicological tests can be conducted *in vivo* and *in vitro*. *In vitro* cellular assays have the advantage of being relatively inexpensive, high-throughput and capable of addressing many mechanistic issues. This project developed an *in vitro* panel of assays to rapidly evaluate RDX and 35 novel replacement formulations for their aquatic toxicity in *Vibrio fischeri* and basal cytotoxicity in human liver cells, neurotoxic function in human neuroblastoma cells, and metabolic fate in human and animal liver tissues. These studies address an urgent need for toxicity information to assess the risks of environmental and human exposure to these new compounds. All these results will assist munition scientists in making health-based decisions regarding the design and selection of new formulas and guide toxicologists in conducting further animal studies for those formulas in development. In addition, the large number of compounds run through the "robust tests" improved the usefulness and practicability of these methodologies for fast screening of a broad range, increased volume of new munition compounds and toxic industrial chemicals/materials for their relative toxicity prior to conducting intensive animal studies.

I-2-076

Preliminary study of the revision of Japanese Pharmacopoeia test for rubber closure for aqueous infusions

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With a view to revising the Japanese Pharmacopoeia General Tests, Session 7.03, specifically for assessing rubber closures for aqueous infusions, we performed a preliminary study of a cytotoxicity test as an alternative to acute toxicity testing using mice. Twelve types of additives (rubber accelerators and antioxidants) that may be used in the manufacture of rubber closures for aqueous infusions were evaluated. The cytotoxicity test involved determination of IC_{50} values in a colony-forming assay using Chinese hamster V79 cells. The additive-derived solutions, prepared based on greater or equal IC_{50} concentrations, were compared to the sensitivity results of acute toxicity studies. Cytotoxicity assay sensitivities for all additives were found to be at least equal to the sensitivities derived by current acute toxicity testing methods. Thus, cytotoxicity assay is a valid option for assessing rubber closures for aqueous infusions.

I-2-082

Toxicogenomics to assess pesticide mixture toxicity

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Pesticides are chemical substances which include fungicides, herbicides and insecticides, some of which pose significant risks to human health. Regulatory authorities mandate submission of toxicity data (e.g. Directive 98/8/EC) prior to marketing approval of such chemicals, particularly with respect to their toxicological and ecotoxicological profiles. Such data are normally obtained on the basis of short term (repeat dose), subchronic and chronic toxicity involving a rodent and non-rodent species (e.g. dog) for mammalian toxicology. For the purposes of environmental toxicology, aquatic species are normally used. Whereas toxicity data have traditionally relied on exposure of test animals to single chemicals, the health risks posed by pesticide combinations have become increasingly evident. The large number of pesticide chemicals in use today in various combinations has created a logistical challenge for manufacturers and regulators alike, with respect to toxicity testing. The advent of toxicogenomics in combination with high throughput robotic systems could provide a replacement to animal use and cope with the large number of pesticide mixtures. The results of a pilot study will be presented to illustrate the principle of using toxicogenomics as part of a tiered testing strategy to replace the use of animals in toxicity testing of pesticide mixtures.

I-2-089

Non-animal approaches for consumer safety risk assessments: Unilever's scientific research programme

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Assuring all aspects of human safety associated with the inclusion of new ingredients into consumer products without the generation of any new animal data on these ingredients currently poses a considerable challenge. To meet this challenge, Unilever initiated a research programme in 2004 to critically evaluate the feasibility of a new conceptual approach for assuring consumer safety without animal testing (Westmoreland et al., 2010).

Unilever's current approach for safety assessment is risk-based, meaning that all available data on a new ingredient (including predicted levels of consumer exposure during product use) are used to assess the level of risk posed by its proposed consumer use. The scientific challenge we are investigating is how, in the future, novel *in vitro* and *in silico* data may be used within this risk-based framework. Our areas of focus are:

1. Risk-based approaches to assuring safety in the area of skin allergy (underpinned by a systems approach to understanding the mechanistic basis of skin sensitisation).

2. A case study (DNA damage-induced carcinogenicity) to evaluate the potential application of a toxicity pathways-based approach (TT21C) (Krewski et al., 2010) to risk assessments for repeat dose toxicity.

It is a significant scientific challenge to understand how safety may be assured for such complex endpoints, using data derived from a pathways-based approach that is rooted in mechanistic understanding of the underlying biology. An equally important challenge is how, if successful, such a pathways-based approach to safety assessment could ultimately receive regulatory acceptance.

References

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I-2-098

Accelerating the transition to 21st century toxicology: outcomes of a workshop organized by the Human Toxicology Project Consortium

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In 2007 the U.S. National Research Council (NRC) published "Toxicity Testing in the 21st Century: A Vision and a Strategy". The report calls for a fundamental shift in the way that chemicals are tested for human health effects and are evaluated in risk assessments. The new approach would decrease the current reliance on animal studies and move towards *in vitro* methods, typically using human cells in a high-throughput context. The *in vitro* methods would be designed to detect significant perturbations to "toxicity pathways", i.e., key biological pathways that, when sufficiently perturbed, lead to adverse health outcomes. To explore progress on the report's implementation, the Human Toxicology Project Consortium hosted a workshop entitled "Accelerating Implementation of the NRC Vision for Toxicity Testing in the 21st Century" on November 9-10, 2010 in Washington, DC. The goal of the workshop was to identify ways to accelerate implementation of the NRC vision for toxicity testing in the 21st century. The workshop format consisted of plenary presentations, break-out group discussions, and concluding commentaries. The speakers and session chairs were drawn from industry, academia, government, and public interest organizations. The workshop identified a number of recommendations for accelerating implementation of the NRC vision, including the need for overarching strategic planning, coordination, and communication, as well as the development of pilot projects with more direct approaches to implementation. The principal recommendation was the urgent need to establish a steering group to help manage the planning, coordination, and communication needed to make the NRC vision a reality.

