

Welcome address to the 15th Congress on "Alternatives to Animal Experimentation" – Linz 2008



Dear colleagues,

On behalf of the organisers zet, the Austrian Centre for Alternative and Complementary Methods to Animal Testing, the Austrian National Platform on Alternatives and of MEGAT, the Middle-European Society for Alternatives to Animal Experiments, I cordially welcome you to this year's congress "Linz-2008".

We are quite happy that again a large number of high quality abstracts have been submitted both for oral presentations or posters. For the third time the congress in Linz will be held in English and we are proud that you, the scientific community, have accepted the change from German to English as the official congress language. Our congresses in Linz are now a European forum for the exchange of new ideas on progress in the development and validation of alternatives.

We are particularly proud that we have attracted key area leaders as speakers, who update the participants on the European Commission's and member states' efforts to implement the 3R principles into basic biomedical and applied research. This year updates will focus on ethical and scientific aspects of stem cell research, on the 7th amendment of the EU Cosmetics Directive and in particular on the application of skin models, on computer assisted procedures, on alternatives in reproductive toxicology and on genetic technology and transgenic animals. In Linz we have always held sessions on good cell culture practice and this year we will for the first time also cover the challenge of using *in vitro* methods in the safety testing of nanomaterials.

As in the past, we are strictly avoiding parallel sessions at the Linz-2008 congress and we have reserved sufficient time for intensive discussions during the poster sessions. In Linz we have always tried to motivate young scientists by giving awards to young authors, who are presenting the most innovative and promising posters. Thus we are looking forward to another exiting award ceremony with new faces at the conference banquet.

Linz, the capital of Upper Austria, is a lively modern city, situated on the river Danube, with a Baroque city core. It is famous for its many modern museums and the *ars electronica* centre. The surrounding Austrian countryside offers unique historic sights as well as cultural and gastronomic highlights.

We hope to provide you with an attractive, stimulating and interesting programme in Linz 2008. I am looking forward to seeing you there.

On behalf of the organisers I welcome you

Sincerely

Horst Spielmann, President of MEGAT

The Congress organisation, MEGAT and ALTEX Edition wish to thank the Foundation Animalfree Research (Zurich, Switzerland) for sponsoring this supplement.

Poster: skin models as alternatives to animal testing

Reconstructed human epidermis (RHE): an *in vitro* skin irritation model for full replacement of the Draize test

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Efforts to fully replace the Draize skin irritation test on rabbit *in vivo*, according to Method B.4 of Annex V to Directive 67/548/ECC or OECD TG 404 have been expended for many years in Europe. The issue has become still more critical as a result of the 7th Amendment of EU Cosmetic Directive and EU regulation for the Registration, Evaluation and Authorisation of Chemicals (REACH).

To date, following an ECVAM-managed skin irritation validation study, an *in vitro* test method using reconstructed human epidermis model EpiSkin has been scientifically validated as the only stand alone method to discriminate skin irritants (I) from non-irritants (NI) according to the EU 67/548/ECC classification. Furthermore a performance standard document has defined the procedure whereby the accuracy and reliability of a Me-too new test method could be evaluated (ECVAM SIVS, 2007).

The purpose of the present study was to assess an *in vitro* reconstructed Human Epidermis (RHE) model from SkinEthic Laboratories (Nice, France).

A multicentre study was performed on 20 reference chemicals under blind conditions (chemicals coded by Vitroscreen) in three independent laboratories (L'Oréal, Coty and Oroxcell) using three different batches of RHE model. The reference test chemicals were selected so as to cover a broad range of physico-chemical properties and of *in vivo* Draize test irritancy scores according to the performance standard document. RHE skin irritation test method consisted in applying topically test chemical to RHE samples and leaving on for 42 minutes before rinsing then incubate the tissues for 42 hours at 37°C before endpoint measurement. The main endpoint was cell viability (MTT reduction), with a threshold of 50% viability. Interleukin 1 alpha (IL-1 α) was also measured to determine whether predictive ability of the assay was improved by additional endpoint.

An independent statistical analysis was performed to assess intra and inter-laboratory reproducibility using standard deviation, coefficient variation, 1-way Anova, Bravais-Pearson' correlation, and identical run classification approaches. Good intra and inter-laboratories reproducibility were achieved. Correct predictions of skin irritation potential for the 20 test chemicals were obtained with 90% sensitivity and 80% specificity (MTT endpoint only). Overall accuracy was 85% and was not improved by IL-1 α data. Similar predictions levels on those 20 test chemicals were already described with the validated EpiSkin system, using the two combined endpoints.

Therefore the present RHE assay appears to be a promising *in vitro* test method to fully replace the Draize skin irritation test on rabbits.

Keywords: *alternative, skin irritation, RHE*

Poster: free communications

Human HepaRG[®] hepatocytes for the detection of toxic metabolic pathways

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Human HepaRG[®] cells from a human hepatic cell line are able to differentiate *in vitro* into hepatocyte-like cells and display hepatic functions: HepaRG[®] cells exhibit (i) a hepatocyte-like morphology; (ii) a metabolic competence for phase I and II enzyme activities; (iii) a concomitant expression of hepatic influx and efflux transporters (Le Vee et al., 2006); (iv) a good response to standard inducers of drug metabolizing enzymes (Aninat et al., 2006; Le Vee et al., 2007; Kanebratt et al., 2008).

In this study, HepaRG[®] cells were used to set up a cytotoxicity screening protocol that modulates the metabolic pathways and uses rezasurin transformation as a single toxicity endpoint. Three model xenobiotics, flutamide, tamoxiphen and amitriptyline were chosen to investigate the relevance of the protocol. Differentiated HepaRG[®] hepatocytes were produced following our standard process. Their metabolic pathways were induced or inhibited as follows: CYP1A2 was induced by omeprazole and DMSO. CYP1A2, CYP3A4, phase II enzymes like NAT were inhibited by co-incubation of furafyllin, ketoconazole, salicylamide or acetaminophen with each xenobiotic.

A comparison of the results obtained with HepaRG[®] cells, with primary culture of human hepatocytes or with HepG2 cells (+ or - S9) was also done.

We found: (i) The specific cytotoxic profiles of each xenobiotic were comparable in human hepatocytes, HepaRG[®] cells and HepG2 cells + S9; (ii) The addition of the CYP inhibitors or the phase II enzymes inhibitors either increased or decreased the toxicity of the compounds as was expected from the literature (Seva et al., 2007) and (iii) from our human hepatocyte data.

These results suggest that HepaRG[®] hepatocytes are a promising tool for the screening of toxic metabolism pathways.

Keywords: HepaRG[®]

Poster: 7th cosmetics amendment – can all goals be achieved in time?

***In vitro* skin irritation assessment of colored and colorant-like chemicals using the ECVAM validated EpiSkin™ assay: the need of appropriate and relevant controls**

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The use of *in vitro* reconstructed epidermis as a replacement model for the rabbit skin irritation method has been scientifically validated in April 2007 (ESAC statement, 2007). The test is based on tissue viability assessment by using the MTT test (Mosmann et al., 1983) (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and allows the discrimination between irritant chemicals (R38) and non-irritant (NC) chemicals. The MTT assay is a standard colorimetric method: Yellow MTT is reduced to purple formazan by mitochondrial dehydrogenases of the epidermis living cells. The colored solution obtained after formazan extraction is quantified by absorbance measurement at 570 nm. Therefore, due to this measurement method, any chemical able to strongly color the tissue or induce indirect and non specific coloration absorbing closely to formazan can result in a possible viability overestimation.

The purpose of this study was to investigate the use of the validated EpiSkin™ protocol (colorimetric assay) for the *in vitro* evaluation of colored chemicals irritancy potential (e.g. hair dyes). The assay consists mainly in a standardized topical application and a post-treatment incubation period (Spielmann et al., 2007; Cotovio et al., 2005). Colored chemicals as dyes can be retained in the epidermis or difficult to wash-off and induce a possible residual staining. Consequently, after extraction, non specific remaining color is able to modify final readings. In order to eliminate colorimetric interference, we introduced specific controls fitting to the validated protocol course. Control tissues followed the same treatment steps than treated ones, MTT step excepted. These controls enabled the quantification of non specific optical density (OD). Therefore calculations take into account only the true OD related to mitochondrial activity of living cells. By introducing these adapted controls, we showed that the validated EpiSkin™ MTT assay is a suitable and reliable method for *in vitro* skin irritation prediction of dyes and chemicals likely to color tissues. The applicability domain can therefore be extended to these chemical families.

References

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Spielmann et al. (2007). *ATLA* 35(6), 559-601.

Keywords: *EpiSkin, 3D model, MTT, Skin irritation, ECVAM validation, colorant*

Poster: skin models as alternatives to animal testing

IL-1 α release quantification in culture media: how to ensure standardization?

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The reconstructed human epidermis EPISKIN™ model was involved in the formal ECVAM sponsored acute skin irritation validation (Draize test replacement). The model and the associated prediction model (PM) were validated by the ESAC as stated in April 2007 (Spielmann et al., 2007). The test is based on measurement cells viability by MTT test (ESAC validated) and the release of the pro-inflammatory mediator IL-1 α (recommended by ESAC as a useful adjunct) (ESAC statement, 2007). Indeed the combination of these two end points enabled a sensitivity shift from 75% to 91% (Spielmann et al., 2007). The validated PM relied on specific cut-off values for both viability and IL-1 α . IL-1 α was usually expressed in pg/ml and its quantification was performed by using the R&D Systems ELISA kit. The purpose of this study was to investigate the possibility to use other purchasable IL-1 α quantification systems. In this work, we compared commercially available kits from several suppliers. Dose response linearity of the assay systems was first assessed by using WHO/NIBSC IL-1 α international standard. Good linear responses were observed for all kits with associated specific sensitivities. IL-1 α quantification in test samples also revealed important sensitivity differences related to the kit used. Standardization of the assays and data expression can be obtained by using the common International Unit (IU) value. To make possible results expression in IU for further data comparisons between different laboratories using the validated test, we proposed a 3 steps procedure: 1- Check R&D Systems kit calibration with the reference NIBSC IL-1 α standard in the specific laboratory working conditions and convert pg/ml to IU/ml. 2- Measure IL-1 α in the test samples with the R&D Systems kit and the other ELISA kit to be used as a replacement. 3- Calculate the converting factor between both kits in order to express results in IU (R&D/IL-1 α standard calibration conditions). 3-D tissues being sensitive to mechanical stress during spreading steps, basal levels of IL-1 α in controls can be modulated by operator-dependent rubbing application strength during the process. In order to eliminate operator-dependent basal interleukin release levels, negative control values must be subtracted to test-sample value for calculations. This standardization process helps: 1) to clarify the use of IL-1 α by adjusting and normalizing calculations, 2) to open the possibility to use other supplier measurement kits while respecting the defined PM, 3) to normalize IL-1 α expression as common IU values.

References

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Spielmann et al. (2007). *ATLA* 35(6), 559-601.

Keywords: skin irritation, 3-D models, EpiSkin, ELISA, IL-1 α , MTT

Lecture: free communications

***In vitro* model for evaluation of zineb induced oxidative stress, immunosuppression and apoptosis**

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Pesticides usage has become an ever increasing phenomenon despite their adverse effects on environment. Exhaustive information is available on the pesticides and their deleterious effects on animal and human health, but it is painful to note that very little emphasis has been directed towards studying pesticide induced immunosuppression and apoptosis employing *in vitro* cell culture system of avian splenocytes.

Zineb is a dithiocarbamate fungicide with some insect repellent properties. It is a metabolic poison of low acute toxicity to mammals: Zineb is toxic to zinc sensitive plants. It has also been used extensively in the rubber industry as a promoter of vulcanization. Present communication evaluates oxidative stress, immunosuppression and apoptosis in chicken lymphocytes due to *in vitro* exposure of low level dose of zineb employing nitric oxide estimation, lymphocyte proliferation assay, cytokine assay, annexin V assay and DNA fragmentation assay, respectively.

Splenocytes were isolated and treated with thousand times dilution of No Observable Effect Level (NOEL/103) dose of zineb. Chicken lymphocytes displayed elevated level of Nitric Oxide when exposed to zineb. Lymphocyte proliferation assay revealed significant decrease in B and T lymphocytes levels. In cytokine assay there was down regulation in interleukin I and II levels in zineb treated cells as compared to control cells. Thus zineb was found to be immunotoxic even at low dose level. Annexin V assay revealed increased number of lymphocytes undergoing apoptosis. It was further confirmed by agarose gel electrophoresis of genomic DNA of zineb exposed cells which displayed typical apoptotic DNA ladder pattern. It is also worthwhile to mention that such *in vitro* tests in immunotoxicology can be useful in the quick preliminary evaluation and toxicity assessment of pesticides and will be helpful in reducing the use of laboratory animals.

Keywords: *zineb, immunotoxicity, DNA fragmentation, oxidative stress, cytokine assay, apoptosis, avian lymphocytes, annexin V assay*

Poster: strategies to reduce animal numbers for testing biologicals

Potency testing of inactivated rabies vaccines for veterinary use: correlation of results between mouse challenge test and serological assay

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The potency of inactivated rabies vaccines for veterinary use is conventionally determined by a mouse challenge test. In this test, vaccinated mice are infected with rabies virus, and the potency is calculated from the resulting survival rate. This method causes severe distress to the test animals and is known to be imprecise and time-consuming. According to the European Pharmacopoeia, also a serological assay may be used for batch potency testing after a suitable correlation with the challenge test has been established. In the serological assay, neutralizing antibody titers induced by vaccination of mice are determined using a rapid fluorescent focus inhibition test (RFFIT). As this alternative method is less painful and requires fewer animals, it meets the 3R criteria of refinement and reduction. However, this assay is not widely used yet, and only few data exist concerning the comparability of both methods. Our results demonstrate a good correlation between the antibody titers obtained in the serological assay and the survival rates of the mouse challenge test. We further show that vaccine batches failing the challenge test were also reliably identified as insufficient in the serological assay. We conclude that the serological assay is a suitable method for the potency testing of inactivated rabies vaccines, and that it is able to replace the mouse challenge test in the long term.

Keywords: rabies vaccine, mouse challenge test, serological assay, RFFIT

Poster: strategies to reduce animal numbers for testing biologicals

A combined assay measuring binding and enzymatic activity allows *in vitro* detection of tetanus toxicity in vaccines

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Tetanus neurotoxin (TeNT) consists of two disulfide-linked subunits. The heavy chain mediates the binding to neurons, whereas the light chain cleaves the neuronal protein synaptobrevin and thus causes the spastic paralysis characteristic of tetanus infections. Formaldehyde-inactivated TeNT preparations (tetanus toxoids) are used as vaccines. According to the European Pharmacopoeia, these toxoids have to be tested for “Absence of toxin and irreversibility of toxoid” by injecting them into guinea pigs and observing the animals for tetanus symptoms. Our aim is to develop an *in vitro* method which can replace these animal tests. First we examined whether an endopeptidase assay can be used to detect active TeNT in toxoids. However, we found that most toxoids contain high synaptobrevin-cleaving activities caused by TeNT light chains with intact protease domains. As these molecules lack a functional heavy chain, they are not toxic *in vivo*. Thus, the endopeptidase assay alone cannot reliably detect tetanus toxicity in toxoids. We are now developing a combined method consisting of a ganglioside binding and an endopeptidase activity step. Both steps are functionally linked, so that only molecules which display both activities on separate, disulfide-linked subunits generate a signal. We demonstrate that this combined assay is able to detect toxic TeNT in spiked toxoid samples.

Keywords: Tetanus neurotoxin, synaptobrevin, toxoids, toxicity

Poster: free communications

Protective effects of herbal cosmetic ingredients assessed in cell and tissue cultures and in human studies

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Herbal ingredients are preferentially used in cosmetic formulations intended for consumers with sensitive or dry skin, with the aim to improve skin condition and appearance. They are reported to promote physiological functions of the skin and may offer a balanced complex of health effects as moisturizing, free radical scavenging, calming and anti-inflammatory, improving skin elasticity, anti-aging, healing sunburn or chemical induced irritation (Leung and Foster, 2003).

The study was focused on the significance of addition of selected herbal ingredients, used in formulations of cleaning products in order to minimize possible adverse irritative effects of tensides, e.g. Sodium Dodecyl Sulfate (SDS).

The protective effects of selected active ingredients were tested *in vitro* in the cell culture of 3T3 fibroblasts and in the human reconstructed skin model (EpiDermTM, Mattek, USA), and subsequently evaluated *in vivo* by testing in a group of volunteers by means of a closed epicutaneous patch test according to COLIPA Guidelines (COLIPA, 1997). The advantage of *in vitro* systems for prediction of biological effects in human practice was examined.

Protective effects against SLS cytotoxicity were proved in the cell culture of 3T3 fibroblasts in case of a number of natural substances, e.g. Green Tea, Aloe Vera, Pronalen Sunlife, Pronalen Cereal, Pronalen Sensitive Skin and Chamomile. The effects of the most promising substances were subjected to further testing in the 3D human skin model. Results from the *in vitro* systems were compared to results obtained by means of an epicutaneous test in a group of human volunteers. All the selected herbal substances, in accordance with results obtained *in vitro*, exhibited protective effects against SDS-induced skin irritation in human volunteers. The highest degree of protection *in vivo* was proved for Chamomile and Green Tea in all recorded times of assessment.

The *in vitro* test systems were found to be a useful tool for screening of biological effects and represent a valuable tool for safety and efficacy testing of cosmetics before skin testing in human volunteers is performed.

References

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Keywords: cosmetic ingredients, SDS, skin irritation, cytotoxicity, skin model, human patch test

Lecture: *in vitro* toxicology

Biokinetic considerations in *in vitro* toxicology

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The current practice in toxicological risk assessment is to assess human health or environmental risk of chemicals on the basis of clinical or histopathological endpoints in animal studies. These apical endpoints do normally not take into account the mechanism(s) of toxic action. *In vitro* studies have much better possibilities to study these mechanisms in great detail. Such data are normally expressed as the concentrations giving a certain degree of effect, e.g. the concentration resulting in 50% of the maximal effect (EC_{50}). However, the extrapolation of *in vitro* toxicity data to the *in vivo* situation needs a number of considerations, too. The interpretation of these results in terms of risk requires the “translation” of the data towards the expected exposure in an intact organism. Thus, EC_{50} s, expressed as molar concentrations will need to be converted to the amount (i.e. the dose) to which the organism is exposed.

These biokinetic aspects can now be studied in detail with the help of (physiologically-based) biokinetic (PBBK) models. Such a model allows the calculation of concentrations in tissues given a certain exposure scenario. When used in a reverse way, it thus also allows the calculation of a dose (or exposure scenario) resulting in a concentration in target tissues that would give a toxicologically relevant effect in an *in vitro* system.

Similar models can also be used to study the biokinetic behaviour of compounds in the *in vitro* system, i.e. “biokinetics *in vitro*”. Evaluating the kinetics of a compound in the cell culture is greatly increasing the relevance of the *in vitro* toxicity findings, e.g. by taking into account the actual (free) concentration to which the cells in an *in vitro* system are exposed to. It may also show any relevant differences in the conditions of exposure at a cellular level between the *in vitro* systems and the situation *in vivo*, e.g. in relation to protein binding.

Making use of these models, a conversion was calculated of the EC_{50} values for cytotoxicity to toxic doses (LD_{50} s). It was shown that the correlations with the experimentally determined LD_{50} in rodents improved.

Keywords: biokinetic models, cytotoxicity, risk assessment

Lecture: alternatives & education

Validation of an *ex vivo* human cervical tissue model for the local delivery of nucleic acid drugs

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Subject: The human cervix is the point of origin of cervical cancer, the second most common female tumor entity worldwide. Every year about 230,000 women die from cervical cancer. The necessary cause of cervical cancer is the infection of the cervix with high risk types of human papilloma virus (HPV) (Walboomers et al., 1999). To address a local therapy with nucleic acid drugs it is important to understand the barrier properties of the target tissue to which the drug will be applied. The aim of this study was to establish and validate an *ex vivo* human cervical tissue model for delivery and permeation studies.

Material and Methods: Permeability studies were conducted using the static Franz cell system (Hiller et al., 2007). The cervical biopsies of an area of 0.125 cm² were inserted with the mucosal side oriented upwards. The transport experiments were performed at 37°C applying a pH gradient (donor pH 6.0/acceptor pH 7.4). 40 µM [¹⁴C]Mannitol and [³H]Propanolol were used. [¹⁴C]Dextran 10,000 was used at a concentration of 1.5 µg/ml without the addition of unlabelled Dextran 10,000 and 40 mg/ml FITC-Dextran were used. The diffusion studies were carried out over 24 hours. After a preincubation time of 15 minutes, the acceptor medium was sampled after 0.5, 1, 2, 4, 6, 18 and 24 h.

Results and Discussion: To determine the influence of the menopausal status of the investigated 34 patients (21 pre- and 13 postmenopausal) the Papp values were statistically evaluated for three subgroups of small molecules, hydrophilic molecules and larger molecules. These subgroups show nearly an equal distribution of patients of both menopausal status. The apparent permeability coefficients (Papp) of the various markers demonstrated that with increasing molecular weight the marker permeability decreases, an upper permeability limit between 10,000 to 20,000 Da, no significant intraindividual variability, because the Papp values were comparable among biopsies of the same patient a significant variation of the permeability among different patient samples. A continuous difference of one log value between the Papp of mannitol and dextran 4,000 make them suitable as an internal marker control pair for each biopsy. The Papp values of both markers across fresh and frozen tissue are comparable.

Conclusion: According to the presented data we concluded that the human cervical tissue model has been well characterized and is therefore suitable for delivery and permeation studies with a focus on nucleic acid drugs. To our knowledge the present studies are the first time investigating in a wide range a human *ex vivo* cervical model for permeability studies. The comprehensive aim was to use this *ex vivo* model to support preclinical lead optimization of locally acting medicaments at the cervical tissue. The validation of the *ex vivo* cervical model did not cover all ECVAM recommendations (Hartung et al., 2004) because our principle goal was not to establish a tissue model for the replacement of animal studies.

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Keywords: cervix, permeability studies, *ex vivo* model, nucleic acid drugs

Poster: nanotoxicology / nanobiotechnology

The use of precision cut lung slices to assess nanoparticle mediated toxicity

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The production and use of engineered nanoparticles has dramatically increased due to their various applications such as in medicine, information technology, and chemistry. The unique physiochemical characteristics of nanoparticles as compared to their bulk material challenge their toxicological risk assessment. To date most studies investigate the effects of a specific material on a particular model system separately, making extrapolations of the same material for e.g. different sizes or surfaces difficult. The exposure route of highest concern for occupational and public exposure to nanoparticles in humans is inhalation. Currently, *in vivo* inhalation studies are required for the investigation of nanoparticle induced effects on the respiratory tract. These experiments are resource intensive and, especially for nanoparticulate matter, technically challenging. Therefore fast but reliable and relevant alternative methods that can be standardized are required. In this study, which is part of the FP6 CellNanoTox project, rat precision cut lung slices (PCLS) were investigated as a model for the respiratory tract. PCLS show characteristic responses to typical pro-inflammatory stimuli and thus provide an appropriate *in vitro* technique to predict the immunomodulatory and cytotoxic potency of inhaled substances. This tissue slice culture is a promising tool e.g. for dose finding for inhalation studies. Likewise precision cut lung slices may be employed for grouping approaches in which a limited number of nanoparticles of a physiochemical group are tested *in vivo* and toxic properties for others are extrapolated from the *in vitro* methods. The aim of the study was to investigate the effects of different nanoparticles for their toxic potential. We have assessed the effects of cobalt nanoparticle aggregates, and cobalt ferrites at different sizes with and without serum protein stabilization and possibly to extrapolate from physiochemical properties to toxic effects. We have demonstrated that this *in vitro* system is a suitable tool to investigate the effects on nanoparticles on the respiratory tract.

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Keywords: *precision cut lung slices, nanoparticle, CellNanoTox*

Poster: nanotoxicology / nanobiotechnology

An epithelial airway model to study the effects of gold nanoparticles deposited at the air-liquid interface

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Potential health effects of nanoparticles ($\leq 100\text{nm}$) associated with human exposure are poorly investigated. In order to avoid and to replace toxicity studies with animals, we have established and evaluated a triple cell co-culture system composed of epithelial cells, macrophages and dendritic cells which simulates the most important barrier functions of the lung epithelial airway. With this model we investigated the potential immune response after exposure to gold nanoparticles (diameter of 15nm). Gold nanoparticles are already used in different biological applications *in vitro*.

Cells, cultured at the air-liquid interface, were exposed to two different particle concentrations (2.5×10^9 particles/cm², 2.5×10^{10} particles/cm²) and then further incubated for 4h and 24h. We measured the inflammatory mediators IL-8 and TNF-alpha by qRT-PCR (mRNA) and ELISA (protein). The intracellular particle localization was investigated by transmission electron microscopy (TEM). Additionally we pre-stimulated the cells with lipopolysaccharids (LPS) to simulate a chronic inflammation at the time point of particle exposure.

By TEM we could show that the particles enter the cells as single particles or as smaller agglomerates. Particles were mainly localized in intracellular vesicles. In terms of inflammatory effects, gold nanoparticles did not cause a significant reaction at both time points and exposure concentrations. When priming the cells with LPS, a clear immune reaction due to LPS was observed by elevated IL-8 and TNF-alpha mRNA and protein levels. However, a combination of LPS with gold nanoparticles did not cause any difference in mRNA or protein release compared to the LPS reaction itself.

Using an advanced cell culture model of the human epithelial airway barrier at the air-liquid interface, we have shown that 15nm gold nanoparticles did not induce an immune reaction or to burst a reaction in a pre-stimulated system, although particles clearly enter the cells. Further studies on translocation and retention of particles are still needed to conclude on their safety.

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Keywords: cell culture model, airway epithelium, nanoparticles, inflammatory response

Roundtable: Embryonic or adult stem cells: scientific and ethical considerations

The use of human embryonic stem cells in EU research projects

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The use of human embryonic stem cells (hESCs) in hazard assessments becomes crucial if no available *in vitro* model can detect the toxicological mechanisms under evaluation or if the extrapolation from animal cells or cells of a carcinogenic origin to the human situation is hampered due to species variations. Cardiomyocytes derived from hESCs may be advancing the current situation of pharmacological toxicity and safety assessments of the heart – a major purpose of the StReP: “InVitroHeart”. Currently available *in vitro* cardiac assays are mainly based on primary animal cardiomyocytes or cell lines expressing human cardiac proteins as primary human cardiomyocytes are limited available. This is problematic as non-clinical cardiac toxicological and pharmacological evaluations are strongly hampered by interspecies variations such as hERG channel expression. One of the most frequently-cited limitations of *in vitro* tests for assessing health effects are deficiencies in the bi-otransformation of test chemicals. Major obstacles of using human primary hepatocyte cultures are their loss of metabolic competences in culture and their restricted accessibility. The StReP “Vitrocellomics” is aiming to use hESCs to establish stable cell lines and 3-dimensional *in vitro* models that reflect human hepatic properties. Finally, the area of reproductive/developmental toxicity is requesting scientific solutions for assessing adverse effects on spermatogenesis and embryogenesis. Using hESCs for these purposes are among the main objectives of the integrated projects “ReProtect” and “ESNATS”. EU research projects applying hESCs have to be in compliance with ethical framework set by the European Commission in FP6 and FP7 and by the Member-states in which the research is carried out. The use of hESCs moreover, requests the application of quality controls that are beyond those that have been laid down in the good cell culture practise guidelines.

Keywords: cardiotoxicity, metabolism, reproductive toxicity, interspecies variations, quality aspects of hESC

Roundtable: Embryonic or adult stem cells: scientific and ethical considerations

Can human embryonic stem cells be an acceptable ethical alternative to animal experiments?

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Amongst the proposals for alternatives to animal toxicology testing, perhaps the most ethically radical is to use human embryos to derive different human cell lines as the subjects for testing. Is it valid to replace one big ethical problem, if our alternative poses an equally large issue? How do we weigh the relative merits of two opposing arguments of principle, or is this trying to 'compare apples and oranges'? Most justifications of human embryo stem cell (hESC) research are made on the basis of an appeal to the direct therapeutic benefits expected for currently incurable human diseases or injuries. The ethical argument for their use in toxicity testing of pharmaceutical has been comparatively little discussed so far, but the case seems less compelling. When weighed against our duties to a human embryo with at least some degree of moral status, the compassionate benefit is indirect, it is more complicated by commercial motivations, and some alternative approaches do exist. The use of hESCs for testing cosmetics or ordinary chemicals is still more problematic. Comparable arguments can be made about what constitutes a justification for animal use in research. The underlying value positions of two communities of belief, one opposed to experimental use of animals, the other of human embryos, tend to have a liberal view of the other's ethical objection. For those less fundamentally committed, how do we weigh the merits of the two cases, and can some common ground or acceptable compromise be found? How far should we pursue an aim of essentially risk-free chemical products, if the means to achieve this leads to serious pressures on other ethical concerns?

Keywords: human embryonic stem cells, animal experiments, ethical alternatives

Lecture: skin models as alternatives to animal testing

SkinEthic Laboratories, a validated choice of *in vitro* alternative methods to the animal use for skin corrosion and irritation and other selected choices for eye irritation and skin pigmentation

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SkinEthic is devoted to develop and produce reliable and robust *in vitro* alternative methods to the animal use and to streamline the clinical trials in cosmetic, chemical and pharmaceutical industries. SkinEthic models provide relevant tools for efficacy and safety screening tests in order to support an integrated decision making during research and development phases. Screening tests are referenced and validated as alternatives to animal use (Episkin) for skin corrosion and irritation, others such as a reconstructed human epidermis and a human cornea are in the process of validation under ECVAM. Episkin is under review process to be integrated in the OECD guidelines as a stand alone alternative to the animal use for skin irritation test.

Our product line includes skin models, a reconstructed human epidermis with a collagen layer Episkin, a reconstructed human epidermis (RHE) without or with melanocytes (with a pigmentation degree from phototype II to VI) and, a reconstructed human cornea (HCE) on an inert polycarbonate filter and grown in a chemically defined cell culture medium.

Our philosophy is based on 3 main commitments: to support our customers by providing robust and reliable models, to ensure a training and education in using validated protocols allowing a large of raw materials, active ingredients and finished products in solid, liquid, powder, cream or gel form to be screened, and, to provide a dedicated service to our partners.

The ECVAM international validation study on *in vitro* tests for acute skin irritation: report on the validity of the Episkin and EpiDerm assays and on the Skin Integrity Function Test (Spielmann et al., 2007).

Assessment of the skin irritation potential of chemicals by using the SkinEthic RHE model and the common skin irritation protocol evaluated in the ECVAM skin irritation validation study (Kandarova et al., 2006a).

Assessment of the SkinEthic RHE model for *in vitro* skin corrosion testing of chemicals according to the new OECD TG 341 (Kandarova et al., 2006).

Pre-validation of a new *in vitro* reconstituted human corneal model to assess the eye irritating potential of chemicals (van Goethem et al., 2006).

Reconstituted human corneal epithelium. A new alternative to the Draize eye test for the assessment of the eye irritation potential of chemicals and cosmetic products (Doucet et al., 2006).

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Keywords: skin model, skin irritation, eye irritation, safety screening, human epidermis, human cornea, SkinEthic

Lecture: good cell culture practice

Serum-free cell culture: the serum-free media interactive online database

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Fetal bovine serum (FBS) is an ubiquitously used essential supplement in cell culture media. However, there are serious scientific and ethical concerns about the widespread use of FBS concerning its harvest and production. During the last three decades, FBS could be substituted through other supplements or by the use of defined chemical components in serum-free cell culture. A number of serum-free media formulations have been described for continuous mammalian and insect cell lines as well as for primary cultures. However, switching to serum-free media still needs a time-consuming literature survey and manufacturer search for appropriate media formulations, respectively. In order to make the search for serum-free media easier, we present the second collection of commercially available serum-free media in an updated free accessible unique interactive online database⁵. Serum-free media and continuous cell lines already adapted to serum-free culture can be searched for by means of different criteria. Searchable criteria in the database are the degree of chemical definition, e.g. serum-free (SFM), animal-derived component-free (ADCF) or chemically defined (CD), and the kind of medium, e.g. basal media, media supplements, or full replacement media. In order to specify the cell lines that are adapted for serum-free media, search terms like organism, organ, tissue, cell type and disease can be used. All serum-free media and adapted cell lines commercially available at present are included in the database. Despite extensive search for serum-free media and adapted cell lines, there is still a lack of detailed informations by companies and suppliers, that are specifically highlighted. It is intended to open the database for interactive exchange of informations and experiences by experts in the field in order to continuously improve and extend the serum-free online database. The database will be accessible at <http://www.zet.or.at>

Keywords: FCS, Cell, mAb, SFM

Poster: revision of the directive 86/609/EEC & national reference laboratories

Towards a European science without animal experiments

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The revision of the Directive 86/609/EEC is a key opportunity for the EU to become a world-leader in animal replacement techniques and thereby reflect the wishes of its citizens to spare animals from suffering.

We see the replacement of animal experiments as one of three key themes to be addressed through the revision of the Directive, along with the improved protection of animals and increased transparency, accountability and enforcement.

In order to replace animals in science and develop non-animal techniques, the EU and Member States should have a responsibility to commission, fund and conduct research to replace all scientific uses of animals, in basic medical research as well as in regulatory testing. A clear focus of training provision in the use of Three Rs approaches is essential for all scientists. Three Rs information should be made available through open databases, and databases of negative and unpublished animal studies should be established to prevent duplication.

At the EU level, our vision is to have an over-arching EU Centre of Excellence for Three Rs Research, to co-ordinate and set a strategy for Member States bodies, charitable institutions and industry replacement activity. Along with state-of-the-art laboratory facilities and policy expertise, the centre would also play a key role in disseminating information between national centres and promoting replacement worldwide. Within the new centre, ECVAM should have an expanded role to conduct validation studies, provide training, and promote the acceptance and use of non-animal regulatory tests.

At the Member State level, national centres should exist to interact with the EU centre as well as working flexibly with charities and companies, with funding assistance from national research programmes. With the provision of leadership, co-ordination, training and multi-disciplinary strategies and expertise for animal replacement, the centres would develop a driving force for change at the national level and raise the profile of replacement initiatives in academic and industrial research circles. Ideally, these centres would have the capacity to conduct in-house research and commission key projects to overcome current barriers in animal replacement. A key to the progression of non-animal research is that national centres should be given funding for researchers to develop, pre-validate and validate non-animal replacements for research and regulatory tests.

We see the establishment of EU and national centres within a research and development framework as essential in achieving an EU-wide strategy to replace all animal experiments, to benefit people and animals.

Keywords: replacement, directive 86/609

Lecture: New developments in alternative testing methods

Predict-IV: Developing novel alternative testing strategies for use in pharmaceutical discovery and development

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Predict-IV is a new project supported by the EU research program for alternative testing strategies. The aim of Predict-IV is the improvement of assessing drug safety in the early stage of drug development and late discovery phase. The large-scale integrating project focuses on the development of a better prediction of the safety of an investigational compound. Predict-IV will combine classical *in vitro* toxicology with innovative technologies, profiling and modelling tools in a system biology approach. Partners with complementary expertise from academia, industry and SMEs participate in this project. This allows a highly integrative approach with an expertise in analytical chemistry, biochemistry, cellular model development, toxicogenomics, metabolomics, high-content imaging technologies, bioinformatics, kinetic modelling and toxicology.

Latest improvements in tissue technologies, molecular biology, toxicity modelling, and bioinformatics allow an advanced *in vitro* toxicity testing that increases notably the predictability of toxicity. The project will integrate these new developments to expand cell culture models for toxicity testing and to analyse the kinetics and dynamics of cellular responses to toxic effects *in vitro*. A focus of the applied models lies on the hepatic and nephritic cell culture systems; those two organs are most affected in toxicity. Due to the shortage of predictive assays for neurotoxicity the project also includes a development of new *in vitro* models for CNS toxicity testing.

Keywords: predictive toxicology; non-animal based test systems, cell culture

Lecture: skin models as alternatives to animal testing

The use of the SkinEthic Human Corneal Epithelial (HCE) model to predict ocular irritancy: optimized 1H/16H protocol applied to a large set of 400 industrial chemicals

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The 7th amendment of the EU Cosmetic Directive will lead to the ban of animal testing for cosmetic ingredients in March 2009. Thus alternative strategies and tests are urgently required in order to evaluate eye damage potential of chemicals. Tissue engineering progresses ended up to a better availability of 3D *in vitro* tissues thus participating to efforts that have been done and are still currently made in order to find reliable and relevant alternative methods.

The HCE model from SkinEthic laboratories is a standardized epithelium reconstructed with immortalized human corneal epithelial cells. Its specific structure allows chemicals to be tested in conditions similar to *in vivo* use. We have used the HCE model to evaluate *in vitro* eye irritation potential of chemicals. Because intrinsic cytotoxicity potential of chemicals is decisive factor affecting cornea after contact, we have used cell viability (MTT test) assessment as the main endpoint to classify chemicals.

Adapted protocols were based on specific contact times, adapted applied volume and a specific post-incubation period. The “long” 1 hour/16hours optimized HCE protocol established on a set of 102 chemicals (Cotovio et al., 2008), was applied to an larger set of industrial “real life” chemicals (>400). The previously defined Prediction Model (PM) based on a 50% viability cut off, allowed the drawing up of 2 chemicals classes (irritants and non irritants). Inter-batch and intra-batch reproducibility was assessed by using a specific batch control (Triton X100), positive control (ethanol) and negative control (Phosphate buffer). Predictive capacities based on these data were analyzed and discussed. The overall performances were good (concordance >80%) and well balanced sensitivity and specificity, thus demonstrating the robustness of the protocol when applied to significant very large set of chemicals representative of industrial needs.

Based on these results, the HCE protocol proved to be an efficient *in vitro* tool for the *in vitro* assessment of ocular irritancy potential. Additional work is currently done in order to evaluate the scope of application of the PM in a larger context of integrated test strategies for ocular irritancy

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Keywords: HCE, eye irritancy, validation, integrated test strategy, corneal epithelium

Poster: skin models as alternatives to animal testing

***In vitro* acute skin irritancy of 184 cosmetic ingredients using the validated EPISKIN model protocol**

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Following considerable interest in the development of *in vitro* model substitutes, reconstructed human skin or epidermis available as standardized kits, allow the measurement of parameters linked to the safety of topically applied products. Able to mimic *in vivo* situation, the reconstructed human epidermis EPISKIN was involved in the ECVAM acute skin irritancy validation and has been officially validated as full replacement method by the ESAC in May 2007 (ESAC statement, 2007).

The validated protocol is based on a short treatment time (15 minutes) followed by an extended 42 hours post treatment incubation period. Using viability measurement (MTT assay) (Mosmann et al., 1983) and IL1 alpha release, appropriate cut-off value allowed the drawing up of 2 chemical classes: Irritants (R38 EU risk phrases) and Non Irritant (EU Risk phrases: Non Classified) (Spielmann et al., 2007; Cotovio et al., 2005). Applied to a set of 184 industrial ingredients of diverse physical-chemical categories, sensitivity, specificity and accuracy of the defined predictive model were 85%, 86% and 86%, respectively. These results confirmed the validation performances and demonstrated the usefulness of the tiered strategy MTT + IL1 alpha as a decision making tool for skin irritancy hazard identification. The EPISKIN model is currently used in basic and screening studies of large number of chemical ingredients. The aim is to assess the real performances and usefulness of the *in vitro* methods in order to concretely determine the applicability domains and the remaining gaps acknowledging the fact that the industry is facing a wide chemical-physical diversity of ingredients.

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Keywords: skin irritation, 3D models, EpiSkin, ECVAM, cosmetic ingredients, MTT

Lecture: skin models as alternatives to animal testing

Advantages and difficulties in using 3-dimensional human skin models in toxicology and efficacy studies

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Three dimensional models of the human skin have been commercially available for use in *in vitro* studies for almost 20 years, but they have never reached their projected potential. Expectations were high that the first full thickness (epidermis plus dermis) skin tissues (produced by Organogenesis) could provide an immediate replacement to animal studies, but although the model proved useful for many investigations, it did not have universal application. Since then other manufacturers have brought skin models to the market, each with a slightly different format, each providing some advantage or disadvantage. Some models have survived, others have been discontinued or their companies have gone out of business.

Clearly human skin models are needed in our *in vitro* arsenal, but each manufacturer seems to struggle at one point or another with the same problems: shipping (especially across the oceans), customs regulations, lot-to-lot reproducibility, contamination with microorganisms, inability to cope with fluctuating demand, and many others. Tissue users can be frustrated by the cost of the product and their inability to cost effectively "test drive" and compare the various tissues. This should not continue since skin has the opportunity to act as a clear replacement for animal studies – it is a human organ that can be grown in isolation from the body yet has the potential to provide information on adsorption, irritation, corrosion, genotoxicity, phototoxicity, sensitization, pigmentation changes, etc. We must reach a point where we can use human skin models from various suppliers interchangeably. It is one of the missions of the Institute for In Vitro Sciences (IIVS) to work with many skin manufacturers so that users worldwide can be assured tissue of high quality and a known standard.

The ability to use multiple skin models is also one of the goals of recent Colipa-sponsored programs on genotoxicity and skin metabolism. In an effort to provide solutions to the animal testing ban of the 7th amendment to the Cosmetics Directive, the project is attempting to validate the use of several human skin models for the detection of both micronuclei induction (originally reported in the EpiDerm™ model [MatTek Corp., Ashland, MA, USA]) and DNA strand breaks (Comet assay) after topical application of test materials. Currently three tissue models (produced by MatTek, Skinethic and Phenion) will be compared under similar protocols for their ability to detect known skin genotoxins. Multiple laboratories are testing the different models first with direct acting carcinogens to show interlaboratory reproducibility, and then with chemicals requiring metabolic activation by the skin, e.g. cyclophosphamide and the polycyclic hydrocarbons. Considerable progress in standardizing the models has been made so far, and it is hoped that several skin models will meet the appropriate criteria so that comparable data can be easily generated in many laboratories around the world.