I-2-099

Assessment of cardiac toxicity of doxorubicin on the rat electrocardiogram and its prevention by drugs

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The therapeutic usefulness of doxorubicin (Dox), an anthracycline antibiotic used as an anticancer agent, is limited by its cardiotoxicity. Dox-induced cardiotoxicity is mainly attributed to accumulation of reactive oxygen species and interaction of Dox with cellular iron metabolism. In this study, the potential protective effects of the free radical scavenger, proanthocyanidins (Pro) and the iron chelator, deferiprone (Def) against Dox-induced cardiotoxicity were investigated using rat electrocardiogram (ECG). Cardiotoxicity was induced in rats by single i.p. injection of Dox (15 mg/kg). The effect of Pro (70 mg/kg, orally) or Def (10 mg/kg) on Dox-induced cardiotoxicity was examined. Three days after Dox injection, rats were anesthetized with urethane (1.8 g/kg, i.p.) and electrocardiograms were recorded from standard lead II limb leads using a single channel ECG (Fukuda ME Kogyo Co. Ltd., Model: 501-B III, Tokyo, Japan). Then, the jugular vein was cannulated for aconitine infusion to induce ventricular tachycardia. Aconitine was infused in a concentration of 2.5 μg/ml and a flow rate of 0.5 ml/min. Dox caused significant increase in heart rate (40.76%), elevation of ST segment (95.87%), prolongation of QT interval (23.15%) and increase in T wave amplitude (56.55%). Moreover, Dox caused a significant decrease in the threshold dose of aconitine producing ventricular tachycardia (34.61%). Administration of Pro prevented these alterations except QT interval prolongation, while Def administration prevented change in all the measured parameters. These results suggest that both Pro and Def might be potential cardioprotective agents against Dox-induced cardiotoxicity.

I-2-102

Prototype pathway research for Toxicity Testing in the 21st Century (TT21C) – a case study using DNA damage characterisation

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The NAS/NRC report on TT21C offers an attractive future paradigm for toxicologists based on toxicity pathway perturbations and human exposure, rather than apical end points measured in experimental animals. In the context of a risk-based approach to the safety assessment of ingredients in consumer products, we are exploring the practical application of this paradigm through a collaborative research programme with the Hamner Institutes. Work investigating the individual elements of: (a) exposure and consumer use assessment, (b) fast, high-content-information *in vitro* assays in human cells, (c) dose-response assessments, (d) computational models of the circuitry of relevant toxicity pathways, and (e) pharmacokinetic models supporting *in vitro* to *in vivo* extrapolations, are being brought together to craft novel risk assessments for putative "genotoxic" case-study chemicals, maintaining exposure below the levels that significant pathway perturbations occur. A combination of techniques are being employed and combined, from Cellomics high content imaging for dose-response assessments of DNA damage and underlying threshold characteristics, to bioavailability measures derived from bloodspot and/or micro-dosing determinations in human subjects. We anticipate that this prototype toxicity pathway research will provide scientific evidence to support the future application of the TT21C principles, and will foster greater development of these much-needed methodologies.

I-2-175

Comparative cytotoxicity evaluation of essential oil from *Minthostachys setosa* in CHO cells, NIH/3T3 cells and human keratinocytes

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Minthostachys is a genus of shrubs of the Lamiaceae family, traditionally used as condiments or preservatives in food storage by the communities of the Andes in Peru. Aromatic and antimicrobial properties of the essential oil from *Minthostachys setosa* confer a potential use in cosmetics, leading to the goal of this work to evaluate the toxicity of the essential oil from *Minthostachys setosa*. The use of alternative methods for new ingredients toxicity determination is commended in the 7th Amendment of the European Cosmetics Directive 2003/15/EC. The essential oil from *Minthostachys setosa* was obtained from the aerial parts of the plant through hydrodistillation. The three cells types – NIH/3T3, CHO and human keratinocytes - were seeded in 96-well plates and incubated at 37ºC on 5% CO_2. Eight concentrations – 0.0028 mg/ml to 0.28 mg/ml – dispersed in culture medium with the surfactant Polysorbate 20 were added after 24 h. After one day, MTS was added to the cells and analyzed by spectrophotometer at 490 nm. All the tested concentrations except the last one presented cell viability higher than 90% compared to the control. The 0.28 mg/ml concentration showed cell viability of 30% for human keratinocytes, 87% for CHO and 79% for NIH/3T3. It is worth mentioning that the antimicrobial minimal concentration of *Minthostachys setosa* oil is 0.028 mg/ml, which is in the non-toxic range; therefore, the results suggest that essential oil can be safe to use as a natural preservative in cosmetic formulations.