Keywords: skin models, irritation, corrosion, genotoxicity

Poster: ethical and legal aspects in animal experimentation

Challenging the legal basis of the rodent carcinogenicity test in UK

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The United Kingdom's Animals (Scientific Procedures) Act 1986 contains a legal provision obliging the government to undertake a cost-benefit assessment before licensing any scientific procedure on an animal. The rodent carcinogenicity bioassay has long been identified as a highly problematic animal test, producing data of doubtful reliability and relevance in assessing the cancer risk posed by chemicals. People for the Ethical Treatment of Animals (PETA) examined the utility and welfare implications of the bioassay to assess whether it meets the requirements of the cost-benefit test. Utility was assessed by analysing data from more than 500 studies available publicly on the US National Toxicology Programme's database. The available data indicated poor concordance of rat and mouse data; high number of inadequate studies; and lack of relationship between test results and final classifications of carcinogenic risk awarded to chemicals by regulatory bodies such as the US Environmental Protection Agency and the International Agency for Research on Cancer. Analysis of welfare implications demonstrated aspects of the test that could be classified as causing moderate or severe suffering. We concluded that the scientific failings of the test may justify a conclusion that the cost to the animals involved exceeds any benefit, and identified the assay as a possible candidate to challenge the UK government's application of the law, by means of judicial review. However, meeting the requirements for a successful legal challenge was difficult due to a lack of available information regarding relevant aspects of the use of this assay in the UK, partially due to a specific exclusion for animal experimentation under the UK's Freedom of Information legislation. The value of animal protection legislation is compromised where there is not a concomitant legal right to the information that would allow proper evaluation of its implementation and enforcement.

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Keywords: carcinogenicity, UK legislation, rodent bioassay, transparency

Poster: free communications

Towards a standardized trans-epithelial electrical resistance measurement on *in vitro* cutaneous and ocular reconstructed models

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Context: The barrier function of epithelial tissues is well described with passive permeability assays using hydrophilic or hydrophobic markers. Two major routes are mainly involved in small molecules flux: while trans-cellular route of chemicals depends on their lipophilicity, paracellular route takes place into the intercellular spaces and is under tight junctions (TJs) control (Schäfer et al., 1996; Bazzoni et al., 2002). Together with the proteo-lipidic complex of the cornified layers, TJs are important effectors of the barrier function of tissues. Those junctions are found mainly in the upper layer of the epidermis, especially in the *stratum granulosum* (Furuse et al., 2002). When TJs are not mature or damaged hydrophilic compounds are able to diffuse from the upper layers to the basal one (for both skin epidermis and eye corneal epithelium). Consequently, modifications of barrier function linked structures can influence trans-epithelial electrical resistance (TEER) state of the tissue.

Purpose: Modifications and quality of the barrier function could be detected by using TEER measurements. This parameter could give some information about both the steady state of *in vitro* epidermis/epithelial models and the tissue integrity modifications after chemical treatments (*in vitro* toxicity testing system). We studied three commercially available *in vitro* epithelial engineered models: two reconstructed human epidermis models (EpiSkin^{LM} large model, RHE small) and a human corneal epithelial model (RHCE) supplied by SkinEthic Laboratories. A comparison between models on TEER measurements is made before and after surfactant treatment.

Material and Methods: TEER was measured by using a specific epithelial tissue Volt-Ohmmeter in different conditions. Electrical resistance was measured during a time period in order to define acceptable and standardized steps where TEER could be regarded as stable prior to measurements.

Results: Comparisons between two electrolyte media were done in order to optimize measurements. Subsequently, TEER assays were carried out after topical surfactants treatment: SLS treated EpiSkin^{LM} or RHE small and Triton X-100 treated RHCE. Results showed a sharp TEER decrease since the lowest concentrations tested reaching low resistance values at the highest surfactant concentrations. Complementary cellular viability assays showed clear effects only at the highest surfactant doses.

Conclusion: TEER standardized method could be a useful endpoint for quality control assessments and comparisons between 3-D models. In addition it could be used as a suitable easy to use tool, to describe barrier function, complementary to TEWL and other permeation studies.

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Keywords: electrical resistance, cutaneous, ocular, barrier function, surfactant, standardized method

Lecture: free communications

Growth of squamous carcinoma cells under the influence of chemotherapeutic agents

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Introduction, Background and Aims: Various chemotherapeutic agents were tested for their efficiency in an *in vitro* test system against known cell lines (A431 and RPMI 2650). Here we are developing a test system for known and new chemotherapeutic agents for the treatment of malignant cancers. The thus collected data are to be used as the basis for proposed future clinical testing.

A combination of vitality and growth studies will enable the determination of the compound's effectiveness on the cell line. It has been shown that resistance to chemotherapeutic agents may lead to an increase in motility and invasiveness as opposed to the desired reduction. This may lead to the induction of metastases and not to the desired effect of reducing the cancer. Various chemotherapeutic agents were tested (Cisplatin, Erbitux, Taxotere and NONI PPT) for their efficiency against the cell lines.

Material and Methods: Cell cultures of epithelial carcinomas were confronted with a series of chemicals at various concentrations to determine the change in vitality and motility using time-lapse microphotography. Treatment was with physiological concentrations of the chemotherapeutic agents to be investigated. It has already been shown that a concentration dependent effect on the motility of mouse and human melanoma cell lines can be observed (paper in preparation) with Noni PPT. The lethality is also concentration dependent and it also appears to be growth dependent.

Results: It has been shown that this method is sensitive and demonstrates the ability to distinguish between different mechanisms of action of the chemotherapeutic agents used and indicate the mechanism by which NONI PPT kills cancer cells. The effectiveness of the treatment of cancer cells with chemotherapeutic agents is also seen to be dependent on the biochemical activity of the cells present i.e. growth, proliferation and motility. Combination tests show that the treatment effectiveness increases when two chemotherapeutic agents are used that work using different mechanisms.

Summary/Conclusion: Three different mechanisms of cell death can be visually identified. The unknown mechanism by which NONI PPT kills cells can be visually allotted to a mechanism related to that of Taxotere, a known cytoskeletal inhibitor of cell division. This is a type of cell death that appears to end in autophagocytosis. Investigations are underway to determine whether autophagocytosis and the type of cell death induced by taxotere and NONI PPT are related using the Vitamin K3 induction method.

It has been shown that this method can in a relatively simple way determine the mechanism by which cell death is induced and if the cells are responsive to the agents used.

Keywords: *cell motility, growth, drugs, therapeutics*

Lecture: 7th cosmetics amendment – can all goals be achieved in time?

High-content cellular imaging approach for *in vitro* toxicology

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High-content cellular imaging (HCI) is increasingly applied to the *in vitro* assessment of toxic compounds. HCI is an automated fluorescent cell-based technology, which allows rapid investigation of cellular effects of chemicals or siRNAs *via* imaging at cellular, sub-cellular and molecular levels of individual cells. The method delivers integrated information on morphology, cell movement, co-localization of different parameters, alteration of patterns and changes in specific pathways. In this presentation, HCI will be introduced by results generated from the Cellomics ArrayScan™ system. Applications for assessing general cellular toxicity as well as specific pathological changes, such as cholestasis in the liver, will be discussed.

Keywords: high-content cellular imaging, in vitro toxicology, cholestasis

Welcome address to the 15th Congress on "Alternatives to Animal Experimentation" – Linz 2008



Dear colleagues,

On behalf of the organisers zet, the Austrian Centre for Alternative and Complementary Methods to Animal Testing, the Austrian National Platform on Alternatives and of MEGAT, the Middle-European Society for Alternatives to Animal Experiments, I cordially welcome you to this year's congress "Linz-2008".

We are quite happy that again a large number of high quality abstracts have been submitted both for oral presentations or posters. For the third time the congress in Linz will be held in English and we are proud that you, the scientific community, have accepted the change from German to English as the official congress language. Our congresses in Linz are now a European forum for the exchange of new ideas on progress in the development and validation of alternatives.

We are particularly proud that we have attracted key area leaders as speakers, who update the participants on the European Commission's and member states' efforts to implement the 3R principles into basic biomedical and applied research. This year updates will focus on ethical and scientific aspects of stem cell research, on the 7th amendment of the EU Cosmetics Directive and in particular on the application of skin models, on computer assisted procedures, on alternatives in reproductive toxicology and on genetic technology and transgenic animals. In Linz we have always held sessions on good cell culture practice and this year we will for the first time also cover the challenge of using *in vitro* methods in the safety testing of nanomaterials.

As in the past, we are strictly avoiding parallel sessions at the Linz-2008 congress and we have reserved sufficient time for intensive discussions during the poster sessions. In Linz we have always tried to motivate young scientists by giving awards to young authors, who are presenting the most innovative and promising posters. Thus we are looking forward to another exiting award ceremony with new faces at the conference banquet.

Linz, the capital of Upper Austria, is a lively modern city, situated on the river Danube, with a Baroque city core. It is famous for its many modern museums and the *ars electronica* centre. The surrounding Austrian countryside offers unique historic sights as well as cultural and gastronomic highlights.

We hope to provide you with an attractive, stimulating and interesting programme in Linz 2008. I am looking forward to seeing you there.

On behalf of the organisers I welcome you

Sincerely

Horst Spielmann, President of MEGAT

The Congress organisation, MEGAT and ALTEX Edition wish to thank the Foundation Animalfree Research (Zurich, Switzerland) for sponsoring this supplement.

Poster: nanotoxicology / nanobiotechnology

Primary porcine alveolar epithelial cells (pAEpC) as a model for drug permeability assays

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Subject: The respiratory tract is considered as an alternative to gastrointestinal or dermal drug delivery systems, due to the fast absorption and the absence of first pass metabolism. Permeability and solubility of candidate compounds are useful data that generally speed up the drug development process. For permeability studies, lung epithelial barrier models are needed. As an alternative for animal testing, epithelial cell cultures would provide a good means for rapidly evaluating the drug delivery process to the lung. While *in vitro* systems that reliably mimic the upper airways epithelia are already available (e.g. Calu-3 cells), there is no such a system (a cell line) that properly reproduces the conditions found in the deeper lung, and researchers still have to rely on primary cell cultures (Steimer et al., 2005).

Material and Methods: Pig lungs were minced and bronchioles were removed. Tissue pieces were pre-incubated with a trypsin-elastase combination and then digested. Macrophages and dissociation from blood cells and cell debris were removed and cells were plated at a density of 8×10^5 cells/cm² on permeable fibronectin/collagen coated Transwell Clear Filter inserts.

TEER was measured with an EVOM and estimated relative to the corresponding surface area. Transport studies were performed in triplicate in ab and ba direction, samples were taken at distinct time points and the Papp was calculated. Sodium fluorescein, 3H-propanolol and rhodamine 123 were used as markers for low, high permeability and efflux transporters, respectively. Triamcinolone acetonide was used as an example of a pulmonary delivered drug compound.

For the inspection with a transmission electron microscope EM10C, cells grown in Transwell filters were fixed in 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M cacodylate buffer. Then cells were postfixed with 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer and dehydrated in acetone. Filters were embedded in Epon resin and polymerized 60-80 nm thick sections were taken perpendicular to the filter surface, stained with uranyl acetate and lead citrate.

Results and Discussion: Morphology: On day 2 after seeding, cells of a different shape with numerous filopodia were adhered onto the filter substrate. By day 8 the monolayer appeared flat and a 100% confluency was observed; multilamellar bodies could be detected, as well as tight junctions and desmosomes. On day 13, two morpho-types could be observed: round cells with multilamellar bodies in the apical cytoplasm and flat, spread cells with no particular ultrastructure. This hinted at a mixed type I/type II pneumocyte phenotype, which was also confirmed by immunofluorescent detection of marker proteins. The paracellular integrity of the monolayers was assessed by transport studies with Flu-Na in the absorptive direction (ab). An inverse correlation between permeability coefficient and TEER was observed. pAEpC had the same behaviour, in terms of permeability of triamcinolone acetonide, as hAEpC or two human bronchial epithelial cell lines (Calu-3 and 16HBE14o-), which are already established models.

Conclusion: pAEpC have proven to be a good model for drug permeability assays. They consist of a mixture of type I and type II pneumocyte-like cells, and exhibit good barrier properties (Steimer et al., 2006, 2007).

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Keywords: Calu-3, pAEpC, lung *in vitro* models

Lecture: nanotoxicology / nanobiotechnology

Lab-on-a-chip: a novel approach to monitor nanotoxicology

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As nanotechnology moves towards widespread commercialization, new technologies are needed to adequately address the potential health impact of nanoparticles. It is uncertain whether the same properties that make engineered nanoparticles attractive in nanomedicine could also prove harmful when interacting with healthy cells. Although the benefits are clearly established and exploited, limited attempts in the evaluation of potential undesirable long-term effects have been made.

Over the past decade, the miniaturization of analytical techniques by means of N/MEMS technology has become a dominant trend in research. As demonstrated in genomics research, microanalytical systems have the ability to provide quantitative data in real-time and with high sensitivity. However, microfluidic biochips are also vital for cell analysis where large numbers of single cells or small numbers of cell populations can be tested inexpensively, at high throughput and in a cellular environment of increased physiological relevance.

We have developed a lab-on-a-chip that is capable of non-invasively monitoring *ex vivo* living cells in the absence of background effects. The cell chip is designed to continuously assess cell viability and morphology changes using embedded contact less dielectric microsensors. The integrated nanofluidics allows for controlled administration of nanoparticles to living mammalian cells adhered to modified/activated chip surfaces that are comparable to biological niches. Consequently, the presented work addresses aspects of chip design, sensor characterization and application to nanotoxicology using a variety of nanoparticles.

Keywords: microfluidic biochip, cell chip

Poster: free communications

Cell-growth promoting fractions originated from bovine blood clot in combination with the porcine ocular fluid

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Sera are generally obtained from drawn and collected whole blood from adult, calf or fetal animals. Following the natural clotting process, which may take several hours at 4°C, the blood consists of serum and blood clot containing 95% of red blood cells, 5% platelets, less than 1% and numerous amounts of fibrin strands. In comparison to a PRP (platelet rich plasma), blood clot containing 4% of red blood cells, 95% platelets and 1% of white cells. The specific cell-growth promoting components are the platelet derived growth factor (PDGF) and the transforming growth factor Beta (TGF Beta). Both of them are contained in the Beta granules of the platelets. Fibronectin and vitronectin are also the components of the PRP. They are the cell adhesion molecules found in plasma and fibrin itself. The experiments presented herein were aimed to isolate, characterise and to test in vitro on different cell cultures the growth promoting material from the bovine blood clot. The bovine blood was collected and allowed to form the clot. Afterward the whole content was centrifuged at 2500 RPM for 20 minutes and the supernatant (serum) was aspirated off. The sediment ("clot") was quickly washed with the sterile buffered saline pH=6.9 for 20 minutes, and centrifuged for 25 minutes at 2500 RPM for 25 minutes. The supernatant (Fraction I) was collected and frozen. To the remaining "clot" the PBS pH=7.2 was added and left for 1 hour at +4°C. After the centrifugation of the suspension at 2500 RPM for 25 minutes, the supernatant (Fraction II) was collected and frozen. To the sediment ("clot") the PBS pH=7.4 was then added for 18 hours (Fraction III). All the fractions were sterilised by 0.2 membrane filtration. The content was analysed by PAG-SDS electrophoresis. The cell growth promotion/inhibition activity in the comparison to the SR-2.055P (Serum replacement based on porcine ocular fluid) and FCS (Fetal calf serum) was tested on the Chicken embryonal fibroblasts, WISH, HAC-3/T2 (Human amniotic cell lines), PLA-2 (Adult pig kidney cell line), Bovine intestinal epithelial cell line, WiREF (Wistar rat embrional fibroblastoid cell line) and CaCo-2 (Colon cancer carcinoma cell line). The results of the experiments shows: (1) The attachment and growth of primary cultures (Chicken embryonal fibroblasts) is not affected after the use of bovine blood clot fractions. (2) Fractions (I-IV) shows the growth promotion, but different according to the group of cells used in the test. (3) The strongest growth enhancement was found when transformed cells (WiREF, CaCo-2) were tested. (4) The optimal content was 8-10% in Eagle's medium. In this range up to 95% value of the SR-2.55P could be obtained. (5) When different fractions (I, II, III) were combined with the porcine ocular fluid the slight enhancement can be found only when the ratio 1:2 was used.

Keywords: cell growth promoting fractions, bovine blood clot, porcine ocular fluid

Poster: free communications

The antiproliferative activity of the Holocene grain wash-out can be enhanced by HuIFN-Alpha N3 but not with the rHuIFN-Alpha 2

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Holocene era denotes the time period of 9560 and 9300 B.C. It started by withdrawal of pleistocene glacier. During this time huge amount of holocene sands occurred. For them it was found, that they contain large amount of fairly uniform holocene minerals. When grained, they show quite unusual biological/microbiological activity, like antifungal against *Peronospora* sp., *Phytophthora* sp..

The performed experiments were aimed to find if the holocene grain-wash out shows the antiproliferative activity against CaCo-2 cells, and if this activity can be enhanced by Interferon (IFN). During the experiments the samples from river Drava sands, near Koprivnica were used. To grain them the "Star-mix" technology was used. As the results the fine grain with 60-80 µm size was obtained. The following wash-out ("suspension") were prepared: (1) 10% Monoethylene-glycole; (2) 10% PBS (Phosphate buffer saline), pH = 7.2. Two types of human interferons were used: Hu IFN-Alpha N3 and rHuIFN-Alpha 2. During the experiments different combinations of holocene grain-wash out and IFNs were tested: (1) 10% Monoethylene-glycole; (2) 10% PBS; (3) Hu IFN-Alpha N3; (4) rHu IFN-Alpha 2, (5) combinations with IFN in ratio 2:1, 1:2 and 1:1.

The following results were obtained: (1) AP activity of samples (Monoethylene – glycole : 4,6 (well with ca. 50% growth inhibition), (PBS : 1,8); (2) AP activity of IFNs: HuIFN-Alpha N3 (500 AV units/ml): 4,6; (3) AP activity of rHuIFN-Alpha 2 (500 AV units/ml): 3,8; (4) Sample + HuIFN-Alpha N3: (1:1) = 10,3; (1:2) = 8,5 ; (2:1) = 12 ; (5) Sample + rHuIFN-Alpha 2: (1:1) = 5,4; (1:2) = 3,5 and (2:1) = 5,1.

From the presented experiments it can be concluded: (1) Basically the holocene grain-wash out shows the antiproliferative activity against CaCo-2 cells in vitro. Monoethylene-glycole much higher than PBS. (2) This AP activity can be enhanced (in vitro) up to four times using the HuIFN-Alpha N3. (3) Such an enhancement cannot be obtained in the same system using the rHuIFN-Alpha 2. (4) It seems, that the single component (subtype) of rIFN cannot affect the AP activity of holocene grain-wash out. (5) For the optimal enhancement (in vitro) different IFNs subtypes are needed.

Keywords: holocene grain, wash-out, CaCo-2 cells, antiproliferative activity, interferons

Poster: nanotoxicology / nanobiotechnology

***In vitro* study of the toxicity induced by Nickel and Cobalt particles on human lung cells**

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Epidemiological studies show that human population in the industrialised world is more exposed to particulate air pollution which contributes to respiratory morbidity and mortality. As more particles are released to the environment, research in the area of particles (micro and nano sized) toxicity is highly important. The respiratory system is a primary interface between the organism and its environment and serves as a portal of entry and therefore as a potential site to particles toxic effect. In this study we used Calu-3, a human bronchial cell line, to investigate the effect of Nickel (Ni) and Cobalt (Co) particles (μm and nm size) *in vitro*. Calu-3 cells are known to form a polarized monolayer and retain the characteristics of the native epithelium with the formation of tight junctions, and production of mucous under air-interfaced culture (AIC). Calu-3 cells were grown on transwells for 14 days under AIC condition. Exposure was performed by addition of the particles in solutions to the apical compartment for 72 hours. In parallel the toxicity of the soluble forms (NiCl_2 , CoCl_2) was also investigated. The trans-epithelial electrical resistance (TEER) was recorded after 24, 48 and 72 hours of exposure. Two viability assays, neutral red uptake (NRU) and lactate dehydrogenase (LDH) were performed at the end of the exposure (72h). Results showed a concentration-response effect on cell viability and TEER after treatment with the soluble forms. However, with the particles (μm and nm size) no significant effect was found on the cell viability but a concentration dependent increase of TEER was observed at non cytotoxic concentrations. This effect on barrier integrity was higher with particles of μm size than nm size. The effect of Ni and Co particles on oxidative stress and gene expression is still under investigation.

[The study is supported by the Marie Curie Research Training Network: PULMO-NET]

Keywords: particles toxicity, viability assays, in vitro, Calu-3, human bronchial cell line, trans epithelial electrical resistance, air-interfaced culture

Lecture :

The emerging concept of evidence-based toxicology (EBT)– results of the 1st international forum towards EBT

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The paper will discuss ongoing projects of evidence-based approaches in toxicology coined “evidence-Based toxicology” (EBT) in analogy to “Evidence-based Medicine” (EBM). EBM has, during the last 20 years, gained paradigmatic status as a working framework for unbiased decision-making pertaining to the selection of the most appropriate preventive or interventional action for individuals or specific risk groups.

“Evidence-based” here means the conscientious evaluation of complete sets of information by applying standardised procedures of assessing/judging information quality/reliability using pre-defined criteria. In practical terms, EBM’s main outputs are short “critical appraisals” or comprehensive “systematic reviews” pertaining to a specific medical decision-making point. Evidence-based approaches thus may be seen in contrast to traditional expert-based or consensus-driven decision-making which normally lacks procedural transparency and which hence may or may not be based on a thorough, rigorous, standardised and hence unbiased analysis of the available scientific information.

Toxicology and medicine share many similarities that warrant equivalent approaches in toxicological practice. Both toxicology and medicine are “crafts”, they translate knowledge into practical acts *via* decision-making procedures that are standardised to differing extents. Basic similarities include: 1) their decision-making is relevant for human health, 2) is based on the principles of causation (adverse effects/hazards, curative effects, pathogenesis/toxicogenesis) and probability (risk or effectiveness) and 3) is, in practice, based on the integration of various data of variable quality, reliability and relevance. These similarities warrant considering whether toxicological practice may be reshaped into an evidence-based toxicology.