I-2-195

Alternative *in vitro* phototoxicity test using reconstructed skin model KeraSkin™

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The reconstructed human skin model, KeraSkin™, has similar morphology, characteristics, and even biochemical marker expressions to native human skin. These similarities offer usefulness as an alternative testing method for skin irritation and corrosion, which has been proven by several validation studies. Therefore, this study was conducted to validate the *in vitro* phototoxicity test method using KeraSkin™. Nine phototoxic or non-phototoxic test chemicals were topically applied onto KeraSkin™. After 24 h incubation, the KeraSkin™ was exposed to 20 J/cm^2 of UVA. The test chemicals were removed, and cell viability was quantified by MTT assay after incubation for another 24 h. Phototoxicity was determined by viability (>20%), photo-irritation factor (PIF; >2), and mean photo effect (MPE; >0.1). When only viability and PIF criteria were applied, accuracy was 88.9%, showing 85.7% of sensitivity and 100% of specificity. But adding MPE criteria increased the overall accuracy to 100%, showing 100% sensitivity and specificity. In conclusion, a good *in vitro* alternative phototoxicity test method using KeraSkin™ was successfully established.

I-2-232

Comparison of xenobiotic metabolizing enzyme activities in normal human skin and reconstructed human skin models from skinethic laboratories

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Skin represents the major protective barrier of the body to its environment. Also, skin is an organ involved in the metabolism of xenobiotics, and its ability to metabolize xenobiotics can become consequent when considering its total surface area (2 m^2). Consequently, research on skin metabolism would need a real scientific effort to characterize skin metabolizing enzymes and their activities. In addition, the 7th European amendment to the Cosmetics Directive forbids the use of animal testing to assess the efficacy and safety of new cosmetic ingredients. This policy has forced the cosmetic industry to develop *in vitro* tools such as reconstructed human skin models as alternative methods to animal experiments. For these reasons, these skin models need to be characterized and compared with normal human skin (NHS) samples in terms of metabolic capabilities. This work presents the mRNA expression of several enzymes (CYP450, esterase, ADH, ALDH, NAT, GST, UGT, SULT, etc.) and their apparent catalytic parameters (apparent K_m, V_{max} and the ratio V_{max}/K_m) in skin models compared with NHS. Results showed that all these enzymes involved in the metabolism of xenobiotics are expressed and effective in the NHS and skin models. Also, apparent ratio V_{max}/K_m (estimating the metabolic clearance) and the metabolic abilities were often comparable between skin models and NHS. These results indicate that the skin models can substitute for NHS to select cosmetic ingredients on the basis of their metabolism, efficacy and/or safety.

I-2-233

Skin metabolism

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Skin is mainly considered to be a physical barrier. However, skin is also recognized to be an important site of extrahepatic metabolism and requires to be characterized in terms of metabolic abilities. Moreover, the 7th European amendment to the Cosmetic Directive will totally ban, in 2013, the use of animal testing to develop and trade new cosmetic ingredients in the European Community. For more than twenty years, L'Oréal has been producing and improving reconstructed human skin models (skin models) as *in vitro* alternative methods to animal experimentation. It uses them to predict different toxicological endpoints such as skin corrosion and skin irritation on finished products and cosmetic ingredients in development. For these reasons, it is essential to assess and compare these skin models with normal human skin (NHS) in terms of metabolic abilities.

In this work, the apparent metabolizing enzyme activities of three SkinEthic™ skin models were determined and compared with NHS for the main cytochrome P450-dependent monooxygenases involved in drug metabolism, esterases, alcohol and aldehyde deshydrogenase, glutathione S-, N-acetyl-, glucuronyl- and sulfo-transferases. Results show that the skin is much better equipped in conjugating enzymes and esterases than in P450-dependent monooxygenases and define it as a much more detoxifying organ than a bioactivating one. They demonstrate as well that the skin models are equivalent to NHS in terms of metabolic capabilities. Consequently they are useful as substitutes of human skin samples to assess the local efficacy and safety of new cosmetic ingredients.

I-2-238

Deciphering the mechanisms of action of potassium bromate in human and rat renal proximal tubular cells

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Potassium bromate (KBrO3) is an oxidising agent and is widely used in the food industry as a maturing agent for flour and as a dough conditioner. However, it has shown to be both a nephrotoxin and a renal carcinogen.

Within the carcinoGENOMICS project we conducted a genome-wide transcriptomic screen in NRK-52E and RPTEC/TERT1 cells treated with a sub-toxic concentration of KBrO3 (0.5 and 1 mM, respectively) for 6, 24 and 72 h. Analysis of altered gene expression revealed "NRF2-mediated oxidative stress", "Glutathione metabolism", "Tight junction signaling", and "DNA damage, p53 signaling and cell cycle" as the most enriched pathways. These gene alterations were reflected by increased expression of heme oxygenase 1 and NQO1 proteins, glutathione depletion, cytosolic occludin accumulation and decreased trans-epithelial electrical resistance.

The characterized biological responses to KBrO3 exposures indicate oxidative damage as a primary mechanism leading to DNA damage and the involvement of p53 and cell cycle alterations. A novel finding of this study is that sub-lethal oxidative damage induced by KBrO3 affects the expression of a number of tight junction proteins. The data elaborated here will be useful for screening other compounds for oxidative damage potential.

I-2-262

Alternative approaches for the evaluation of repeated dose toxicity and its use for quantitative risk assessment of cosmetic ingredients

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including repeated dose toxicity, in view of the full marketing ban foreseen in 2013 for cosmetic products and ingredients tested on animals in Europe. For repeated dose toxicity, current animal studies provide information on many endpoints. They allow evaluation of an integrated response and its quantitative aspects, making its replacement very challenging. Alternative methods have been developed mainly as standalone methods for predicting effects in specific target organs. Initial attempts of computer-based modelling/techniques suggest the feasibility of developing models providing meaningful predictions of chronic toxicity. "Omics" technologies have been applied recently to *in vitro* models for the purpose of understanding and ultimately predicting toxicity. However, a major challenge is to develop approaches for combining and interpreting data on multiple endpoints, obtained from several alternative methods. The experts concluded that, for quantitative risk assessment, enhanced scientific knowledge on exposure, toxicokinetics, dose response, mechanisms of toxicity, and extrapolation between exposure routes is needed. Better understanding of mode of action and key events associated with repeated dose toxicity endpoints would support the development of alternative approaches. Biokinetic models need to be improved to support dose response extrapolation from *in vitro* to *in vivo*. Additionally, optimal use of existing data by the Threshold of Toxicological Concern concept, read-across and integrated testing strategies can provide opportunities to avoid *in vivo* testing. Although full replacement will not be available by 2013, possibilities for reduction/refinement for repeated dose toxicity testing were identified and will be described.

I-2-273

Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including toxicokinetics, in view of the full marketing ban foreseen in 2013 for cosmetic products and ingredients tested on animals in Europe. Toxicity risk assessment is moving away from descriptive animal toxicity studies towards mechanistic *in vitro* screening systems and their integration with various types of computational approaches, including modelling and simulation. The use of omics techniques and high-throughput screening are some of the emerging tools to ensure predictive toxicity risk assessment.

Toxicokinetics (TK) is the endpoint that provides information about the penetration into and fate within the body of a toxic substance, including the possible emergence of metabolites, and their absorption, distribution, metabolism and excretion (ADME). Currently, when there is an increasing reliance on non-animal testing approaches, toxicokinetics has been identified as a key element to integrate the results from *in silico*, *in vitro* and *in vivo* studies. There are several crucial contributions from TK knowledge in integrated risk assessment. TK is needed to estimate the range of target organ doses that can be expected from realistic external exposure scenarios. This information is crucial for determining the dose range that should be used for *in vitro* testing. TK is necessary to convert the *in vitro* results, generated at tissue/cell or sub-cellular level, into dose response or potency information relating to the entire target organism, i.e. the human body.

For the optimal use of TK knowledge it is imperative that in all *in vitro* toxicity testing systems the behaviour of the compound

under study is investigated, to produce data on disposition (biotransformation and transport) and concentration-response relationships. *In silico* approaches, such as QSARs, read across, etc., allow further quantitation of specific processes needed for the prediction of TK *in vivo*.

Physiologically based toxicokinetic modelling (PBTK) is currently regarded as the most adequate approach to simulate the fate of compounds in the human body and can be used to estimate the relevant exposure at the organism level from measured *in vitro* concentrations, and *vice versa*. However, validation is currently a bottleneck and novel approaches are needed to ensure that mechanistic and integrative risk assessment can be employed to the full extent.

I-2-290

The Integrated discrete Multiple Organ Co-culture (IdMOC) technology as an *in vitro* model for systems toxicology

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A major limitation of *in vitro* toxicity testing is the use of single cell-type cultures for the evaluation of organ-specific toxicity. Such systems ignore the critical interactions between different cell types within an organ or between multiple organs. The multiple cell type/organ interactions include paracrine signaling, endocrine signaling, as well as xenobiotic metabolism (e.g., hepatic metabolism of a xenobiotic with toxic metabolites exerting effects on extrahepatic tissues). The Integrated discrete Multiple Organ Co-culture (IdMOC) technology is developed in our laboratory to overcome this major deficiency of most single cell type *in vitro* systems. In the IdMOC, multiple cell types are co-cultured as physically separated cultures interconnected by an overlying medium. IdMOC employs the wells-in-a-well concept, with shallow inner wells situated within a larger containing outer well. The dimensions of the inner wells are designed to minimize dilution of critical extracellular signals (e.g. metabolites; cytokines) by the overlying medium. The IdMOC system has been applied to evaluation of toxic potential of xenobiotics towards key human organs including liver (hepatocytes), kidney (renal proximal tubule cells), lung (airway epithelial cells), nervous system (neurons/astrocytes), and vascular endothelium (aortic endothelial cells). The toxicants evaluated include pharmaceuticals, pesticides, environmental pollutants, and cigarette smoke condensates. Endpoints used successfully with the IdMOC include cellular ATP content, MTT metabolism, caspase activation, and gene expression using real-time PCR for the evaluation of cell-type specific functions. Specific examples of the application of IdMOC to evaluate organ-specific toxicity, metabolism-dependent toxicity, paracrine signaling and endocrine signaling will be described. The IdMOC system represents an improved *in vitro* experimental system modeling complex multiple organ interaction in an intact animal/human. The IdMOC system has the potential to be used routinely as a replacement of whole animal studies for the evaluation of xenobiotic properties.