ECVAM’s current work towards EBt (<http://www.ebtox.org>) is based on the First International Forum towards an evidence-based toxicology, held in October 2007 in Como, Italy. Organized by the European Commission supported by an International Scientific Advisory Group, the conference gathered about 170 participants from Europe, the US, Africa and Asia. Major outcomes are 1) the declaration of Como supporting the ongoing development of EBt, 2) a provisional definition of EBt and 3) consensus documents on possible evidence-based tools for the appraisal of toxicological tools (e.g. test methods) and data. The proceedings will be published in Human and experimental toxicology by summer 2008.

In addition, ECVAM has developed an internet-based portal to EBt to support the practical implementation of EBt. The portal will facilitate the virtual interaction of working groups developing evaluation criteria (standards) and drafting systematic reviews or appraisals pertaining to specific toxicological hypotheses and will provide public information on EBt products and events.

Moreover, ECVAM has developed a quality scoring tool for the standardised assessment of toxicological information, a first step towards an approach for assessing the evidential substance of toxicological data. The tool is currently undergoing an assessment and optimisation phase.

Keywords: evidence-based toxicology, evidence-based medicine, systematic review, critical appraisal, validation, toxicology, standardization, evidence

Lecture: computer assisted procedures

Go3R – the knowledge-based search engine for information on animal testing alternatives

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The core of any strategy to reduce animal experiments lies in the availability of relevant information regarding alternative methods. EU Directive 86/609/EEC for the protection of laboratory animals obliges scientists to consider whether a planned animal experiment can be substituted for by another scientifically satisfactory method which is reasonably and practicably available. To meet this regulatory obligation, scientists must consult the relevant scientific literature prior to any experimental study using laboratory animals.

The internet enables access to a huge quantity of information. Nevertheless, it is difficult and time-consuming to select adequate information from this vast amount. Moreover, at the end of a query it remains unclear if all the required relevant information actually has been retrieved. This is where a new generation of knowledge-based search technology take effect.

In April 2008, the beta version of Go3R (www.Go3R.org), the first knowledge-based search engine for alternative methods in agreement with the 3Rs principle, was released. Go3R is free of charge and enables scientists and regulatory authorities involved in the planning, authorisation and performance of animal experiments to determine the availability of alternative methods in a fast, comprehensive and transparent manner.

The technical basis of this search engine is a specific expert knowledge, captured within an ontology. An ontology is a network of – also hierarchically – grouped “concepts” like subject areas, indicative for the respective field of research. It specifies the unambiguous meaning of relevant terms and depicts the complex relationships existing between them. With the help of such an ontology, the content of any document can be semantically determined by the mapping of the unique pattern of concepts and terms utilised in it.

An essential step in the development of Go3R has involved the creation of an appropriate ontology by defining those concepts and terms that are relevant for alternative methods in accordance with the 3Rs principle and inferring the unique relations between them. The engine can now assist searchers by pre-sorting the retrieved documents according to their respective pattern of concepts and by attributing them to delimited topics. The result is an “intelligent table of contents” representing the hit list of relevant concepts and terms used in the documents, which the searcher can then use to navigate through the “thicket of information” of his query result.

In the presentation, concrete step-by-step examples show how the use of Go3R speeds up and improves the procedure of information retrieval – making it more comprehensive and transparent. Go3R improves animal protection in accordance with the 3Rs principle by reliably revealing alternatives to animal experiments documented in the literature.

Keywords: animal testing alternatives, knowledge-based search engine, Go3R, internet, information retrieval, semantic, ontology

Roundtable: Embryonic or adult stem cells: scientific and ethical considerations

Biological differences between embryonic and adult stem cells

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Characteristics of embryonic and adult stem cells. Stem cells are unique cell populations that retained the capability of either self-renewal in nearly infinite cell divisions or to differentiate into one or several cell types. Embryonic stem cells are pluripotent, i.e. they retained the potential to differentiate into one of the approx. 220 somatic cell types of the mesoderm, endoderm or ectoderm. In contrast, adult stem cells are multipotent, harbouring the ability to differentiate into specific cell types of the tissue of origin in which they reside. Thus, adult stem cells are the *in vivo* source for cell renewal during tissue turnover or in tissue repair. However, broad plasticity of adult stem cells have been described, allowing the cells to differentiate across tissue lineage boundaries to give rise to cell types of other lineages.

Source and origin of embryonic and adult stem cells. Embryonic stem cells are isolated from the inner cell mass of mouse or human blastocysts. The generation of human embryonic stem cell lines from preimplantation blastocysts and their use in basic research as well as in future therapeutic application has raised considerable ethical concerns. The source of a human blastocyst are exclusively fertilized eggs that have been grown *in vitro* for 5 to 6 days. Fertilized eggs may be provided from surplus zygotes after *in vitro* fertilization (IVF). However, the isolation and preparation of cells from the inner cell mass results in the destruction of the blastocysts and is thus considered an embryo-consuming technology, which by this reason is prohibited in many European countries. Alternative technologies, like somatic nuclear transfer, have been developed in order to avoid to waste fertilized human embryos, however, ethical concerns still exist, since enucleated human oocytes are needed. The latest development are induced pluripotent stem cells (iPS), obtained by direct reprogramming of somatic cells after retroviral transfection. Adult stem cells are easy to obtain, although they reside in low abundance in adult tissues and organs, so-called stem cell niches. Any ethical concerns about harvest and isolation of adult stem cells are neglectable. The sources for adult stem cells are manifold and include easily available human tissue, like blood, bone marrow or adipose tissue, or human material, which is normally unused or even discarded, like umbilical cord, placenta, or deciduous milk teeth. Also amniotic fluid has been recently described as potential source of stem cells.

Pitfalls and mistakes in culturing embryonic stem cells. When human embryonic stem cells (hESC) are expanded *in vitro* in tissue culture, it is critical to maintain their self-renewal and differentiation capacity. In present state-of-the-art culture protocols, hESC are cultured on mitotically inactivated mouse embryonic fibroblast feeder layers, which serve three important functions: (1) they support hESC growth, (2) prevent spontaneous differentiation of hESC during culture, and (3) maintain hESC pluripotency. Mouse embryonic stem cells can be maintained *in vitro* in undifferentiated state, when the culture medium is supplemented with leukemia inhibitory factor (LIF), or when LIF is secreted in sufficient amounts by mouse fibroblast feeder layers. LIF, however, does not act on hESC, which must be cultured in high amounts of fetal bovine serum (FBS) supplementation. The use of animal-derived feeder layers and culture medium supplements, like FBS, turned out to pose the risk of cross-species contamination of hESC with animal pathogens, thereby greatly limiting the future clinical application of the cells. Recent studies have indicated the expression of immunogenic nonhuman sialic acid residues by hESC, due to their culture in the presence of FBS, and cells would be rejected when transplanted for therapeutic purposes. Thus, great efforts have been undertaken to develop xeno-free and feeder-free culture conditions for hESC, however, with limited success.

Keywords: pluripotency, blastocyst inner cell mass, IVF, iPS, stem cell niches, stem cell culture

Lecture: good cell culture practice

Alternatives to the use of fetal bovine serum (FBS): A survey of recent strategies to reduce or replace FBS in cell and tissue culture

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Fetal bovine serum (FBS) is commonly used as an essential supplement to cell culture media. FBS is a cocktail of most of the factors required for cell attachment, growth, and proliferation *in vitro* (Gstraunthaler, 2003). However, the use of animal serum also bears a number of disadvantages. These can either be seen from (a) a theoretical, cell biological point of view, since serum in general is ill-defined, (b) from ethical perspectives in terms of animal protection arguments about harvest and collection of FBS from bovine fetuses (van der Valk et al., 2004), and (c) in terms of recent concerns about the global supply vs. demand of FBS. It is estimated that about 500,000 litres FBS are produced per year for the world market. This means, that more than 1,000,000 bovine fetuses have to be harvested, and it is expected, that these numbers will continue to increase annually. As a consequence, a number of strategies have been developed to reduce or replace the requirement for FBS in cell culture media. As a major goal of these initiatives, any efforts shall be undertaken in order to decrease the global demands for FBS and thus to decrease the number of bovine fetuses needed (ESAC, 2008). At the 11th Congress on Alternatives to Animal Testing in 2003, a session on alternatives to the use of FBS in cell and tissue culture was held. Several strategies were presented, that in the past five years have all been published elsewhere (Falkner et al., 2006; Gonzalez Hernandez and Fischer, 2007; Gstraunthaler, 2003; Pazos et al., 2004; van der Valk et al., 2004). The collection of fetal sera may impose harm to the bovine fetuses. To explore this topic in detail by experts in the field, a workshop was held in Utrecht, NL, in 2003. A comprehensive report of this workshop was given (van der Valk et al., 2004). Further presentations dealt with the use of plant extracts as FBS alternatives (Pazos et al., 2004), testing synthetic surfactants in serum-free bioreactors (Gonzalez Hernandez and Fischer, 2007), and a free access online database, searchable for commercially available serum-free culture media (Falkner et al., 2006). Most of this work was recently included in a statement by ESAC, the ECVAM Scientific Advisory Committee, on the use of FBS and other animal-derived supplements, that has been endorsed at the 28th ESAC Meeting in May 2008 (ESAC, 2008).

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Keywords: fetal bovine serum, FBS, alternatives, cell and tissue culture, 3R

Poster: free communications

Characterisation of a new human renal proximal tubular cell line (RPTEC/TERT1), generated by the introduction of telomerase activity

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Telomeres are tandemly repeated hexamers at the end of mammalian chromosomes which shorten at each cell division and act as a replicative clock. Primary cells in culture eventually enter replicative senescence due critically short telomeres. It has been shown for some cell types that the introduction of human telomerase reverse transcriptase (hTERT), an enzyme which extends the telomere, can confer cells with unlimited life span. In addition, hTERT extends the life span of cultured cells far beyond normal senescence without causing neoplastic transformation. Here we carried out extensive characterisation of a human renal proximal tubular cell line (RPTEC/TERT1), immortalised by hTERT transfection.

The cells exhibit an almost normal karyotype, with 47 chromosomes compared to 58 for the HPV transformed cell line HK-2 cells. RPTEC/TERT1 show morphological properties of proximal tubular cells including cobble stone morphology, contact inhibition and the expression of numerous microvilli. They exhibit polarised transport of water and solutes and exhibit transepithelial electrical resistance similar to that of primary proximal tubular cells. The cells possess pH dependent ammonia genesis. They increase cAMP upon parathyroid hormone, but not arginine vasopressin stimulation. And they express alkaline phosphatase and gamma glutamyl transpeptidase. Interestingly these cells also appear to further differentiate over 1-2 weeks at confluence, dramatically reducing their glycolytic metabolism. In conclusion these cells offer a real alternative to primary cells for in vitro studies of the proximal tubule and may prove to be an invaluable tool for in vitro nephrotoxicity studies.

Keywords: telomerase, hTERT, RPTEC, cell line, nephrotoxicity

Lecture: Skin models as alternatives to animal testing

An initial evaluation of the CellSystems EST-1000 reconstructed human skin model for distinguishing R34 and R35 corrosives *in vitro*

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The human epidermis model Epidermal Skin Test 1000 (EST-1000; CellSystems® Biotechnologie Vertrieb GmbH) can be used in the assessment of skin corrosivity potential as indicated in the OECD Guideline for the testing of chemicals No 431. Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material (as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS)). The potential for chemical induced skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. Validation studies have shown that tests employing human skin models are able to reliably distinguish between known skin corrosives and non-corrosives.

The purpose of this validation programme was to assess whether the EST-1000 model is capable of distinguishing between known R35 (UN packing group I) and R34 (UN packing group II & III) chemicals. 6 coded test substances of varying classes of corrosivity (2 R34, 2 R35, and 2 NC) were evaluated using treatment periods of 3, 60 and 240 minutes. The results of the study concluded that the EST-1000 was able to correctly classify all the tested chemicals and was able to distinguish between R35 and R34 substances. With the data we demonstrated the EST-1000 as a promising candidate to fulfil the demand of hazard identification. In respect to *in vivo* tests this adaptability is one major advantage of *in vitro* skin models.

Keywords: reconstructed epidermis, EST-1000, in vitro skin corrosion, transport labeling, R34, R35

Poster: new developments in alternative testing methods

Development of a three dimensional trachea model as an alternative for testing toxicity

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Introduction: Three dimensional cell systems have been already established for the cornea and the skin. These models are – due to their organ-specific properties – suitable for the analysis of different substances with regard to their biocompatibility and toxicology which up to now could be examined only by animal experiments. The airway represents also an important entry gate for many different pathogenic agents and environmental impacts. A three dimensional model of the trachea becomes also more and more important as an alternative for animal experiments because it shows similarities in structure and function of the human trachea.

Aim: Our aim is to develop a functional trachea test system in a bioreactor system in which the cells are cultivated under physiological conditions and simulation of the respiration. This model could be used as a test system for the analysis of different substances (e.g. nanoparticles or other aerosols) as an alternative for preclinical animal testing and it could be also used as a biological graft.

Materials and Methods: For the assembly of a 3D-model of the trachea, we use human respiratory epithelium cells. The isolation occurs with the sprouting method from small tissue pieces of a biopsy. According to confluence the cells were trypsinated and used for the construction of a three dimensional model. For further colonization the cells were seeded in Collagen-I coated inserts and cultivated in an air-liquid interface on different membranes to mimic the native conditions. The cultivation occurs with special Airway Epithelial Cell Growth Medium. The cells were characterized via histological and immuno-histological methods.

Results: The picture below shows a piece of the biopsy with grown-out respiratory cells. In preliminary tests human respiratory cells were cultivated on inserts with a PET- and a biological membrane. Most of these cells are vital and show a high rate of proliferation. There are ongoing experiments with a co-culture of fibroblast cells and respiratory epithelium cells. The cells were also cultivated in a special trachea-reactor with simulation of the respiration to stimulate the ciliary activity to adjust the mucociliary clearance which is one of the major tasks of the trachea.

Perspectives: The cultivation of functional epithelium cells with active cilia and mucus secretion is very important for the development of a functional test system. Therefore a better understanding of cells and matrix and also the stimuli for the differentiation of these cells is immanently necessary. The next step is the cultivation of the respiratory cells in co-culture with fibroblasts in a bioreactor system under mechanical stimulation and simulation of the respiration.

Acknowledgments

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Keywords: *trachea, three dimensional test system, primary cells*

Lecture: computer assisted procedures

One step further towards computer assisted simulation of percutaneous absorption to avoid animal experiments – the combination of experiment and simulation effectively helps to identify new important parameters

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In the prospect of the EU-chemicals policy REACH and the 7th cosmetics amendment from March 2009 mathematical modeling to predict skin permeability of chemicals, drugs and cosmetics becomes more and more a focus. In contrast experiments with human skin are expensive and time consuming. They are also limited by a lack of sufficient skin resources. Therefore a number of equations have been established to predict skin permeability based on physicochemical molecular characteristics. Usually molecular weight and the octanol-water partition coefficient are used to estimate quantitative structure activity relationships (QSAR) assuming that skin permeability is conclusively determined by a compound's affinity to lipids and its size (Potts and Guy, 1992; Magnusson et al., 2004). Meanwhile, QSAR-software is readily available on the internet for free use.

However, these predictive models have several major drawbacks that are often not sufficiently respected. These are: (i) since additional datasets are scarce they mostly rely on the heterogeneous Flynn database comprising the skin permeability data of 97 compounds; (ii) they only apply to aqueous dispersion media; and (iii) they repeatedly failed to predict experimentally measured permeabilities (Schaefer-Korting et al., 2007).

Therefore, we are using an alternative strategy. Heisig et al. developed a diffusion model of human stratum corneum to predict non-steady state stratum corneum-depth profiles and to estimate permeability parameters like apparent permeability coefficient and lag-time (Heisig et al., 1996). This model has now been adapted to an experimental database (Naegel et al., 2008). We have developed experimental methods to provide the model with all relevant input parameters and validated the predictions with experimental data (Hansen et al. 2008). In the context of this work the corneocytes were found to influence both the skin penetration of a hydrophilic and of a lipophilic, however ionizable, test compound significantly. We could now specify the mechanisms of interactions dependent on the physicochemical characteristics of the compound. Hydrophilic water soluble compounds will be taken up into corneocytes by dissolution in water that is present due to physiological hydration or artificial occlusion. Lipophilic compounds with the potential to protein binding may adsorb to stratum corneum proteins such as intra-corneocyte keratin or proteins of the cornified envelope or inter-cellular desmosomes. These findings may have significant consequences for the risk assessment of compounds as protein adsorption may lead to depot formation within the stratum corneum and sustained release of bound substance long after removal of the topical hazard (Vieth and Sladek, 1965). Additionally, for hydrophilic compounds their skin penetration may strongly be increased by swelling of the skin. This may readily occur during occupations where the skin of especially the hands is in prolonged or repeated contact with water or under occlusive conditions.

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Keywords: skin, partition, hydration, keratin, in silico diffusion model

Lecture: *in vitro* toxicology

Food for thought ... on the future of toxicology

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Toxicology is in a crisis – this holds especially true for its regulatory /applied part, while the modern, mechanistic part is indistinguishable from other sciences lacking unique topics or methodologies. Major limitations as the science of risk assessment are:

- It is too slow to deal with the number of questions posed, e.g. for chemicals we lack 86% of the essential information for the more 100,000 chemicals on the market;
- concepts have not changed for decades, in part because they were “put into stone” in international guidelines;
- the public is less and less willing to accept millions of animal used every year;
- costs of animal testing are high (about 10 billion \$ per year);
- the predictive value for humans is not clear;
- the exposure information is typically poor;
- the precautionary approach taken is not suitable for valuable existing chemicals and many classes of chemistry do not pass to clinical trials because of false allegations;
- despite having passed large toxicity testing requirements, up to 30% of substances going into human trials fail because of toxicological side-effects, questioning the predictive value of current strategies;
- many new products such as nanoparticles, genetically modified organisms, biologicals or cellular therapies can not been dealt with using current approaches.

At the same time, society is less and less accepting risk. The globalization of trade furthermore requires international agreements based on sound, defensible science. This crisis situation represents the driving force for change. At the same time there are enormous opportunities:

- The biotechnology and informatics revolution have delivered novel tools;
- there is increasing awareness of the limitations of toxicology;
- the political pressure forces investments from companies of novel scale and prompt funding programs;
- the process of formal validation of new approaches has shown to work and build the confidence for change even in international agreements.

The paper will discuss the emerging opportunities of an evidence-based toxicology (www.ebtox.org) and systems toxicology.

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Keywords: toxicology, genomics, bioinformatics, alternative methods

Poster: skin models as alternatives to animal testing

Drug metabolizing enzyme activity in human *in vitro* dermal (EpiDerm™) and airway (EpiAirway™) epithelial models

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Background: Human dermal and airway epithelia contain xenobiotic metabolizing enzymes (XME) including a variety of phase I (oxidative) and phase II (conjugative) activities. These XMEs play an important role in biotransformation of drugs and environmental/occupational chemicals. Biotransformation of chemicals may lead to altered drug activity or formation of toxic/mutagenic metabolites. The present work evaluated expression of XMEs in highly differentiated *in vitro* models of human dermal (EpiDerm™) and airway (EpiAirway™) epithelia that are cultured at the air-liquid interface to facilitate *in vivo*-like chemical exposures.

Materials and Methods: EpiDerm™ and EpiAirway™ cultures were prepared by standard procedures in the MatTek GMP *In Vitro* Model Production Facility. RT-PCR experiments were conducted to evaluate baseline and inducible expression of CYP isoforms in the epithelial cultures. CYP1A1 activity was determined by fluorescence microscopy utilizing ethoxyresorufin as the substrate. Total GST activity in the epithelial models was also evaluated by measuring conjugation of glutathione with 1-chloro-2,4-dinitrobenzene and UDP-Glucuronyltransferase activity was determined by 4-methylumbelliferone conjugation.

Results: In EpiDerm™, CYP1B1, CYP2C19, CYP2D6, CYP3A4 (weak) and CYP3A5 were constitutively expressed. 3-Methylcholanthrene (3MC) strongly increased expression of CYP1A1 and CYP 1B1 in EpiDerm™. EpiAirway™ cultures constitutively expressed CYP1A1 (weak), CYP1B1, CYP2A6, CYP2B6 (weak), CYP2C8 (weak), CYP2C19, CYP2D6, CYP2E1 and CYP3A5, while CYP3A4 and 3A7 were not detected. 3-Methylcholanthrene (3MC) strongly increased expression of CYP1A1 and slightly increased CYP2B6 and CYP2C8 expression in EpiAirway™. Enhanced metabolism of the CYP1A1 and CYP1B1 substrate ethoxyresorufin confirmed increased activity following treatment with 3MC. High baseline GST and UDP-glucuronyltransferase activity in both models was not further enhanced by 3MC treatment.

Discussion: CYP expression in EpiDerm™ and EpiAirway™ showed a high concordance with CYP expression reported for *in vivo* human dermal and airway epithelia. The results demonstrate that the EpiDerm and EpiAirway *in vitro* human epithelial models possess *in vivo*-like XME activities and may thus be useful for evaluating epithelial metabolism of drugs and environmental/occupational chemicals.