I-2-294

Implementation of a redesigned preclinical biocompatibility testing program to support personal lubricant medical device products

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A traditional biocompatibility testing battery for a personal lubricant medical device consists of *in vitro* cytotoxicity testing, systemic toxicity testing in mice, irritation testing in male and female rabbits (RPI and RVI, respectively), and evaluation of contact sensitization potential in guinea pigs. A redesigned preclinical biocompatibility testing program to support personal lubricant medical device development has been developed in the spirit of the 3Rs; reduction of the number of animals used, refinement of the data derived from the animals used, and replacement of a non-relevant model. The RVI and RPI tests have been refined by expanding the scope of the protocols to evaluate both genital irritation potential and systemic toxicity endpoints concurrently, thereby making more efficient use of the animals on study, exhibiting greater relevance than mouse IV or IP exposure, and still yielding some degree of exaggerated exposure relative to intended consumer use. Furthermore, the standard

agar overlay cytotoxicity assay in monolayers of L-929 mouse fibroblast cells will be replaced with the Epi-Vaginal assay model, a human 3D vaginal-ectocervical tissue construct, to measure cytotoxic potential. Additionally, there is greater reliance on raw material selection and paper toxicology assessment when possible, to support the progression of the personal lubricant directly to human clinical RIPT testing instead of conducting the guinea pig sensitization testing. In summary, this redesigned testing program is being implemented with the intention of providing more predictive results with greater suitability to the test material, while reducing and refining the use of animals for biocompatibility testing.

I-2-310

Taking a mode-of-action approach to designing a hepatotoxicity screening strategy using the HepaRG cell model and high content imaging

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The liver is central to the metabolism of xenobiotics and faced with harmful effects of toxic substances. Evaluating the risk of liver toxicity is a major issue and there is still no established *in vitro* screening strategy to reliably identify potentially hepatotoxic chemicals. In the approach described here, a mode-of-action targeted analysis of the literature has been used to identify toxicity pathways and the key biological events associated with them. This knowledge has then been used to design a multiparametric HTS experiment to classify chemicals based on their likely association with a specific mode-of-action.

We used a metabolically competent cellular model, HepaRG, and high content imaging implemented on a HTS platform. The HepaRG cell line expresses the major liver functions, including P450s, phase II enzymes, transporters and nuclear receptors at levels comparable to those found in primary hepatocytes.

The high content screening approach we adopted is based on automatic analysis of image-sets acquired with an epifluorescent microscope, for the quantification of immuno-fluorescently stained biomarkers expressed by treated HepaRG cells. A quantitative high throughput screening format was employed using a 96-well plate format, which facilitated the testing of a set of 92 reference chemicals and drugs with known hepatotoxic activity. Multiple cellular phenotypic changes were analysed by staining with fluorescent dyes for identification and quantification of response parameters. A biostatistical model was then developed to associate the test chemicals with different mode-of-action based categories. A systematic comparison of the classification results with literature findings allowed a preliminary validation of the approach.

I-2-325

Toxicity of unfiltered and filtered diesel exhaust under various loads

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Diesel engine exhaust contains numerous gas pollutants and particulate matters which may pose adverse health risks. The use of diesel engines for powering passenger cars has gained more popularity due to diesel fuel containing more energy per litre than petrol. In addition, combustion inside a diesel engine is more complete, hence there is less emission of pollutants such as CO and HC.

The aim of this study was to develop and validate the use of a direct dynamic method for exposing human cell lines (A549 and HepG2) directly to filtered and unfiltered diesel exhaust at a range of exposure of 7.5 to 15 min, and post-incubation periods of 0-24 h. In summary, cells were grown on porous membranes and placed inside dynamic exposure chambers connected to a diesel engine exhaust. The cytotoxicity of the exhaust was analysed using ATP, MTS and NRU assays. The exhaust was also analysed for pollutants such as CO, CO_2, NO_x, HC and diesel particulate.

Results of this study indicated that human cell lines (A549 and HepG2) were sensitive to diesel exhaust pollutants at all exposure

times and loads. The 0 h post-incubation period assay showed greater reproducibility, while there was no statistical significant difference in cell viability between filtered and unfiltered diesel exhaust. The results suggest that the gaseous component of diesel exhaust causes cell death rather than the particulates.

I-2-339

A virtual liver for pharmacological and toxicological investigations: multiscale, location dependent, xenobiotic hepatocyte response mechanisms

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We describe experiments on iteratively improved, multizone *in silico* livers (ISLs) (e.g., Park et al., 2010) that are designed to improve instantiated, predictive, mechanistic insight into hepatic disposition, enzyme induction details, and hepatotoxicity patterns (Sheikh-Bahaei et al., 2010) that influence safety and efficacy testing of new xenobiotics (XBs). ISLs are examples of a new class of biomimetic, discrete event, object and agent oriented, multilayered, multiscale, physiological models (Hunt et al., 2009). Hepatocytes within liver lobules are quasi-autonomous; the mechanistic details of XB clearance and enzyme induction are location dependent. A project goal is to make the same true within ISLs: intralobular, XB-specific metabolic clearance, enzyme induction, and biliary elimination patterns quantitatively mimic literature data. ISL experiments use independent XO objects that carry a list of physicochemical properties (PCPs) of the referent compound. Upon dosing, XOs percolate through ISL spaces. Most events are stochastic. An XO that encounters an enzyme within an ISL hepatocyte (Hc) may be metabolized, and that can initiate enzyme induction events that are specific to that Hc and location. Absent XO exposure, enzyme number per Hc is within a "normal", zone dependent range. An XO-enzyme interaction can initiate a metabolic event and/or an induction event. A metabolite that maps to a toxic counterpart can subsequently initiate a toxicity event. There is a location signal dependent enzyme removal rate (rule) that maps to a blood oxygen gradient. Rules are placeholders for more fine-grained mechanisms. Simulations were conducted on Amazon's EC2 cloud platform using a validated protocol (Ropella and Hunt, 2010).

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I-2-368

COSMOS – A new European project to develop computational models for the repeat dose toxicity of cosmetic ingredients to humans

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The COSMOS (Integrated *In silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety) Project is funded by the European Commission and COLIPA. It is part of the Seurat Cluster (start date 1 January 2011), which aims to assess the safety of cosmetic ingredients to humans using non-test methods. The focus of the COSMOS Project is to develop an integrated suite of open source and open access computational models to predict human repeated dose toxicity for cosmetic ingredients. This suite of models will form a flexible and transparent tool within an integrated computation-

al workflow. The *in silico* workflows will allow for the prediction of repeated dose toxicity to humans through the integration of models based on threshold of toxicological concern (TTC), innovative chemistry and physiologically based pharmacokinetics (PBPK). The workflows will be adaptable and form a set of building blocks, allowing users to incorporate their own data and search existing data compilations. The specific objectives of the COSMOS Project are to:

Collate and curate new sources of toxicological data and information from regulatory submissions and the literature.

– Create an inventory of known cosmetic ingredients and populate with chemical structures.
– Establish thresholds of toxicological concern for endpoints relating to human repeated dose toxicity.
– Develop innovative strategies based around categories, grouping and read-across to predict toxicity and relate to adverse outcome pathways where possible.
– Establish kinetic and PBPK models *in vitro* and *in silico* and other relevant data to predict target organ concentrations and long term toxicity to humans.
– Integrate open source and open access modelling approaches into adaptable and flexible *in silico* workflows using the KNIME technology.

The funding of the EU COSMOS Project (Health-F5-2010-266835) is gratefully acknowledged.

I-2-426

The platform on science within the European Partnership for Alternative Approaches to Animal Testing (EPAA)

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative between the European Commission and industry, created in November 2005 to promote the development and implementation of novel 3Rs approaches to regulatory testing. The partnership focuses on identifying research needs, developing novel approaches and strategies, promoting communication, validation and acceptance of alternative approaches. Its activities are coordinated by three platforms: Science, Regulation and Communication/Dissemination. Current activities within the EPAA Platform on Science include:

– Building up a research consortium bringing together computational chemists and system biologists to evaluate whether special aspects of liver toxicity can be identified without animal studies as was recommended during the 2010 workshop "Harnessing the chemistry of life".
– A gap analysis in current stem cell research with focus on toxicological pathways and applications for systemic toxicity testing. It is planned to stimulate collaboration of experts who would not traditionally apply their work to toxicology.
– An *in vitro* and *in silico* ADME project addresses one of the greatest unmet challenges for complete replacement.
– Methods used in different sectors/companies are being compared to optimize the current toolbox and identify gaps.

Full replacement of animals with alternative approaches is not achievable in the short term and unpredictable in the long term. However, as new *in vitro/in silico* approaches become available, they are considered for inclusion in integrated testing strategies (ITS). Therefore, all the EPAA projects are being considered for their possible impact in integrated testing strategies.

I-2-450

Data integration and analysis approaches for toxicogenomics applications in the 3Rs: McDSA, ConXbase and ToxProfiler

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To reduce, refine and replace animal testing in toxicology, it is increasingly important to integrate information from heterogeneous data sources. The toxicity of a substance may be learned from integration of data from animal or non-animal studies, which can be improved with increasing availability of omics data, allowing for understanding of mechanisms of toxicity. In addition, information improving chemically and biologically-based grouping of chemicals contributes to the 3Rs. This complexity of different data resources necessitates a system that allows integration of this information. To this end, a framework for Metadata Capture and Data Storage and Analysis (McDSA) to integrate toxicological knowledge with toxicogenomics data is implemented. Metadata capture entails the systematic description of the experimental setup in a database. Besides metadata, project data need to be brought together with the actual measurements, as well as with the biological context of the results. To achieve this, we have developed ConXBase. ConXBase is a web-based tool that connects projects, researchers, studies, biological source, experimental conditions, chemicals, chemical groupings, genes, pathways, and experimental results. ConXBase is integrated with ToxProfiler, a data analysis and database tool for the analysis of toxicogenomics data at the level of biologically relevant gene sets. The benefits of this infrastructure will be illustrated using datasets from the Netherlands Toxicogenomics Centre (NTC), the EU Project Carcinogenomics, as well as the ASAT dB project, in which human disease mechanisms are explored to improve animal-free chemical hazard assessment.