Keywords: *in vitro* skin model

Poster: skin models as alternatives to animal testing

Healing of dermal wounds in the EpiDerm-FT™ *in vitro* human skin model

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Background: Dermal wound healing involves interactions between dermal fibroblasts and epidermal keratinocytes, as well as cell and extracellular matrix interactions. The current poster describes wound healing experiments conducted with a full-thickness *in vitro* human skin model (EpiDerm-FT™).

Material and Methods: Normal human epidermal keratinocytes (KC) and dermal fibroblasts (FB) were cultured to produce the highly differentiated full-thickness skin model. Small wounds of several mm in diameter were induced in the epithelial model by means of a battery operated cauterizer or a dermal biopsy punch. The wounded EpiDerm-FT™ cultures were fixed at various time points and H&E stained paraffin sections were prepared to evaluate the wound and the wound healing process.

Results: Immediately after burn wounding, necrotic epithelium and denatured collagen dermal matrix were evident. Within one day, the denatured collagen matrix began to degrade and epithelial KC were observed migrating inward from the wound edges. Over a time course of seven days, migrating KC repopulated the wounded area to form a fully covered epithelium. Dermal fibroblasts were also observed to be proliferating within the wound area and generating new dermal matrix material. Biopsy punches were used to produce wounds that removed only the epidermis. These wounds also healed within a timeframe of 3-7 days. Increased FB proliferation in dermal areas directly adjacent to migrating KC was observed.

Discussion: These results demonstrate that EpiDerm-FT™ is a useful model for applications designed to elucidate dermal-epidermal interactions during wound healing and to evaluate the role of specific growth factors or new therapeutics in the dermal wound healing process.

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Keywords: *in vitro* skin model, wound healing

Poster: alternative testing methods for toxicity to reproduction

Development of a predictive *in vitro* test module for developmental neurotoxicity testing using mouse embryonic stem cells

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The development of the nervous system is a rather complex process known to be affected by different drugs and chemicals. Therefore, regulatory test guidelines have been adopted for the prediction and assessment of developmental neurotoxicity (U.S.EPA OPPTS 870.6300 and OECD TG 426). However, current *in vivo* test methods are laborious, costly and necessitate use of considerably high numbers of laboratory animals. Validated alternative methods for developmental neurotoxicity testing are not available. Thus, standardised, predictive screens for the evaluation of developmental neurotoxicity need to be available with the ultimate goal of increased efficiency in terms of reduced animal use and higher throughput compared to whole-animal testing using the existing guidelines.

In a newly established joint project funded by the German Ministry for Research and Education our final goal is to develop standardised predictive cell-based *in vitro* assays for developmental neurotoxicity testing. Different complementary cell models which represent selected developmental stages of the developing brain *in vivo* will be investigated to predict developmental neurotoxicity *in vivo* from *in vitro* data. In the context of a complex modular test strategy, we are developing a predictive *in vitro* test module using the mouse embryonic stem cell line D3. We established a simple and fast method for differentiation of mouse embryonic stem cells into neurons, astrocytes and oligodendrocytes suitable for testing of chemicals and other compounds. The differentiation of D3 cells into neural cells was determined by analysis of neuron-specific as well as glial-specific marker protein expression using flow cytometry and western blotting, respectively. In terms of a higher throughput, the protocol was adapted to a 96-well plate format. Here, we present our achievements in establishing predictive toxicological endpoints regarding proliferation as well as neural cell differentiation. Moreover, preliminary dose response profiles of selected developmental neurotoxicants are shown.

Keywords: in vitro, developmental neurotoxicity, embryonic stem cells, neurons, differentiation, test method

Lecture: strategies to reduce animal numbers for testing biologicals

An up-date of three Rs progress in the area of vaccine quality control

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At the 2006 MEGAT conference I gave a presentation entitled "Towards eliminating the use of animals in regulatory required vaccine quality control". Two years have gone since then. The question one might ask is: "Where are we now?" Well, certainly not at a total replacement. The fact is that laboratory animals are still extensively used for this area of testing, and also will be used in the near future. However, significant progress has been made the last few years. My presentation will provide an up-date. Part of it will deal with some case studies coming from my own area of interest. Information will be given about an ECVAM sponsored pre-validation study and an up-coming international validation study, commissioned by the European Pharmacopoeia, of a serological alternative to the lethal challenge and animal demanding (Kendrick) test in the estimation of batch potency of whole cell pertussis vaccine. Also, I will address a promising *in vitro* alternative to the heavily criticised Histamine sensitisation (HS) test in the safety evaluation of a-cellular pertussis vaccines. I also will focus on the consistency approach; a new strategy in vaccine quality control that ultimately might result in vaccine quality control without the need for animal use. Reference will be given to two ECVAM workshops on this issue and to the outcome of a recent EDQM conference in Dubrovnik.

Is it all positive news I have to tell. Well, there are some developments that need to be monitored in a critical way, such as the extensive use of fish for fish vaccines and the lack of harmonisation in vaccine quality control. However, the general conclusion of my presentation will be one of moderate optimism.

Keywords: vaccines, quality control, European Pharmacopoeia

Lecture: free communications

The reference laboratory concept in the United States: successfully combining *in vitro* testing services and an education program in a non-profit institute

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Introduction: The Institute for In Vitro Sciences, Inc. (IIVS) is a non-profit organization dedicated to the advancement of alternative testing methods. IIVS was organized in the US for the purposes (among others) of: 1) "... reducing or eliminating the use of animals in biological research and experimentation through education, training and the development, promotion and performance of non-animal research and analysis" and 2) "...publishing the results of non-animal research and experimentation to establish the validity and reliability of the methods of biomedical research and experimentation...".

Activities: The approach that IIVS uses to meet the above-mentioned goals is to operate a high quality laboratory facility that conducts *in vitro* safety and efficacy testing for industrial and government clients (including validation authorities) worldwide. Knowledge of the performance of various test methods and protocols that is gained from having this unique hands-on familiarity is then shared with others through education and outreach programs.

Research and Testing: Many different types of *in vitro* assays are conducted in the IIVS laboratory including those using organotypic models, engineered human tissues, or traditional monolayer culture. Currently acute skin and eye irritation assays are most often conducted.

A responsibility that correctly falls on the Institute is to provide information about the relative merits of new methods and models to the scientific community. IIVS strives to gain experience with all types of *in vitro* models, especially those that may commercially compete with one another, so that unbiased information about the performance of the various systems is available. This is especially useful for international researchers who may only be able to conveniently purchase from a supplier in close geographic proximity.

Participation in national and international validation studies (dozens since our founding) – especially those conducted by ECVAM and ICCVAM – is a prime example of an area where IIVS' *in vitro* experience is beneficial. Because of our strong belief in the use of Good Laboratory Practices (GLP) for *in vitro* studies, we have created GLP-compliant protocols for nearly all our studies, and have well over a decade of experience conducting assays in this manner. As we participate in such studies we are providing reference standards which become goals for laboratories newly entering the field.

Education: IIVS provides *in vitro* standards through hands-on training sessions conducted several times each year. These courses are taught not only by IIVS staff but also by outside experts. Due to our non-profit status, scientists from other contract research facilities often attend so that they can expand their own non-animal testing services. *In vitro* testing information is also provided through our web site (www.iivs.org), newsletters, and scientific publications.

Conclusions: IIVS's not-for-profit status enables us to promote *in vitro* methods and provide reference laboratory activities throughout the scientific community. We are not only a significant technical resource through which industry can meet many of its testing needs, but we also supply unbiased technical information to numerous constituencies world-wide. The overall goal is to accelerate the use and acceptance of *in vitro* methods.

Keywords: reference laboratory, *in vitro* testing, GLP's

Poster: strategies to reduce animal numbers for testing biologicals

The hen's egg testing on the chorioallantoic membrane – a model for multiple purposes

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The chorioallantoic membrane of the fertilized and incubated chicken egg emerges around embryonic day 6, fusion of chorion and allantois are forming a membrane, which primarily serves as a primitive respiratory system for the chick embryo, releasing CO₂ and absorbing O₂. Due to its function as respiratory organ the chorioallantoic membrane is highly vascularised, providing a network of fine capillaries in which gas exchange occurs. Tests conducted on the chorioallantoic membrane can be regarded as painless for the chick embryo for two reasons: the absence of neural cells in the chorioallantoic membrane itself and the fact that the neural system of the chick embryo has not been completely developed until embryonic day 11 (fusion of the neural tube).

Testing of the potential irritancy of different chemicals for the human eye is an established model of tests conducted on the chorioallantoic membrane and is one alternative to animal testing (Draize test). The tested substance is directly applied onto the chorioallantoic membrane and reactions, such as haemorrhage, intravasal coagulation or lysis of blood vessels, are investigated in course of time. In a slight modification of this test different materials as used for medical implants can be tested for their biocompatibility.

Another field of application using the chorioallantoic membrane is cancer research and the study of angiogenesis. We are testing the growth behaviour of different tumour cell lines on the chorioallantoic membrane in order to establish an alternate model to animal testing. Therefore the following protocol was developed: On embryonic day 5 a hole is drilled at the pointed pole of the egg shell and 3 ml albumen are removed by a syringe. The remaining hole is sealed with wax. At the following day the egg is carefully cracked at the blunt end and the shell membranes are removed with sterile forceps. A silicone ring is placed onto the chorioallantoic membrane in which 2 x 10⁶ tumour cells suspended in 20 µl medium are given. The egg is sealed with autoclaved aluminium foil and incubated at 37°C and 95% rh until day 11. Pictures are taken daily to document tumour growth. At day 11 the chorioallantoic membrane are fixed *in ovo* with 4% formalin and the tumour areal is excised, embedded in paraffin and investigated histologically and immunohistochemically.

Until now we tested different cell lines in this model:

- HoMel A1 and L1 (horse melanoma) and MelJuso (human melanoma) are unable to invase the chorioallantoic membrane, but remain swimming in the thin layer of albumen which covers the chorioallantoic membrane.
- 22RV1 cells (human prostate carcinoma) form solid tumours on the chorioallantoic membrane, but are unable to transcend the basal lamina and grow invasive.
- SaOs 2 cells (human bone sarcoma) form solid tumours and grow invasive, but very slowly, so only very few cells enter the chorioallantoic membrane.
- MCF 7 cells (human breast carcinoma) form solid tumours and grow invasive.

Furthermore SKBR3 (human breast carcinoma), Lovo, sw620 (both human colon carcinoma) cells are tested in this model system.

Keywords: HET-CAM, angiogenesis, 3D-tumor model

Poster: strategies to reduce animal numbers for testing biological

A new human hepatocyte cell line as a vantage point for a new generation of organoid liver test systems?

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The human liver is the main organ of drug biotransformation. To analyze substance activity or the generation of toxic by-products can be generated animal experimentation is routinely applied. These experiments are ethically critical and the results cannot be exactly transferred to the human organism because of significant differences in drug metabolism. Thus, a model has to be developed that meets the requirement of being comparable to the complexity and functionality of the human liver. At the same time such a model has to be applicable and reproducible.

At the Fraunhofer IGB a liver module based on an acellularised porcine jejunal segment with a maintained vascular system has been established (Schanz et al., 2007). This module allows a physiological co-culture of endothelial cells and hepatocytes. Initially, because of their better availability porcine hepatocytes were used for system development and now a possible human cell source has to be evaluated. Furthermore, the liver module has to be compared with established hepatocyte culture systems like sandwich and monolayer culture.

Primary human hepatocytes cannot be obtained in an adequate quantity and reproducible quality. Most hepatocyte cell lines are dedifferentiated and thus do not exert liver-specific functions. We compared a new human hepatocyte cell line in sandwich and monolayer culture with primary human and porcine hepatocytes.

Immunohistological experiments showed that the cell line does not feature all hepatocyte specific markers although its morphology is similar to primary hepatocytes. Nevertheless, the cell line performs liver-specific functions as urea and albumin synthesis as well as biotransformation. These functional results indicate that this cell line could be used as a human hepatocyte source.

Further functional testing will show if the cell line integrated in the vascularized liver module could be a vantage point for a new generation of organoid liver test systems, which could minimize animal experiments in terms of the 3R principles.

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Keywords: vascularised liver model, human hepatocyte cell line

Lecture: free communications

MucilAir: a novel human 3D airway epithelium model for acute or long term toxicity testing of chemicals

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Our company, Epithelix, has developed a novel *in vitro* cell model of the human airway epithelium, MucilAir. It is not only morphologically and functionally differentiated; but it can also remain at a homeostatic state for more than one year. MucilAir has already being successfully used by some university laboratories, pharmaceutical companies for research and drug development. With the advent of European legislation on chemicals, namely REACH, it becomes an urgent matter to develop more alternative methods to cope with the increasing demands of the chemical industry for chemical assessments. The alternative methods need to be validated by an official organization such as ECVAM.

Our goal is to push our *in vitro* cell model MucilAir into pre-validation process. With the support of 3R Foundation, we assessed the toxicity of 8 chemical compounds using MucilAir. Cell viability (resazurin test) and Trans-epithelial electric resistance (TEER) have been used as endpoints. The EC₅₀ values for each chemical have been determined on 3 independent batches (3 donors) of MucilAir. These tests showed that the EC₅₀ values obtained with the two endpoints from 3 different batches of MucilAir are quite similar, suggesting a good reproducibility. The particularities of MucilAir and the relevance of these results will be presented and discussed.

[This work is supported by 3R Foundation.]

Keywords: 3D, human airway, acute, long-term, toxicity, inflammation, cytokines

Poster: current activities regarding the EU-chemicals policy (REACH)

Alternative endpoints in the LLNA in order to reduce false positive results

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Background: According to the EU-chemicals policy (REACH), the murine Local Lymph Node Assay (LLNA) is the first-choice method for *in vivo* testing of skin sensitization properties. Compounds inducing lymphocyte proliferation in lymph nodes draining the site of topical exposure are regarded as sensitizers in the traditional LLNA. However, some non-sensitizing irritants have been shown to induce false positive results in the traditional LLNA. In this study, we investigated if a method using alternative endpoints is able to discriminate between sensitizers and irritants in order to reduce the number of false positive results.

Methods: Mice were treated with vehicle (actone:olive oil, 4:1 v/v), a sensitizer (0.5% DNCB) or an irritant (20% SDS or 1.0% BZC) on the dorsal surface of both ears for 3 consecutive days. Both traditional LLNA endpoints (ear irritation, lymph node (LN) size and cell count) and alternative endpoints (ear weight, LN weight and B220+ cell population in the draining LN) were analyzed one day after the last treatment.

Results: DNCB induced an increase in LN weight, size and cell count. Furthermore, a trend towards an increased B220+ cell population was observed after DNCB treatment. SDS induced slight erythema and increased ear weight and LN cell counts. BZC caused severe erythema and increased ear weight, LN weight and cell count. Both irritants did not induce an increase in the B220+ cell population.

Discussion: The traditional lymphocyte proliferation, measured by LN size and cell counts, was not able to discriminate between the sensitizer and irritants. Although analysis of the B220+ cell population was suboptimal and further optimization is necessary, all three compounds were correctly classified as sensitizer or irritant using B220+ cell populations and ear weights.

In conclusion, analysis of B220+ cell populations and ear weights proved to be promising alternative endpoints in the LLNA in order to reduce the number of false positive results of irritants.

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Keywords: LLNA, B220, sensitization

Lecture: free communications

Transcriptomic profiling of the effects of cadmium chloride and diquat dibromide on renal proximal tubular cells reveal a role of Nrf2

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Nuclear erythroid 2 p45-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor that is involved in the transcriptional regulation of many antioxidant genes. Since toxic compounds often elicit their deleterious effects through the build up of free radicals and oxidative stress, this pathway may be of major relevance to the prediction of toxicity in vitro. In this study we examined the effects of sub cytotoxic doses of cadmium chloride and diquat dibromide in a human proximal tubular cell line, HK-2. Utilising genome wide DNA microarrays (Affymetrix HGU 133 plus 2) we discovered a surprisingly large overlap between transcriptomic changes due to both compounds. Additionally genes downstream of the transcription factor Nrf2 such as heme oxygenase-1 (HO1) and activating transcription factor 3 (ATF3) were regulated by exposure to these compounds. Western blot analysis demonstrated that Nrf2 was indeed up-regulated on exposure to these compounds and silencing by siRNA increased the toxicity of both cadmium chloride and diquat dibromide. This study demonstrates that the Nrf2 pathway plays an important role in proximal tubular cells survival following exposure to certain toxins.

Keywords: in vitro, nephrotoxicity, renal, proximal, cadmium, diquat, Nrf2, microarray

Lecture: alternatives and education

Three's a crowd: the 1R of replacement for education and training

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The ideal “replacement alternative” is defined within the 3Rs philosophy of Russell and Burch (1959) as “non-animal”. However, the nature of knowledge and skills acquisition within the life sciences highlights a shortcoming of the 3Rs approach for education and training. Despite the widespread success of replacement of harmful animal use by non-animal alternatives such as multimedia, virtual reality, training mannekins and simulators, they may not be sufficient for full knowledge and skills acquisition in all courses. Specifically, some students and professionals should work with animals, animal tissue and clinical procedures. There is widespread evidence of the ability to meet such teaching objectives in ways that are neutral or beneficial to individual animals and that do not involve animal experimentation or killing. The use of ethically sourced animal cadavers for dissection and skills training, and apprenticeship into clinical practice with animal patients, are examples. Such approaches are themselves ideal replacement alternatives, and complement the non-animal alternative methods. Furthermore, countless examples of successful replacement can be identified across the world within all life science courses. It is argued that the 1R of replacement, when broadened from its non-animal focus, is sufficient to ensure ethical and effective acquisition of knowledge and skills in life science education and training. Policy and practice should therefore move beyond the 3Rs.

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Keywords: replacement, 3Rs, alternatives, education, training, InterNICHE

Lecture: alternatives & education

Alternatives across Latin America: catalysing change in the curriculum

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A 6-week series of seminars addressing replacement of harmful animal use in education and training was held across Latin America in April-May 2008. Organised by InterNICHE and partner organisations in Bolivia, Peru, Brazil, Argentina and Mexico, the events built on the experience of previous major outreach tours in Russia and India. Up to 6 day-long seminars were held at universities and independent venues in each country. Smaller meetings with campaigners, teachers and professional bodies complemented the large presentations. All events included spoken presentations, demonstrations and free trial of a wide range alternatives. Speakers included InterNICHE experts and those from the host countries who are involved in replacement work. Partner organisations were empowered through the process of planning and execution of the seminars; and the tour helped identify and provide support to others who are progressing humane education initiatives. Further collaboration is now planned. Information on the current situation concerning animal use and alternatives was also gathered from each country. The tour succeeded in raising awareness and generating interest in replacement alternatives, including through national-level interviews and media coverage. Information and freeware alternatives were widely distributed, and further translations of material into Spanish and Portuguese are in progress. To continue the hands-on access to alternatives provided at the seminars, a Central American Alternatives Loan System was established in Mexico, and plans have been made for other new libraries of resources. The donation of computers and alternatives to selected universities is also being planned in order to establish multimedia laboratories that will promote alternatives through example.

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Keywords: replacement, Latin America, alternatives, education, training, InterNICHE

Roundtable: Embryonic or adult stem cells: scientific and ethical considerations

The technology of human embryonic stem cells

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The first embryonic stem cell lines from mouse were first derived in 1981 and have allowed a remarkable progress in biomedical research. They enabled the making of so-called knock-out mice and allowed the study of gene function in a more targeted way. Many disease mechanisms have been elucidated thanks to these mice. Soon it became clear that the pluripotent embryonic stem cells could be used to derive differentiated cells of all 3 germ layers and be used for regeneration in vivo. Accordingly, researchers attempted to derive embryonic stem cells from human blastocysts. After several fruitless efforts to establish stable human embryonic cell lines the break-through happened in 1998 with the derivation of several cell lines. Since then, the stem cell field has exploded, over 500 lines have been derived from human blastocysts produced during IVF treatment and would have been discarded otherwise. The potential applications of human embryonic stem cells have evolved far beyond regenerative medicine. For the first time in history, researchers have the capabilities to establish robust models for human diseases, and have abundant human cells in culture that are karyotypically normal, which will allow to characterize cellular functions in the different human organs. It is now even possible to model early human embryonic development, until now a black box. It is expected that human embryonic stem cell technology will have an enormous impact on society and day-to-day life in the next 1-2 decades.

Keywords: hESC lines, karyotypically normal human cells, in vitro fertilization, blastocyst, human embryonic development, human disease models, regenerative medicine

Lecture: skin models as alternatives to animal testing

***In vitro* topical toxicity testing in line with requirements of EU and US regulators: reconstructed human tissue models**

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The potential for substances to cause effects such as corrosion or irritation to skin and eye is a concern of industrial toxicologists in their assessments of possible worker and consumer safety issues. Moreover, national and international regulatory agencies (e.g. ECA, EPA, US DOT), require that substances are labelled as to the toxicity potential to skin or eye. To prevent the unnecessary use of animals for the above-mentioned purposes, EU as well as US regulations recommend the use of alternative tests methods “whenever appropriate and feasible”.

Since reconstructed human tissue (RHT) models closely mimic native tissues, they can be used for reliable estimation of hazard (and in some cases also risk) related to human health. Tests with RHT models for topical toxicity testing are cost-effective and deliver faster and more reproducible results than many of the traditional *in vivo* assays. Another advantage of the commercially available RHT models is that their characteristics can be precisely controlled by established Quality Assurance procedures to insure long-term reproducibility, which is important in the regulatory toxicology (Rispin et al., 2006).

RHT-based assays for skin corrosion and skin irritation testing are validated, moreover, skin corrosion test with RHT models has reached full regulatory acceptance at the OECD level as OECD TG 431: The Human Skin Model Test. A number of *in vitro* RHT-based methods have completed pre-validation testing (photo-toxicity, eye irritation, genotoxicity) or are ready to enter the pre-validation process in the near future. They enable testing without excessive need for laboratory animals, which is of great importance for REACH as well as for EU cosmetic legislation. This presentation will describe currently available RHT-based assays for topical toxicity testing (with discrimination between risk and hazard). Approaches to the development, validation and implementation of these assays into regulatory systems and testing strategies will be discussed.

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Keywords: topical toxicity, reconstructed human tissue models, in vitro

Poster: skin models as alternatives to animal testing

Phototoxicity of essential oils intended for cosmetic use

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The phototoxic potential of chemicals, cosmetics, dietary supplements and pharmaceuticals is a growing concern in the consumer products industry. The determination of phototoxicity in the 3T3 Neutral Red Uptake Phototoxicity Test (3T3-NRU-PT), an *in vitro* test accepted in the EU Member States since 2000 and in the OECD Member States since 2004, is the first step in the sequential phototoxicity testing strategy. The reconstructed human skin model assays represent an important supplement to the 3T3-NRU-PT which may be used in order to obtain additional information on bioavailability of the chemical in human skin and phototoxicity risk related to topical exposure. The 3D human skin models overcome many of the limitations of the 3T3-NRU-PT, as they employ multi-layer tissues that closely parallel human skin morphology instead of a fibroblast monolayer, the human primary keratinocyte-based tissues are a more relevant model than a mouse tumor cell line, and non-aqueous soluble formulations can be tested. However, further investigations are needed in the extrapolation of *in vitro* results to the human situation.

The aim of this study, linked-up with a previous study on bergamot oils (Kejlová et al., 2007), was the evaluation of phototoxic potential of a group of essential oils used as cosmetic ingredients. The applied tiered testing strategy included chemical analysis of the substances (by means of capillary gas chromatography/mass spectrometry), the *in vitro* 3T3 NRU Phototoxicity Test and the EpiDerm™ Skin Phototoxicity Assay, performed according to the pre-validated phototoxicity test design (Liebsch et al., 1999). In order to clarify the situation in man, the negative results or the first non-phototoxic concentrations determined by the EpiDerm skin model were confirmed *in vivo* by means of the human skin photopatch test in a limited group of volunteers.

The study revealed, that phototoxicity of the selected essential oils was dependent on the content of photoactive substances (namely geranial and neral) and the solvent used. Using aqueous dilutions, a phototoxic classification was obtained *in vitro* and experienced also *in vivo* in the human photopatch test. In higher concentrations, the phototoxic effect was in some cases accompanied by skin irritation reactions.

The 3D human skin model test seems to be a useful tool for consideration of initial concentrations for confirmatory human photopatch tests to prove product safety, however, a safety factor of 10 might be considered for extrapolation.

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Keywords: essential oils, phototoxicity, 3T3 NRU PT, reconstructed human skin model, human photopatch test

Lecture: nanotoxicology / nanobiotechnology

Nanotoxicity: application of atomic force microscopy and novel nanoparticle measurement technology

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Within the field of scanning probe microscopy, atomic force microscopy (AFM) is extensively used in a wide range of disciplines such as life science, solid-state physics, and materials science. The AFM has evolved into an imaging method that yields structural details of biological samples such as proteins, nucleic acids, membranes, and cells in their native environment. AFM is a unique technique for providing subnanometer resolution at a reasonable signal-to-noise ratio under physiological conditions. As a result of continuous developments in sample preparation, imaging techniques, and instrumentation, AFM is now a companion technique to X-ray crystallography and electron microscopy (EM). It complements EM by allowing visualization of biological samples in buffers that preserve their native structure over extended time periods. AFM has been used in various bio-medical applications including the testing of cellular toxicity of nanoparticles and carbon-nanotubes. Complementary to AFM we introduced recently novel particle analysis technology allowing reproducible measurements of nanoparticle size, aggregation, and zeta-potential. Reliable particle size and zeta potential results help ensure top-quality emulsions (such as fat particles in milk) and dispersions (such as silicon dioxide particles in toothpaste) for foods and personal care products. With their high sensitivity to small particles, the newly introduced technology is ideal for measuring drug suspensions, protein aggregations and agglomerations, liposomes, and intravenous emulsions particularly relevant for pharmaceutical analysis and quality control. In summary, the presented technology allows to characterize the physico-chemical effects of nanomaterials on biological systems including cell samples and tissue samples efficiently, rapidly, and very accurately. Current EU research projects and project proposals deal with carbon nanotubes and nanoparticles toxicity analysis using novel and validated technologies.

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Keywords: atomic force microscopy, nanoparticles, nanotoxicity, cell biology

Poster: alternative testing methods for toxicity to reproduction

Towards an *in vitro* model for xenobiotic passage at the materno-fetal interface based on permanent cell lines

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Currently, studies on transplacental passage are largely performed in or *ex vivo*. However, species differences are known and in humans, studies can only be performed using at term placentae with limited relevance for embryo-/ early fetotoxicity and teratogenicity. Liu et al. (1997) and others have suggested syncytia-forming human BeWO choriocarcinoma cultures grown on permeable filter inserts (Transwells) as models for transport in the developing placenta. It was the aim of this study to investigate the experimental conditions for producing reproducible and stable monolayer cultures with a controlled paracellular permeability.

BeWO cells obtained from DSMZ were seeded on uncoated polyester or collagen-coated Transwell filter inserts (Corning) at densities from 25,000-200,000 cells/cm². Medium (Ham's F12+10% FCS) was exchanged and transepithelial electrical resistance (TEER) was measured daily to assess paracellular permeability for up to 10 days. For induction of syncytia, forskolin (10-100 µM or di-butyryl-cAMP (1 mM, db-cAMP) was added. The effect of DMSO was tested at concentrations between 0.1 and 3%.

Uncoated polyester membranes with a diameter of 12 mm were found superior to smaller or collagen-coated filters with regard to monolayer homogeneity and development of TEER. Seeding densities between 100,000 and 200,000/cm² resulted in TEER values 50-70 Ohm x cm² above background within 3-4 days, while up to one week was required after seeding of 75,000 or 50,000/cm² to reach a similar level of paracellular permeability. The maximum of TEER was usually observed 1-2 days after the cell layer reached confluency and was concurrent with a reduction of cross-sectional area per cell. Cells remained mono-layered thereafter for at least 2 further days.

Addition of 30 or 100 µM forskolin or 1 mM db-cAMP induced syncytia formation of BeWO cells grown on plastic as well as on filter support. This was confirmed by trypsinisation of cells and subsequent counting with cell size classification. However, no syncytia formation could be observed at seeding densities above 50,000/cm² and for confluent cell layers in general. Importantly, addition of forskolin to BeWO cultures grown on Transwells reduced cell-cell contacts and prevented development of low-permeability monolayers. In contrast, the solvent DMSO (1%) promoted TEER development.

Therefore, a strategy based on seeding of pre-formed syncytia onto filter inserts was evaluated. Treatment of cells seeded at ~25,000/cm² on culture dishes with 30 µM forskolin for 6 days produced the highest yield of syncytia with 38% of all particles. After careful resuspension, these adhered well to Transwell filter inserts, but permeability of the resulting monolayers remained high with stagnant TEER values below 20 Ohm x cm². Seeding of a second layer of forskolin-induced cells or addition of 1% DMSO did not improve the result.

Induction of syncytia formation in BeWO cells grown on filter inserts by forskolin or db-cAMP was not compatible with formation of monolayers with controlled paracellular permeability as indicated by the absence of an increase in TEER. Similarly, pre-formed BeWO cell syncytia failed to form low-permeability monolayers upon single or repeated seeding onto Transwells. Surprisingly, DMSO stimulated TEER development, but only in the absence of forskolin (pre)treatment.

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Keywords: placenta, cell culture, BeWO, permeability, transport, teratogenicity, transwell

Lecture: ethical and legal aspects in animal experimentation

The beginning of the end for chimpanzee experiments?

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The advanced sensory, psychological and social abilities of chimpanzees confer upon them a profound ability to suffer when born into unnatural captive environments, or captured from the wild – as many older research chimpanzees once were – and when subsequently subjected to confinement, social disruption, and involuntary participation in potentially harmful biomedical research. Justifications for such research depend primarily on the important contributions advocates claim it has made toward medical advancements. However, a recent large-scale systematic review indicates that invasive chimpanzee experiments rarely provide benefits in excess of their profound animal welfare, bioethical and financial costs. The approval of large numbers of these experiments – particularly within the US – therefore indicates a failure of the ethics committee system. By 2008, legislative or policy bans or restrictions on invasive great ape experimentation existed in seven European countries, Japan, Australia and New Zealand. In continuing to conduct such experiments on chimpanzees and other great apes, the US was almost completely isolated internationally. In 2007, however, the US National Institutes of Health National Center for Research Resources implemented a permanent funding moratorium on chimpanzee breeding, which is expected to result in a major decline in laboratory chimpanzee numbers over the next 30 years, as most are retired or die. Additionally, in 2008, The Great Ape Protection Act was introduced to Congress. The bill proposed to end invasive research and testing on an estimated 1,200 chimpanzees confined within US laboratories, and, for approximately 600 federally-owned, to ensure their permanent retirement to sanctuaries. These events have created an unprecedented opportunity for US legislators, researchers, and others, to consider a global ban on invasive chimpanzee research. Such a ban would not only uphold the best interests of chimpanzees, and other research fields presently deprived of funding, but would also increase the compliance of US animal researchers with internationally-accepted animal welfare and bioethical standards. It could even result in the first global moratorium on invasive research, for any non-human species, unless conducted in the best interests of the individual or species.

Keywords: great ape protection act, directive 86/609, animal experiment, animal research, chimpanzee, Pan troglodytes, Pan paniscus, great ape

Poster: free communications

Non-animal methodologies within biomedical research and toxicity testing

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High-throughput chemical testing programs within Europe and the US face marked logistical challenges associated with laboratory animal use, which is also limited by scientific constraints on human applicability, and expanding regulatory restrictions, driven by increasing social concerns about biomedical animal use. However, a range of non-animal methodologies are available within biomedical research and toxicity testing. These include: mechanisms to enhance the sharing and assessment of existing data prior to conducting further studies; physicochemical evaluation and computerized modelling, including the use of structure-activity relationships and expert systems; and the use of minimally-sentient animals from lower phylogenetic orders, or early developmental vertebral stages, as well as microorganisms and higher plants. A variety of tissue cultures, including immortalised cell lines, embryonic and adult stem cells, and organotypic cultures, are also available. *In vitro* assays utilising bacterial, yeast, protozoal, mammalian or human cell cultures exist for a wide range of toxic and other endpoints. These may be static, or perfused, and used individually, or combined within test batteries. Human hepatocyte cultures and metabolic activation systems offer potential assessment of metabolite activity. Microarray technology may allow genetic expression profiling, increasing the speed of toxin detection, well prior to more invasive endpoints. Enhanced human clinical trials utilising microdosing, and surrogate human tissues, advanced imaging modalities, and human epidemiological, psychological and sociological studies, may all increase understanding of illness aetiology and pathogenesis, and facilitate the development of safe and effective pharmacologic interventions. Particularly when human tissues are used, non-animal models may generate faster, cheaper results, more reliably predictive for humans, yielding greater insights into human biochemical processes. Greater commitment to their development and implementation is necessary, however, to efficiently meet the needs of high-throughput chemical testing programs, additional important emerging testing needs, and the ongoing development of human clinical interventions.

Keywords: 3Rs, alternative, animal experimentation, animal model, toxicity testing, biomedical research

Poster: skin models as alternatives to animal testing

***In vitro* differentiation of skin sensitizers by cell signaling pathways**

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Introduction: The EU-Guideline (76/768/EEC, Feb. 2003) prohibits the use of animal testing for the assessment of toxicological data for cosmetic ingredients as of 2009/2013. In contrast, the resolution of the REACH-program will increase the number of animals needed since approximately 30,000 chemicals have to be evaluated. For some test areas adequate *in vitro* tests to replace animal testing are missing. In this study, we focused on the test area of skin sensitization and investigated whether analyses of cell signaling pathways can provide a methodology for the detection of sensitizing compounds *in vitro*. For this purpose a differentiation between non-specific immune reactions (skin irritation) and skin sensitization was of major importance. For the induction of a local immune reaction an intact skin barrier plays a key role, since compounds need to be able to penetrate this natural barrier before reaching living immune competent cells. To mimic this situation best, human and murine skin explants were chosen and compared with the reconstituted skin models EST-1000 and AST-2000 (CellSystems, St. Katharinen, Germany).

Methods: Murine and human skin explants as well as reconstituted skin models (epidermal model EST-1000 and full-thickness model AST-2000) were exposed to different concentrations of sensitizing (Oxazolone and DNFB) or irritant compounds (SDS and TritonX-100). The lowest observed effect level (LOEL), defined as the concentration resulting in a decrease in viability of about 10% after 24h of exposure, was determined for each compound and skin model by using the MTT viability assay. Each skin model was then exposed to the appropriate concentrations of the LOEL for 1h or 3h. Phosphorylation of MAP-kinases (p38, ERK1/2 and JNK1/2), STAT1 and PLC γ were determined by cytometric bead array (CBA).

Results: In skin explants all three MAP-kinases were exclusively activated after exposure to sensitizing compounds. Differences were obtained regarding the time points of activation. Whereas in murine skin explants phosphorylation was generally detected after exposures for 3h, cell signaling proteins in human skin explants were already activated after 1h. Regarding the reconstituted skin models, exclusive phosphorylations of p38 and JNK1/2 were obtained after 3h stimulations with allergens. In contrast to skin explants, treatments with irritant compounds lead to an ERK1/2 activation after 1h exposure in the EST-1000 and AST-2000. Inductions of STAT1 and PLC γ were not detected in any of the skin models analyzed.

Discussion: In our study, MAP-kinase activation was shown to provide a promising *in vitro* tool for the discrimination between sensitizing and irritant compounds. The reconstituted skin models AST-2000 and especially the EST-1000 showed high induction levels of phospho-p38. The inductions were comparable to those found in skin explants, i.e. complex immune competent tissues, and specific for an exposure to sensitizing compounds. With respect to availability, variability and simplicity in handling, the EST-1000 turned out to be the model of choice for further analyses of compounds.

Keywords: skin explants, reconstituted skin models, irritant compounds, sensitizing compounds, cell signaling pathways

Poster: Current activities regarding the EU-chemicals policy (REACH)

Functional assays are mandatory for a correct prediction of immunotoxic properties of compounds *in vitro*

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Subject: An increasing aim in safety assessment of chemicals and drugs is to reduce, refine and replace animal testing, especially in the context of the new system for the Registration, Evaluation and Authorisation of Chemicals (REACH). REACH will require the re-assessment of about 30,000 existing substances currently marketed at volumes greater than 1 t per year. Great efforts were and still are made to evaluate potential adverse effects of compounds on the immune system *in vitro*. Regarding immunosuppression, most methods are based on mitogen stimulation assays. To our knowledge the *in vitro* antibody response (Mishell-Dutton culture) has never been considered as an *in vitro* alternative to the existing animal tests nor has its potential of correctly predicting different immunosuppressant compounds been analyzed. Therefore, we designed a study comprising seven immunosuppressant (Benzo(a)pyrene, Cyclophosphamide, Cyclosporine A, Dexamethasone, Methotrexate, Rapamycin, and Urethane) and four negative compounds (1-Bromo-4-Chlorobutane, Heptanal, Mannitol and SDS) and compared the results to data obtained from rat mitogen stimulation experiments.

Methods: For the mitogen stimulation experiments rat splenocytes were cultured with either ConA or LPS under exposure to immunosuppressant or non-immunosuppressant compounds. As endpoints, proliferation (BrdU-ELISA) and cytokine release (TNF α - and IFN γ -ELISA) were assessed. Cytotoxic effects of compounds were determined on non-stimulated cells by measuring LDH release or Resazurin conversion. For the *in vitro* antibody response murine splenocytes were immunized with SRBC under exposure to the different compounds. After 5 days of culture, viability was measured by Resazurin conversion and cells were plated on petri-dishes with complement under a second exposure to SRBC. Plaques (areas of hemolysis) were counted per dish.

Results: Using rat spleen cell mitogen stimulation assays, problems arose in discriminating non-specific cytotoxic from specific immunosuppressive effects due to opposed results of the two performed viability assays for Rapamycin and Dexamethasone. A weak immunosuppressant compound like Urethane could not be detected. Other compounds like Methotrexate or Benzo(a)pyrene were correctly predicted by one endpoint only. However, the *in vitro* antibody response showed a high sensitivity and specificity. All four negative compounds were correctly predicted, whereas among the seven immunosuppressants only one false negative compound was obtained. The incorrectly predicted compound was Cyclophosphamide, so this misclassification was not surprising due to the known requirement of metabolism.

Discussion: The *in vitro* antibody response is a promising assay for the prediction of immunosuppressive properties of chemicals and drugs, whereas rat spleen cell mitogen stimulation assays are rather poor in respect thereof. Immunosuppressive effects of compounds can be limited to certain cell types and endpoints. Since mitogen stimulation assays are also restricted to certain cell types and the chosen endpoints, some compounds might not be correctly predicted. Regarding the *in vitro* antibody response, such limitations do not exist. In a functional assay, where several immunocompetent cells have to cooperate to result in the humoral response analyzed, any compound-induced alteration is likely to be detected.

Keywords: immunosuppressant compounds, cytotoxic compounds, mitogen stimulation assays, *in vitro* antibody response, functional assay

Lecture: gentechonology & animal experiments / transgenic animals

Animal testing of genetically modified food and feed: can it be justified?

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In Europe, a political controversy about the bringing onto the market of food and feed based on genetically modified plants has been going on for years. One main aspect of this controversy is the potential risk for human health. The placing on the market of genetically modified food or feed products is governed by Regulation 1829/2003/EU. According to this regulation, such food and feed must not show any harmful effects. The European Food Safety Authority (EFSA) is the keystone of European Union (EU) risk assessment regarding food and feed safety. EFSA has published a guidance document for the risk assessment of genetically modified plants and derived food and feed that inter alia explicitly demands animal data, so animal experiments are carried out or commissioned by the applicants. However, the interpretation of the results of such experiments is highly controversial. This is due to the fact that a great uncertainty exists to whether the effects observed in animal studies can be regarded significant as such, whether they can be transferred to humans, and which conclusions could be drawn with regard to potential effects in the food chain. Therefore, such animal tests are questionable, and initiatives to even increase animal testing for genetically engineered products, e.g. by introducing long-term studies, must be regarded critically.

A similar situation exists concerning the risk assessment of milk or meat derived from cloned or genetically manipulated animals. Animal experiments for this purpose are carried out in a smaller scale compared to those for crops but deserve attention particularly with a view to the current discussions in the EU around the authorisation of cloning of animals for food production purposes.

All above issues have in common that the question whether the animal experiments can be regarded ethically acceptable needs to be asked. According to legislation in Europe, an animal experiment is regarded ethically acceptable if the benefit resulting from it outweighs the suffering of the animals involved. In this case, however, like in many fields of animal experimentation, it is neglected that the benefit of proving the safety of genetically manipulated food and feed is merely a commercial one.

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Keywords: animal testing, genetically modified food and feed, Regulation 1829/2003/EU

Poster: alternative testing methods for toxicity to reproduction

Generation of functional astrocytes from embryonic stem cells for drug testing

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Background motivation

Deleterious effects of neurotoxicants in the brain are not only caused as a result of direct neurotoxicity, but are also the result of inflammatory processes caused by glial cells activated by the toxicant (Wyss-Corray et al., 2002). The main players involved in inflammation in the brain are microglial cells and astrocytes (Falsig et al., 2006).

Embryonic stem cells (ESC) are a promising source for reliable and reproducible cell culture systems. Cells generated from ESC have the potential to reduce the use of primary cells and possibly animal experiments. Recent advances in the directed differentiation of murine ESC have led to robust methods for the derivation of neuronal cultures. However, no method for the efficient generation of astrocytes from murine ESC has yet been published.

We are interested in developing methods that allow generation of cultures containing functional astrocytes. The astrocytes are to be used to characterise toxicants.

Material and Methods

Mouse ESC (CGR8) were pre-differentiated and subsequently plated and further differentiated.

The astrocytes were characterised as to the expression of specific markers and compared to primary astrocytes and astrocytes differentiated from a neural stem cell line. Furthermore, the astrocytes were functionally characterised with regards to their inflammatory reactivity. Briefly, the astrocytes were incubated in the presence of proinflammatory cytokines. Then, IL-6 and NO concentrations in the supernatants were measured.

Results

During the differentiation of both, embryonic and neural stem cells, astroglial markers (GFAP, S100b, A2B5, CD44) were upregulated while the neural marker Nestin as well as neuronal markers (NCAM, bIII-Tubulin) were downregulated. In the stimulation experiments, we observed nuclear factor κ B (NF κ B) translocation into the nucleus upon stimulation as well as an increased release of IL-6 and NO in the supernatant.

Discussion

The presence of astroglial markers (GFAP, S100b, A2B5, CD44) in about 80% of the cells of the culture and the absence of the neuronal markers NCAM and bIII-Tubulin indicate a successful differentiation process.