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I-2-470

The FP7 Project AXLR8 – Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally coordinated research and technology development

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The EU FP7 coordination support action project AXLR8 (=accelerate) aims to support the transition to a toxicity pathway-based paradigm for quantitative risk assessment. To reach this goal, AXLR8 is conducting the following activities: 1) organize a series of annual workshops to map research progress, gaps and needs in the FP6/FP7 program on alternative testing strategies; 2) provide a forum for enhanced interdisciplinary and international communication, coordination and collaboration in order to maximise the impact of available resources; 3) work to improve acceptance procedures to provide for the uptake of validated 3Rs methods, including the transition to 21st century systems as they become available; 4) produce annual progress reports on the state of the science, including recommendations on priority research and funding targets to ensure a prominent role for European science in this rapidly developing global research area.

In 2010 and 2011 the AXLR8 workshops (AXLR8-1 & AXLR8-2) have focused on progress made in the EU FP6/FP7 projects funded by the health theme of the DG Research and Innovation "Alternative Testing Strategies: Replacing, reducing and refining use of animals in research". The results of the discussions and recommendations of the AXLR8 Scientific Panel at the AXLR8-1 workshop 2010 have been published in the AXLR8 Progress Report 2010. The results and the recommendations of the 2011 AXLR8-2 workshop on a "Roadmap to innovative toxicity testing (ITT)" will be presented.

I-2-545

Displacement of test chemicals from serum constituents in mixtures and possible effects on free concentrations and *in vitro* assay results

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In vitro assays may be used to estimate toxicity of mixtures of chemicals. The concentrations of chemicals in these assays are normally expressed as nominal concentrations. However, the freely available concentration may be much lower than the nominal concentration because the chemical may bind to serum constituents in the culture medium. When chemicals are exposed to an *in vitro* assay in a mixture, one chemical that is normally bound to serum protein (and thus has a low free concentration) may be displaced from serum protein by another chemical that binds more strongly to protein, thus increasing the free concentration and response of the first chemical in the assay. When nominal concentrations are used, one could falsely attribute this increase in response as being a direct effect of the second chemical. Therefore, the aim of this study was to measure the free concentration of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), individually and in a mixture, in a CAFLUX and a cytotoxicity (AlamarBlue) assay, using solid phase microextraction (SPME). Results indicate that the extent of synergistic and antagonistic effects attributed to non-AhR agonists may in part change when considering displacement of AhR agonists from serum constituents by more lipophilic non-AhR agonists.

I-2-546

In vitro kinetics of chlorpromazine after repeated exposure in primary rat hepatocytes and human HepaRG cells

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In the extrapolation of *in vitro* data to the *in vivo* situation, it is important to take the *in vitro* kinetics of a compound into account. In this project, part of the FP7 project Predict-IV, the *in vitro* kinetics of chlorpromazine hydrochloride (CPZ) were determined in two different liver cell systems. Primary rat hepatocytes (sandwich cultured) and human HepaRG cells were exposed daily to two concentrations of CPZ for 14 days. Samples were taken from medium, cells and plastic at five different time points after the first and last day of treatment. These samples were analyzed by HPLC-UV to determine the total concentrations of CPZ.

The concentration of CPZ in the supernatant decreased over time. This decrease was more pronounced in the primary rat hepatocytes. CPZ was taken up by the cells, as shown by an initial increase in the amount of CPZ inside the cells, while at later time points, the amount of CPZ decreased. More CPZ was found on the last day of treatment, indicating accumulation of CPZ inside the cells. Plastic binding of CPZ was only found in the HepaRG cell cultures. After 24 h, all CPZ had disappeared from the rat hepatocyte culture compared to only half in the HepaRG cell culture.

The *in vitro* kinetics of CPZ were different in the two *in vitro* liver systems. Furthermore, a difference was seen between the first and last day of exposure. Therefore, the kinetics of a compound in an *in vitro* cell system should be taken into account for a reliable interpretation of toxicity results.

I-2-577

Experiences with cytotoxicity assays to select starting doses for acute oral toxicity testing

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With the objective of reducing and refining animal experiments for determination of acute oral toxicity, we have implemented cytotoxicity data to estimate acute oral starting doses in rats. We used the neutral red uptake (NRU) method in Balb/c 3T3 fibroblasts (ICCVAM, 2006) to determine the cytotoxicity of about 120 test substances including chemicals and formulations. The estimated predicted starting doses were then used in rat acute oral toxicity studies.

Comparing the predicted LD_{50} and the *in vivo* determined GHS classification, the cytotoxicity assay showed good prediction only for low toxic substances (83%, GHS Cat. 4, >300-2000 mg/kg body weight). The overall concordance was rather low (36%), mainly because 76% of the tested substances were classified as low toxic *in vitro*, but only 34% *in vivo*. Expanding the prediction +/- one category greatly enhanced the overall concordance to 82% with only 8% overpredicted (*in vitro* Cat. 3, >50-300 mg/kg, *in vivo* Cat 5, >2000 mg/kg) and 10% underpredicted test substances (*in vitro* Cat. 4, *in vivo* Cat 1-2, ≤50 mg/kg).

The use of cytotoxicity data to predict starting doses did not sufficiently contribute to the refinement and reduction in acute oral toxicity testing. As the predictivity of the *in vitro* test highly depends on specific properties of the tested substances, further analysis considering metabolic breakdown and mode of action are necessary. Using this data the applicability domain of the cytotoxicity assay for the prediction of acute oral toxicity in rats might be refined.

I-2-594

Autonomous virtual hepatocyte micromechanisms learn to respond to compound physicochemical properties (PCPs): clearance from simulation experiments, given new compound PCPs, predicts *in vitro* clearance

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We demonstrate the feasibility of using *in silico* hepatocyte cultures (ISHCs) to provide predictions of the intrinsic clearance (CL) of compounds in hepatocyte cultures. We compare results to predictions obtained using a multiple linear regression method. Our expectation is that the method can influence safety and efficacy testing of new xenobiotics by, for example, being extended to predict *in vivo* clearance of new compounds in humans. Within ISHCs, mobile "compounds" carry referent compound's physicochemical properties (PCPs). We used an Iterative Refinement Protocol for ISHC refinement and development of parameterization methods. Quasi-autonomous "hepatocytes" and their components (including "transporters" and "enzymes") use a small, event-specific subset of PCPs to interact with mobile "compounds" each simulation cycle. The probability of occurrence for each event type is specified by a rule based on a subset of PCPs known to influence that event counterpart *in vitro*. ISHC experiments mimic *in vitro* counterparts. *In silico* clearance is measured the same as *in vitro* and used to predict a corresponding CL value. For 39 of 73 compounds having calculated CL standard deviations (SDs), 79% of ISHC predictions and 23% of regression predictions were within CL ±2 SD. For all 73 compounds, 38% of ISHC predictions and 32% of regression predictions were within a factor of two of the referent CL values. ISHC details during simulations stand as a mechanistic hypothesis of how clearance phenomena emerge during *in vitro* experiments.

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I-2-639

Effect of Trichostatin A on miRNA expression in cultures of primary rat hepatocytes

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In the present study, the effect of Trichostatin A (TSA), a histone deacetylase inhibitor, was investigated on the microRNA (miR, miRNA) expression profile in cultured primary rat hepatocytes by means of microarray analysis. Simultaneously, albumin secretory capacity and morphological features of the hepatocytes were evaluated throughout the culture time. In total, 25 out of 348 miRNAs were found to be differentially expressed between freshly isolated hepatocytes and 7-day cultured cells. Nineteen of these miRNAs were connected with "general metabolism". MiR-21 and miR-126 were shown to be the most up and down regulated miRs upon cultivation and could be linked to the proliferative response triggered in the hepatocytes upon their isolation from the liver. MiR-379 and miR-143, on the other hand, were found to be the most up and down regulated miRs upon TSA treatment. Together with the higher expression of miR-122 observed in TSA-treated versus non-treated cultures, we hypothesise that the changes observed for miR-122, miR-143 and miR-379 could be related to the inhibitory effects of TSA on hepatocellular proliferation.

I-2-642

Detection of endpoints and biomarkers of repeated dose toxicity using *in vitro* systems

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DETECTIVE is part of an integrated research strategy towards the replacement of animal testing set up by the European Commission within the FP7 Health Programme and supported by the European Cosmetics Association. Within this collaborative project, 15 partners address the development of biomarkers of long-term toxicity in human target cells.

As from 1 January 2011 and for a duration of 5 years, emphasis will be put on the systematic exploitation of a battery of complementary functional and "-omics" readouts, including high content and high throughput screening platforms to identify and investigate human biomarkers in cellular models for repeated dose *in vitro* testing. While functional parameters give more insights into the effects of toxicants on specific cell functions of interest, "-omics" techniques will deliver data on the entire cellular situation at the molecular level. Importantly, DETECTIVE will perform for the first time an in-depth investigation of repeated dose effects on epigenetics and microRNA (miRNA) expression, thus exploring whether such analyses deepen our understanding of toxic modes of action.

Upon combination and subsequent integration of the various readouts, biomarkers of optimal predictivity for human long-term toxicity *in vitro* can be obtained. Based on integrative statistical analysis, systematic verification and correlation with *in vivo* data, the most relevant, highly specific, sensitive and predictive biomarkers will be selected. DETECTIVE concentrates on hepatoxic and cardiotoxic, and – to a smaller extent – nephrotoxic effects, representing three common target organs of repeated dose toxicity. Ultimately, developed concepts will also be applicable to other organs or organ systems affected by systemic toxicants such as the nervous system. Furthermore, it is expected that DETECTIVE will be able to define human toxicity pathways relevant for all organs.

I-2-722

Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals – COACH

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The European Commission and the European Cosmetics Association (COLIPA) have launched a research initiative to improve current safety assessments and to accelerate the development of alternatives for the complex human health endpoint "systemic repeated dose toxicity testing" by making a total funding of € 50 million available. As a 1st step towards a vision of a "Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)," six individual research projects SCR&Tox, HeMiBio, DETECTIVE, COSMOS, NOTOX and ToxBank kicked off in January 2011 in order to develop technologies and to gain necessary scientific knowledge relevant for assessing repeated dose effects of cosmetic ingredients.

The collaboration within the cluster of six research projects (SEURAT-1) is facilitated by the coordination action COACH ("Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals") which will constitute the secretariat of the cluster in order to:

- monitor progress of the cluster towards the goals of the SEURAT initiative
- facilitate the information exchange and collaboration between the different cluster projects
- disseminate results of the cluster via annual strategy books, leaflets, in meetings, etc.
- cooperate with other international research teams
- prepare a strategy for future research activities in an international context

The poster will provide an overview of the objectives of COACH in order to facilitate the activities of the SEURAT-1 cluster.