Overall, mESC-derived astrocytes and neural stem cell derived astrocytes showed the same response pattern as astrocytes isolated from mouse brain. The differentiation process can be affected by toxicants.

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Keywords: astrocytes, embryonic stem cells, neurotoxicology

Poster: skin models as alternatives to animal testing

Development of a new reconstituted human corneal model to assess the ocular irritating test

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Alternative methods to the Draize eye irritation test, such as the BCOP, HET-CAM, ICE, and IRE, are used to evaluate the ocular irritation potential of cosmetic, livelihood articles or industrial chemicals.

In order to improve the sensitivity and specificity of alternative eye irritation test, we developed a novel three-dimensional human corneal model that uses normal human corneal epithelial cells. In this study, two laboratories, have tested 20 reference chemicals. The results were compared to previously published *in vivo* eye irritation as well as existing data obtained in the other three-dimensional corneal model test. A good intra/inter-laboratory reproducibility and correlation with *in vivo* and other *in vitro* model results were obtained.

Our new three-dimensional model, developed from normal human corneal epithelial cells, is reproducible and is accurately predicting model of eye irritation test.

Keywords: eye irritation, cornea model



The biological basis of the use of human embryonic stem cells for *in vitro* test systems

Introduction to the roundtable "Embryonic or adult stem cells: scientific and ethical considerations"

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Human embryonic stem cells (hESC) are presently being cultured in many laboratories, and differentiation protocols are available for a large variety of cell types. The most immediate use of these cells may not be therapeutic applications, but more immediately, the design of test systems for toxicological and pharmacological research (UKTI, 2005; Bremer and Hartung, 2004; Vogel, 2005). The use of such human-based test systems would contribute to a bottom-up test strategy for new chemicals, where initially the mode of action is explored, and animal experiments are only used as a last resort and in special situations (Leist et al., 2008a). One obstacle to the broad use of hESC for experimental test systems are ethical issues that have different legal implications in different countries (MBBNET, 2008).

What are embryonic stem cells? Before entering a bioethical debate it is important to create a common platform of biological facts important for such a debate. We will first take a look at natural conception and embryo development as basis for the overall understanding of the technology. After fertilization of the *oocyte* ("egg") in the oviduct by a *sperm*, a *zygote* is formed. This cell, which contains genetic information from two parental gametes (*i.e.* the oocyte and sperm) starts dividing while migrating down the oviduct, and gives rise to a tiny (less than 0.1 mm), blackberry-shaped, compact cell clump termed the *morula*, which eventually enters the uterus at around day 4. By day 5, a cavity is formed in the "ball" and the resulting structure of embryonic cells is now termed *early blastocyst*. The blastocyst is initially surrounded by a trans-

lucent structure (the *zona pellucida*) that protects it and prevents it from attaching to incorrect structures. In the uterus, the *blastocyst* begins to form different cell types - the outer cells (about 200), which will later become the placenta, and the inner cell mass (ICM) of about 30 cells which will later develop the embryo. Eventually it "hatches" *i.e.* it breaks out of the *zona pellucida* and attaches to the wall of the uterus. This process of "*implantation*" of the late blastocyst occurs around day 9 post-fertilization and represents the first physical connection between the early embryo and the fertilized woman. This step, also termed "*nidation*", is crucial for the development of embryo polarity (body axes/up and down-definition). Finally, at day 12-14, a dramatic morphological restructuring occurs when the embryonic cells form a double-layered structure - the *gastrula*. This is the start of primitive tissue formation (*primitive streak*) in the *embryo proper* (the real embryo). At day 14, therefore, pregnancy is established, the embryo has a close connection to the womb, it has developed polarity as well as "inside" and "outside" directionality, and, importantly, the three germ layers (primordial tissues) begin to form early organs such as the primitive gut and neural system. In parallel the placenta forms from formerly external cells of the blastocyst.

Couples facing problems in conceiving naturally (in some countries also women that can get anonymous sperm) now have the option to increase their chances of pregnancy by undergoing an *in vitro* fertilization (IVF) procedure. This process differs from a naturally-occurring pregnancy only in the initiation phase where

sperm and oocyte meet. Oocytes are harvested from the woman, and this process is facilitated by ovarian stimulation with hormones. Then they are fertilized with a sperm sample from the potential father to form zygotes. Typically, 10-15 early pre-implantation embryos are generated in such a process and propagated *in vitro* up to the blastocyst stage (day 5). In some countries (e.g. Germany), national regulations forbid selection of the embryo after nuclear fusion and preclude the cultivation of more than three embryos at a time (Zollner et al., 2003). After positive selection of the best blastocysts, 1-3 (depending on the country) are re-implanted directly into the uterus, where they have the chance to attach to the uterine wall and form an embryo just as it occurs in a natural conception. IVF procedures result in a successful pregnancy in about 15-25% of the procedures. If the first attempt is unsuccessful, a second and third round of re-implantation of blastocysts may be initiated, since the initially superfluous blastocysts are typically cryopreserved, *i.e.* stored in a liquid nitrogen tank where they can be maintained for several years. It is not known when a blastocyst under conditions of cryopreservation loses its potential to form an embryo. Albeit one case study reported a successful pregnancy after implantation of a blastocyst stored for 12 years (Revel et al., 2004), it is generally observed that the quality is strongly falling after 5-10 years. Many supernumerary blastocysts accumulate in fertility clinics and will be ultimately destroyed. It is estimated that 400,000 fertilized oocytes were stored in 450 fertility clinics in the USA alone in 2003. Tens of thousands are also frozen in Germany,

Switzerland, Austria, the UK and many European and Asian countries. Thus, it is reasonable to assume that by now over one million pre-implantation embryos are stored in the USA and elsewhere. A very small minority of these (less than 1%) is typically donated for research purposes, including hESC generation.

The *in vitro* generation of hESC starts with thawing and propagating a superfluous donated day five pre-implantation blastocyst. The ICM is isolated and placed in growth medium. These cells can be cultured *in vitro*, but they require a supportive “feeder” cell layer to provide them with nutrients and hormonal signals. Typically, mouse or human fibroblasts (connective tissue cells) are used as feeders. If the procedure is successful, the cellular outgrowth of this ICM will form the “passage 1” of a new hESC line, and once the cell culture dish is densely grown with hESC, the cells will be harvested and transferred to five fresh cell culture dishes. There they grow again to cover the whole dish (passage 2), and be transferred to five new dishes, and this procedure can be repeated continuously to generate more cells. It is now possible, in principle, to expand hESC indefinitely, and hESC have been cultured as stable cell lines up to passage numbers far beyond 150 using methods developed originally in 1998 in the laboratory of James Thomson (Thomson et al., 1998). All hESC harbour the potential to form a diversity of different cells – theoretically any cell type of the about 200 existing in the human body. Therefore, they are called “pluripotent” cells. Pluripotent means that the cells can form any known cell type, but they cannot generate a whole organism, because they are lacking the capacity for implantation and formation of a placenta. This capacity is only found in the zygote and the cells up to the 8-cell stage of the morula, and these cells are called “totipotent”. It is important to emphasize here that hESC themselves are not totipotent and they are therefore unable to generate a new embryo.

The use of hESC in research requires the generation of hESC lines. The lines currently used were mostly produced from fertilized oocytes that had undergone about 7-8 divisions. The major objection to the use of ESC is that their generation is purported to involve an ‘act

of killing’. To evaluate this point of view it is best to look at a typical example of cell line generation: A couple wishing to have children decides to try *in vitro* fertilization (IVF) – oocytes are removed from the woman, fertilized with sperm, and two embryos are implanted into the woman. The remaining embryos are stored, frozen in liquid nitrogen, and the woman is lucky to become pregnant on the first attempt – possibly even giving birth to twins. Life goes on and the couple is content with their two kids. After more than five years of storage, the quality of the stored blastocysts starts to deteriorate, and the storage is expensive as well. Presently, at least half a million such left-over zygotes are stored in the US alone. None of these blastocysts has any potential at all to ever develop to a human being without a foster mother. After parental consent of our happy couple, the left-over material will either be destroyed (moved to a trash can), or it may be donated for research purposes (only in some countries). Embryo adoption schemes as an alternative to discarding them were discussed, but this obviously does not appear to be a realistic option for all stored blastocysts at annual storage costs of several hundred million \$. In the case of research donation for hESC research, the blastocyst will be allowed to be thawed, and hESC will be generated. When the procedure is successful, this will result in the generation of a single continuously growing hESC line that can provide pluripotent cells indefinitely. For instance the first such cell line, generated around 1998 (Thomson et al., 1998) has now been spread to hundreds of laboratories worldwide; its usage has generated a tremendous amount of novel scientific knowledge which may enable future therapies, and it is still being used extensively today. Most laboratories working with hESC nowadays use such cell lines for their research, but were never involved in the generation of cell lines, *i.e.* the use of any blastocyst.

Concerning the issue of the “use of human beings for research”, the definition of “human personhood” is the most crucial issue, and a wide range of viewpoints exists:

- On one side of the spectrum one can find views that human life and personality begin and reach their full extent within a

single moment, when the nuclei and chromosomes of the gametes combine.

- On the other side of the spectrum one finds views that the first days after the zygote formation merely result in the formation of a “pile of cells” (morula/blastocyst stage) which cannot yet be assigned full human rights and therefore does not deserve the specific ethical consideration of a person (Schuklenk, 2008).

- In between these extremes, there are also views that acknowledge the zygote as being unique (as opposed to other cells) in its natural, but theoretical, potentiality to become a human being, but that full human personhood and the rights associated to it are acquired gradually. Here, metaphors are often evoked of the embryo being a walnut, a cherry or a caterpillar, which may or may not become a walnut tree, a cherry tree or a butterfly – but they certainly are none of the latter, and therefore they deserve a different status. No one would, for instance, argue that each walnut deserves the same protection as a full-grown walnut tree.

We have no authority of deciding who is right in the above issue, but we can look at the basic concepts associated with these different views.

One line of thought builds on the concepts of continuity, identity and potentiality. Obviously, a zygote does not fit our intuitive and accustomed picture of a human being. Still, it clearly belongs to the human race (biologically). In order to assign the status of personhood to a microscopic pile of cells, it has to pass at least the test of the following characteristics:

- A defined succession of events leading **invariably** from the zygote to a human person (= continuity);
- A situation in which **one** zygote only becomes **one** person and **one** person can only be derived from one single zygote (= identity);
- The full capacity of a zygote to become a human person **without further manipulation or help** (= potentiality).

If one accepts that these three requirements are met, **and** if one assumes that fulfillment of these requirements is both necessary and sufficient for the personhood definition, then a zygote indeed **is** a human being.

However, biological research in mammals has shown that these assumptions

may not hold true: the continuity criterion is not fulfilled, because *most* zygotes (70%) never become persons (because of implantation failure), the identity criterion is not met, because one zygote can produce twins, and *vice versa* some persons are made up from two fused zygotes, and the full potentiality is not found in the zygote, as an embryo can never develop to a person in the absence of the mother's womb, *i.e.* without maternal signals received upon implantation. It may also be argued that these requirements are necessary, but not sufficient (e.g. time as additional factor, see: gradual acquisition of personhood).

It has been argued that hESC can by no means be used to substitute animal experiments. This would mean that experiments are instead performed on "humans". However, this argument contains a misconception. The testing of toxicity on human cells allows man to take responsibility for himself without relying on animals and their suffering. It would not be a substitute, but an entirely different, and possibly better approach. Human cell-based *in vitro* methods would fully correspond to the new vision of a mechanistically based toxicology (Leist et al., 2008b; Hartung and Leist, 2008) that implies higher financial efficiency of the procedure and improved safety for mankind. For instance the EU project ESNATS has been started with the goal to develop novel hESC-based alternative test systems for drug profiling. This is a logical consequence of a line of research that has shown that 3R (reduction, replacement, and refinement of animal experiments) methods have been astonishingly successful and can sometimes yield better data than animal based tests (Hartung, 2001, 2007a, 2007b, 2008; Leist et al., 2008b). hESC-based test systems could contribute to such a strategy of safety testing with fewer animals, and with data more relevant to humans, and more based on sound science. This concept should by no means be mistaken (or distorted) to imply that animal experiments would be substituted by embryo research. Typical examples for the application of hESC at the moment are in the field of developmental neurotoxicity. The

thalidomide catastrophe showed that animal models can be very poor predictors of human developmental toxicity. Accordingly, a lot of focus has been on the development of ESC-based test systems, initially on murine cells, and in recent years also using human cells (Pellizzer et al., 2005). Research on human neurons is extremely difficult due to the poor availability of the material. Therefore, most experiments to test toxicity have relied on animals or animal material, and this has sometimes led to detrimental erroneous conclusions (see thalidomide). Another illustrative example is cardiotoxicity (in particular toxicity of drugs that cause changes of the heart beat). This is frequently associated with drugs that interact with the so-called hERG potassium channel on the heart. It is likely that in the near future, hESC-derived cells may be substituting the current assays performed with primary porcine or rabbit cardiomyocytes (heart muscle cells). Such cells can now be generated from hESC with almost 100% purity, and these cells have been shown to function in the hERG test (Xu et al.).

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Lecture: 7th cosmetics amendment – can all goals be achieved in time?

Reduction of animal testing by development of a novel exposure chamber to expose cells to nanoparticles at the air-liquid interface

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Due to the wide range of potential applications, the use of nanoparticles will increase dramatically in the near future and by this also occupational and public exposure. Screening strategies have been developed to assess nanoparticle toxicity by *in vitro* and *in vivo* studies. Traditionally, *in vitro* exposure experiments have been performed which incubate cells with particles in liquid suspensions (“submerged”). Recently, more advanced air-liquid particle exposure systems have been introduced that more realistically mimic exposure via inhalation. In these systems cells are exposed to airborne particles, but they typically require profound technical skills for nanoparticle generation/characterization and the nanoparticle deposition rate (onto the cells) may be very low (2% for diffusional deposition). In order to minimize the need for animal testing it is desirable to find a simpler and economic, but still reliable *in vitro* exposure method for nanoparticle toxicity. We have developed such an exposure chamber in which a human epithelial cell line (A549; other cell lines could also be used) is exposed to nanoparticles at the air-liquid interface. Nebulization of a nanoparticle suspension forms micron-sized droplets containing nanoparticles that deposit onto cells via sedimentation (not diffusion). This method is technically simple and it allows both accurate nanoparticle dosimetry and a very efficient use of expensive test materials (30-70% deposition efficiency).

First evaluations with a cylindric prototype device showed a highly uniform and reproducible particle deposition on the bottom of the chamber with a total base area of 225 cm². Nebulization of 1ml of a 10% NaCl solution yielded a deposited mass of 1.327±0.047 mg/cm² (±3.5%) corresponding to about 60% deposition efficiency. When the A549 cells, were placed in the chamber and exposed to 0.9% NaCl, the cells did not show any reduction in cell viability. This is a first hint that it is possible to use this chamber for cell exposures without damaging them.

The prototype of this novel exposure chamber shows very promising features but needs to be further improved.

Keywords: nano-zinc particles, air-liquid interface, novel exposure chamber

Poster: strategies to reduce animal numbers for testing biologicals

Prevalidation study for testing the toxic effects of inhalable substances (gases) on human lung cells using an air/liquid culture technique

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To date, studies of inhalation toxicity of air contaminants are restricted to animal experiments, mainly because of difficulties in exposing cell cultures directly to these substances. The increasing demand for assessing inhalation toxicity hazards calls for development of new testing strategies which comprise both in vitro and in vivo tests. For this purpose, we are evaluating a direct exposure strategy of cultured human lung cells to diverse inhalable substances at the air/liquid interface. In this approach, the well characterized alveolar cell line A-549 is grown on microporous membranes and exposed to test atmospheres. Appropriately designed systems for medium supply and gas support enable the nutrification and humidification and a direct contact of the exposed cells to the test gases at the same time.

Currently, four partners (Fraunhofer ITEM, ZEBET, UFZ and BAuA) are funded in part by the German Federal Ministry of Education and Research (BMBF) to assess in frames of prevalidation study the intra- and interlaboratory reproducibility and predictive capacity by testing the toxic effects of gases of well known in vivo toxicity (NO₂, SO₂, formaldehyde, ozone) after exposure of human lung cells at air/liquid interface. Particularly, at present time all four partners are working on optimisation and improvement of A-549 cell culturing on the microporous membranes, cell exposure to the test gases, as well as establishment and transfer of tests for the toxicological endpoints. The Neutral Red Uptake assay was chosen as a cytotoxic endpoint and Comet assay as a genotoxic endpoint. The aims of this prevalidation study are: 1) optimisation and refinement of experimental protocols; 2) generation of standard operating procedures (SOPs) according to GLP; 3) assessment of transferability and reproducibility within and between laboratories; 4) determination of the in vitro vs. in vivo dosimetry relations; 5) Development of a first prediction model that enables predicting in vivo toxicity from in vitro data.

In the presentation the concept of the prevalidation study as well as some preliminary data will be discussed.

Keywords: inhalation toxicity, air/liquid culture technique, prevalidation study

Lecture: skin models as alternatives to animal testing

Follow-up validation of the modified EpiDerm Skin Irritation Test (SIT): results of a multi-centre study of twenty reference test substances

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In April 2007, ECVAM endorsed two alternative *in vitro* test methods (EPISKIN and EpiDerm Skin Irritation Tests (SIT)) as replacements of the *in vivo* rabbit skin irritation test. While EPISKIN was recognized as a stand alone method, the EpiDerm SIT was endorsed for use in a tiered testing strategy, where irritating results are accepted and non-irritating results may require further testing by another method (e.g. QSAR).

Based on results published by Faller and Bracher (*Skin Pharmacol. Appl. Skin Physiol.* 2002, 15, suppl. 1, 74–91), and analysis of the results of the ECVAM validation study, there was evidence that differences in the barrier properties between the two models were responsible for the lower sensitivity of EpiDerm SIT when using an identical protocol as used for EPISKIN. Therefore, modifications of the exposure conditions were introduced to the EpiDerm SIT protocol: a) exposure time was increased from 15 min to 60 min; b) the temperature during the exposure was increased to 37°C. With these modifications, when testing chemicals from the pre-validation and validation studies, a significant increase in sensitivity (84%) was obtained, while maintaining an acceptable specificity of the method.

In autumn 2007, an international multi-centre validation study employing ZEBET (D), BASF (D), IIVS (USA) and ZET(A) was performed to evaluate reproducibility of the modified EpiDerm method. Overall, sensitivity and specificity of 80% were obtained, which is comparable to results for the EPISKIN SIT for the same set of chemicals (sensitivity of 70%, specificity 80%). The inter-laboratory reproducibility of the modified EpiDerm SIT and its concordance with the *in vivo* rabbit data was also very good. The modified method and validation data were submitted to ECVAM for scientific review in April 2008. It is foreseen to be completed in October 2008.

Keywords: human 3D skin model, in vitro skin irritation testing, validation

Lecture: good cell culture practice

New developments in commercial supply of serum-free media

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The culture medium is an essential component of the *in vitro*-environment of cultured cells and should be best designed for the special purposes of the respective cell type.

Serum addition, especially fetal bovine serum, to a chemically defined medium was routine until in the late sixties Sato and others propagated serum-free media. It was thought, that the thorough and complete analysis of the sera's protein components would lead to a unique approach, where all cell types would grow in a defined manner with the addition of a limited number of defined proteins from the serum. But just ten years after, this approach was given up, due to the complexity of the sera's components, which are responsible for all aspects of growth and proliferation of cells *in vitro*.

Since then, the approaches for culturing cell without sera are expanding in some aspects slowly but steadily, especially for some transformed cell lines, for stem cell research, for the production of cell components for therapy and for selected primary cell cultures.

But on the other hand for many individual cell types, the use of serum-free media is difficult and not always straightforward (maintenance of selected cell type, risk of transformation, growth characteristics).

There are some common aspects to consider, when one uses serum-free media:

- 1) Choosing the appropriate chemically defined media, which have to be more enriched than serum-containing media.
- 2) Some defined additional components are widespread used and are available as a combination package: Insulin, transferrin and selenium (ITS-System) and/or insulin, albumin and soya lipids. But these constituents are only useful for a limited number of transformed cell lines.
- 3) For all other cell types, there is a growing number of more or less individual recipes of serum-free media available, which are marketed commonly as a complete media approach. The number of these formulations are increasing steadily and it is not wise to develop own formulations from all yet known components (about 100 known components and an unknown number of additional constituents). Even the known components are not completely well defined and well known to keep in every respect the cells healthy and in a proliferative state.
- 4) In the meantime, however, there are some fifty different complete media already on the market, which can be used directly without adding additional components. Information given by the companies should be comprehensive and concise, and cautions may be appropriate when companies' media are not completely biochemically defined for whatever reasons.

Taking together all reasons and benefits for the use of serum-free media, there are certainly great advantages of this approach for those cell lines, which are designed for the production of cell proteins or especially in the stem cell research, but when cells are just used for basic research, one has to judge very carefully, if serum-free media should be used.

Keywords: commercially available serum-free media, serum substitutes, adaption to serum free media, recommendations for cell lines

Poster: strategies to reduce animal numbers for testing biologicals

Use of *in vivo* bioluminescence imaging for the investigation of bacterial infection courses and heterologous gene expression of bacterial vectors with small groups of mice

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The elucidation of complex bacterial pathogenicity mechanisms and the testing of antimicrobials often necessitates experimental infection of model animals. In addition, the evaluation of novel live attenuated bacterial vectors for vaccination and therapy requires careful analysis of the safety and efficacy profile of the recombinant microorganisms in the mammalian host. The mouse is an important animal model for infectious disease research and testing of biological medicines. The course of bacterial colonization is followed conventionally by plating of organ homogenates from infected animals at consecutive time points. For this approach several animals have to be sacrificed for analysis at each time point. Similarly, large numbers of mice are needed for the conventional determination of inducible gene expression profiles of bacterial vectors for therapeutic factors. Here, we have used *in vivo* bioluminescence imaging in order to follow the colonization course of light-emitting strains of *Escherichia coli* and attenuated *Salmonella enterica* serovar *Typhimurium* in tumor bearing mice. This method allows the analysis of bacterial colonization during the whole infection course in the same small group of living mice. Additionally, we have used this method to investigate the kinetics of induced transgene expression in bacteria residing in tumors of living mice. In both applications, *in vivo* bioluminescence imaging was suited to reduce considerably the number of experimental animals as compared to the conventional approach.

Keywords: in vivo bioluminescence imaging, bacteria, bacterial vectors, inducible gene expression

Poster: free communications

Caco-2 adenocarcinoma on chicken embryos chorioallantoic membrane (CAM) as an alternative to mammalian models for preclinical tests of new cytostatic drug formulations

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In the development of new cytostatic drug formulations targeting plays a prominent role. To test the efficacy of targeting strategies a frequent approach is to use immune-deficient nude mice grafted with tumors. The chicken embryo's chorioallantoic membrane (CAM) is a known alternative to mammalian *in vivo* models (Armstrong et al., 1982; Kunzi-Rapp et al., 2001) and a boarder-case between *in vitro* and *in vivo* model. It combines the advantages of *in vivo* models, that are to allow (simultaneous) investigating activity, biodistribution, pharmacokinetics, biocompatibility and toxicity of the drug and/or drug carrier, with some *in vitro* model advantages, like being quicker, less labor-intensive and pain-causing. However, the CAM model have not yet attracted that much interest in its role as test system for tumor-targeting drug delivery systems. This is possibly due to two major limitations: First, the difficulties to induce well-defined, reproducible damage of the CAM epithelium to enable reproducible tumor invasion and -growth also by moderately or well-differentiated tumor cell lines. Second the cumbersome quantization of tumor cells in contrast to CAM cells.

Caco-2 cell line, clone C2BBel, was chosen as model for colorectal adenocarcinoma with limited tumorigenic potential. Without damaging treatment of the CAM epithelium Caco-2 cells were not able to penetrate into the CAM mesenchym. Mechanical treatment of a small CAM area and the supplementation of the inoculation sample with Matrigel® (BD Biosciences) lead to a reproducible tumor growth. Histological sections of these tumors showed typical growth patterns for colon cancer. In order to quantify the number of tumor cells, the tumors with surrounding CAM were cut, treated mechanically and subsequently enzymatically to produce single cell suspensions. A staining with tumor-specific EpCAM (CD326) antibody allowed distinguishing between tumor- and CAM cells in flow cytometer analysis.

In conclusion, the developed method enables the culturing and quantization of Caco-2 adenocarcinomas on the CAM. Therefore, this model should be a very useful tool to investigate drug delivery systems for cytostatic drugs.

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Keywords: chorioallantoic membrane, CAM, Caco-2, preclinical model, cytostatic drug delivery systems

Poster: strategies to reduce animal numbers for testing biologicals

ARPE-19 retinal pigment epithelia cells as an *in vitro* model for the examination of light induced cell damage in the eye

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Introduction: Photochemical damage to retinal pigment epithelial (RPE) cells and photoreceptors is involved in the pathogenesis of age-related macular degeneration (AMD). Numerous studies have suggested that photochemical damage includes oxidative events by which retinal cells die of apoptosis. Heme oxygenase (HO-1), a stress-responsive protein is induced by a variety of stimuli. HO-1 and its products are also known to contribute to diverse physiological cytoprotective mechanisms against oxidative injury and cellular stress. Transcriptional activation of the HO-1 gene was observed after light damage which is considered as an adaptive response to oxidative and cellular stress. Because ascorbic acid (AA) is known to prevent or delay retinal degenerative processes we investigated the effects of AA on photochemically damaged retinal pigment epithelial cells especially in respect of affecting HO-1 gene expression.

Methods: ARPE-19 cells were plated at a density of 25,000 cells/cm² and maintained in culture for 6-8 weeks for differentiation. Light damage was induced by preincubating the cells with the photodynamic active substance Merocyanin 540 (MC540) for 4h with subsequently illuminating the cells with a light dose between 1-4 J/cm². Uptake of AA in ARPE-19 cells was measured by HPLC-analysis. Gene expression profiles of light damaged cells were assessed by microarray analysis. The expression of HO-1 mRNA was measured by quantitative PCR (qPCR). HO-1 protein expression and PARP cleavage were detected by Western blot analysis. Cell viability was determined by LDH-release and MTT-assay.

Results and Discussion: Light induced cell damage resulted in an upregulation of the oxidative stress defence enzyme, HO-1 in differentiated ARPE-19 cells. As expected, preincubation with AA attenuated significantly light induced cell damage. Not expected was the increase of HO-1 expression more than 2-fold on the protein level in AA-supplemented cells after light-induced cell damage. AA upregulates HO-1 significantly only under conditions in which the light stress response is provoked. The inhibition of HO-1 activity by ZnPPiX showed that the main cytoprotective effect of AA was caused by the induction of HO-1 and not by its role as an antioxidant. The *in vitro* RPE model of light-induced cell damage and the new detected survival pathway induced by AA may be of interest in the understanding of the pathophysiology of retinal degenerations and in exploration of new therapeutic modalities.

Keywords: *ascorbic acid, HO-1, light-induced cell damage, in vitro RPE model*

Poster: nanotoxicology / nanobiotechnology

Nanotechnology and contact allergy

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Nanotechnology is an emerging technique used in the cosmetic and pharmaceutical industry. The benefits of using nanotechnology in skin products include increased delivery of active ingredients to skin, protecting product from degradation, and giving improved cosmetic performance.

However, when active ingredients are formulated in nanoparticles, such as polycaprolactone, this could theoretically increase the sensitization and elicitation potential. Different tests have been developed for investigating the risk of sensitization, and some of these are based on the use of laboratory animals. When using laboratory animals, it is our obligation to use the least straining test for the animal. Traditionally the Guinea Pig Maximization (GMT) test and Buehler test have been used for testing sensitization. The Local Lymph Node Assay (LLNA) has the potential of detecting sensitization with a significant lower strain for the animals, since no injections with adjuvants, as in the GMT, or restraint, as in the Buehler test, are required. Therefore this test was chosen as the panel to test sensitization for nanoparticles, beside the patch test in human volunteers.

Two different types of nanoparticles, used in cosmetics (liposomes and polycaprolactone), were selected for experimental studies in mice (LLNA) and human volunteers (patch test). The nanoparticles were manufactured and loaded with three different contact allergens, being potassium dichromate (hydrophilic), isoeugenol (slightly hydrophilic) and oxazolone (lipophilic).

The liposomes were produced by extrusion through a 50 or 100nm filter. The allergen was placed in the lipophilic or hydrophilic compartment. Extraliposomal allergen was removed by ultrafiltration. Size and stability was measured by light scattering and encapsulation efficiency of the allergen was performed by HPLC. Polycaprolactone was produced by the solvent displacement method. Incorporation of allergen was achieved by dissolving the allergen in either the organic or the water phase. Hereafter, water was evaporated to the desired end-volume. Stability, size and encapsulation efficiency was analysed as for liposomes. Sensitization animal experiments are ongoing and methods and data will be presented.

Keywords: nanoparticles, sensitization, local lymph node assay

Poster: alternatives & education

Training the animal doctor: caring as a clinical skill

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The veterinary profession requires that students are well trained in clinical skills and have a solid basis of theoretical knowledge. Furthermore, the guardians of patients – especially in small animal practice – and society as a whole, expect the veterinarian to treat patients with care and compassion. The concept of care as a central skill is not always emphasised in veterinary education, but a caring approach may better enable the veterinarian to diagnose and treat his or her patients. This presentation discusses the reasons to look upon care and compassion as essential clinical skills to be developed and prioritised within veterinary education. The different teaching tools and approaches that are, in the author's opinion, best suited to develop such skills are discussed, as well as teaching methods that may be counter-productive to these objectives. In particular, the role of animal experiments within education is challenged, and their role in potentially limiting the number of motivated students wanting to enter veterinary education and the profession is discussed.

Keywords: clinical skills, care, veterinary, alternatives, education

Poster: free communications

Assessment of corneal surface impairment and early signs of eye irritation on reconstructed Human Corneal Epithelium

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Background: ESAC (Ecvam Scientific Advisory Committee) has validated in April 2007 two replacement Alternatives to assess the eye irritation: the Bovine Corneal Opacity Permeability (BCOP) and Isolated Chicken Eye (ICE). The 2 methods will formally replace the Draize test for and will be introduced in the Annex V of Dir. 67/548 /EEC of dangerous substances .

Nowadays the need of pharmaceutical and cosmetic industry beside the regulatory requirements is to discriminate between low, very low or even non irritant products, stressing the concept of eye compatibility more than eye irritation. Two main categories of products represent a real challenge: cosmetics that could strongly differ for ingredient's type and concentration, technical form and mode of application and the long terms ophthalmological treatments (i.e. dry eye) involving repeated application without clinical signs as it is the case of preservatives toxicity .

Material and methods: The HCE model-SkinEthic Laboratories (Nice, F) has been used in this study : Human 3D Tissue cultures of Corneal Epithelium may support basic research investigation and they are versatile for the set-up of modified protocols allowing objective and reproducible quantification of complementary testing parameters.

The Multiple Endpoints Analysis (MEA) Protocol, proposed several years ago, that includes complementary parameters (cell viability, morphology, release of soluble factors as IL-8) has been modified by including the transcriptional regulation of a structural component of the tight junctions as early marker of the effects of sub toxic doses linked to infra-clinical reactions. The mRNA expression has been monitored after different exposures by quantitative RT-PCR with Taqman[®] assay. The modified protocol has been applied to study a category of potentially non toxic products as the commercially available tears substitutes. Toxic and non toxic doses of well known eye irritant as BAK (Benzalkonium Chloride) were used as positive controls.

Results: The results reported in this study have shown that this modified procedure of the MEA approach was able to better discriminate between products that seems to be identical after an acute exposure of 24h. By using the modified protocol that includes a post incubation, a better understanding of the interaction with the corneal surface has been possible. Globally by scoring the different parameters a first discrimination has been done between a strong irritant that is responsible of the degradation of the tissue and a low irritant able to allow tissue recovery or finally to distinguish between different levels of low irritation. The reactivity of corneal epithelium has been also modified with specific conditions of hydration and osmolality in order to study its reactive defense mechanism.

Discussion: Thanks to its sensitivity the HCE model seems to be promising in predicting the impairment of corneal surface with adapted toxicological protocols.

Keywords: ocular surface, eye irritation, HCE, BAK

Poster: ethical and legal aspects in animal experimentation

Swiss court bans work on macaque brains

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The Canton of Zurich (Switzerland) is the only place in the world where it is possible (since 1992) to appeal against licences for animal experimentation which have already been accorded by the veterinary office. The possibility is given to the eleven members of the governmental advisory committee on animal experimentation, provided that at least three of them act jointly. In 2006 the Zurich canton's veterinary office approved two experiments on rhesus monkeys, both in the field of neuroinformatics. This was against the advice of the animal experimentation committee and so the committee appealed against the licences. In doing so it at least temporarily inhibited the start of the projects which had also been approved by the Swiss National Science Foundation (SNSF), one of the most important Swiss funding agencies. As a consequence, the researchers concerned, the Swiss Federal Institute of Technology Zürich (ETHZ), the University of Zürich, representatives of the SNSF, politicians and others publicly commented on the almost unbelievable fact that a most important scientific branch should be hindered or even made impossible in this way. In spite of these harsh reactions, in 2007 the local court decided to ban both experiments. The researchers refused to accept this decision and appealed to the next level of jurisdiction, the canton's administrative court. However, in May 2008 they failed a second time. They are now appealing to the country's Supreme Court in a final attempt to be right after all. Let's hope that the Supreme Court supports the decisions of the two lower courts.

It is not for the first time that members of the animal experimentation committee – first and foremost the three official animal welfare delegates – appealed against licenses. But it is for the first time that the majority of the committee members supported this ultimate step and that the commission appealed to court in a body; this gave the legal action more weight and reflected what was called in the press a possible change of paradigm. Furthermore, it was for the first time that the dignity of animals (i.e. the violation of dignity) played an important role in the substantiation of the appeal. This argument was raised in addition to classic arguments judging the pain and suffering of the animals as being too severe and the cost-benefit equation as being against the experiment (Note: The dignity of animals is protected by the Swiss constitution since 1992; the concept of dignity was also adopted in the animal welfare law in the course of its 2005 revision). Finally and most importantly, it was for the first time that the appeal against harmful experimentation on primates – our next relatives – was successful at two levels of jurisdiction. This is a promising indication that a change of paradigm is indeed taking place.

Keywords: primates, dignity, legal procedure

Poster: 7th cosmetics amendment – can all goals be achieved in time?

Dynamic 3D *in vitro* intestinal test system

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Background: For the investigation of the intestinal epithelial barrier regarding its permeability for different test substances highly simplified test systems are used at present. Established 2D Assays based on cell lines and porous membranes reflect the *in vivo* situation not sufficiently yet.

Our aim is therefore the development of a 3D *in vitro* model which represents the physiological barrier of the small intestine epithelium in relation to the blood vessel system. This model provides us an exact classification of different substances regarding their effectiveness and toxicity.

Methods: The scaffold we use for our intestinal test system consists of an acellularised porcine jejunal segment. Currently we reseed the collagen matrix with human primary microvascular endothelial cells (mEC) and luminal segments with primary enterocytes or cells with similar characteristics (e.g. Caco-2 cells). The intestine model is cultivated in a simplified bioreactor system under dynamic conditions (media flow). In preliminary experiments we try to compare the absorption of different substances with the conventional 2D Assay (Insertmembrane) over the permeation coefficient. The evaluation of the epithelial barrier function takes place over immunohistological methods and quantitative high performance liquid chromatography.

Results: Caco-2 cells and endothelial cells could be successfully co-cultivated over a 14-day period on the dynamic system. Exclusively Caco-2 cells which were cultivated under dynamic conditions show typical, high prismatic enterocyte morphology.

Perspectives: Our aim is the establishment of a physiological 3D intestinal *in vitro* test system. This model is to limit animal experiments in the future and make it possible to transfer data from oral bioavailability studies better to humans.

Keywords: *in vitro* test system, small intestine, scaffold

Poster: free communications

Confrontation cultures of embryonic stem cell-derived embryoid bodies and multicellular tumour spheroids: a novel *in vitro* model for the study of tumour-induced angiogenesis

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Background: Vascularization is a prerequisite for tumour growth and metastasis. Therefore anti-angiogenic therapy is one of the most promising strategies to defend against cancer. Consequently a large number of anti-angiogenic agents is currently tested in animal experiments that include the rabbit corneal assay, the mouse cheek pouch assay, and the mouse and rabbit cranial and skin window. Anti-angiogenic substances, such as thalidomide, are nowadays routinely used in several cancer therapies, for example the treatment of multiple myeloma. Preclinical studies of such substances require a large number of animal experiments. Here we introduce an *in vitro* model for anti-angiogenic screening based on co-culturing of embryonic stem cell-derived embryoid bodies and multicellular tumour spheroids, in order to reduce the number of animals needed for *in vivo* testing.

Methods: Embryonic stem cells and tumour cells were cultivated in separate spinner flasks to form 3-dimensional cell aggregates which are called embryoid bodies (EBs) or tumour spheroids respectively. 4 day old EBs were brought in close contact with tumour spheroids using the hanging drop technique. Once the aggregates grew together, they were transferred into bacteriological dishes or plated on gas-permeable cell culture dishes, and cultivated for additional time needed for the experimental setup. Analyses were performed using confocal laser scanning microscopy and the Leica analysis software, as well as flow cytometry and the CellquestPro software.

Results: We observed that blood vessels produced in co-culture are denser than in EBs grown alone, and also show directed growth of vessels towards the tumour. The anti-angiogenic and dose-dependent effect of three substances (thalidomide and the tyrosine kinase inhibitors SU5614 and ZM323881) was shown by immunocytochemistry and flow cytometry, based on staining for PECAM-1 (CD31) and VE-Cadherin (CD144). FACS analyses indicated that the number of CD31+ cells was not significantly changed, but the degree of vascularization was reduced upon treatment with anti-angiogenic agents. It is also shown that tyrosine kinase inhibitors have anti-inflammatory properties besides being anti-angiogenic, which was visualised by staining for leucocyte specific markers CD45, CD68 and neutrophil antigen. Preventing inflammation is of importance, since it often occurs next to the tumour, thus inducing a pro-angiogenic micro-environment.

Discussion: The EB derived from embryonic stem cells mimics early stages of embryogenesis, facilitating research on various differentiation processes of all three germ layers. This system combined with multicellular tumour spheroids simulates interactions between healthy and pathological tissue, thus mimicking the process of tumour-induced angiogenesis. In summary we demonstrate that confrontation cultures of EBs and multicellular tumour spheroids are suitable to replace or reduce the number of animal *in vivo* studies, e.g. the rabbit cornea assay, the hamster cheek pouch assay, the cranial window assay or the skin chamber assay. Our novel *in vitro* confrontation culture model can be used for screenings of various substances as well as for examinations of signalling pathways involved in host tissue-tumour interaction.

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Keywords: angiogenesis, confrontation culture, embryoid bodies, multicellular tumour-spheroids

Poster: computer assisted procedures

New animal model for objective pain research: non-invasive functional imaging in anesthetized animals by BOLD fMRI to study initial processes of chronic pain

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The experience of acute pain is an elementary sensation necessary for maintaining individual integrity and well-being in interaction with the environment. However, repetitive noxious input can induce chronic pain states without any biological advantage. Long-term changes in the excitability of neurons have been shown at the peripheral and spinal cord levels. Maladaptive supraspinal reorganization is thought to play an important role in central sensitization, reconfiguring the central pain-processing matrix through learning, extinction, and memory processes. But it is a major challenge to investigate cerebral mechanisms and structures that contribute to sensitization of pain.

Traditional behavioural pain examinations in animals are highly stressful and subjective. Contributing to the 3R's non-invasive imaging approaches like fMRI in anesthetized animals would significantly reduce the stress for animals and simultaneously refine and improve objective measurements of chronic pain. Moreover, a model which would allow the investigation of chronic pain processes would open a new avenue in pain research.

Having established a model for acute heat pain in fully anesthetized animals we now seek to identify brain areas that may be involved in initial processes of chronic pain using an experimental model of repetitive pain exposure in healthy rats which results in reliable and quantifiable BOLD responses in pain related brain areas.

fMRI data were acquired in slightly anesthetized rats with mild noxious heat stimulation (max. 48°C, 12 repetitions over 1h) every second day over 6 days. Highly specific activity of the pain pathway was found (e. g. thalamus, primary and secondary somatosensory cortex, cingulate cortex, insular cortex, frontal cortex and parietal cortex). The comparison of fMRI data of the first *versus* the last stimulation indicated increased activation in terms of increased stimulus coupling in cingulate cortex, entorhinal cortex and hippocampus. This finding nicely compares to a human study (Valet et al., 2006) suggesting that in these structures first processes of pain chronification take place. Interestingly, no significant increases for response amplitudes in these structures could be found. Structures reported to be involved in pronounced chronic pain like parietal cortex and structures of the medial prefrontal cortex (Baliki et al., 2006) also showed increased stimulus coupling between the first *versus* last session. These results could neither be found comparing the first versus earlier days nor during innocuous heat stimulation. Moreover, optimized data-analysis strategies reduced the number of experiments needed to obtain statistically significant results.

In conclusion, our topical heat stimulation of the rat hind paw is a robust paradigm leading to reliable BOLD signals well suited for repetitive stimulations. This non-invasive animal pain model at minimal animal stress level due to the anaesthesia of the animal is highly objective and well qualified for studying chronification of pain responses.

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Keywords: non-invasive imaging, fMRI, heat, rat, image processing, chronic pain

Lecture: free communications

Improved toxicity prediction for safety assessment of drugs during preclinical drug development using relevant hepatic and cardiac cell lines

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Preclinical drug testing for efficacy and safety represents a lag phase in drug development where most time is spend. With strict regulations and exigent requirements from the regulatory authorities such as the FDA and EMEA, it is not only a challenge but sometimes a problem for the pharmaceutical industry which has to kill thousands of potential drug molecules due to safety concerns.

Lately huge progress has been achieved for the safety issues and side effects monitoring and understanding of toxicity mechanisms of drugs. However, the methods of safety assessment have been lagging behind. This has led to an urgent need to improve the preclinical testing so as to avoid late drug attrition and clinical trial failures where most of pharmaceutical costs are concentrated. In addition, post-marketing withdrawal due to side effects is becoming a nightmare for the pharmaceutical industry. This is despite the extensive use of animal test systems which shows that these systems have poor clinical relevance.

Better cell based assays incorporating human relevant cells such as the hESC derived cells as well as innovative non invasive assays in an integrated approach hold a great promise for the improvement of toxicity prediction. We present here a novel integrated approach of a kinetic respiration assay (Deshpande et al., 2005) and the techniques of metabolome and flux analysis (Gouder et al., 2006) for toxicity assessment of drugs and NCEs (new chemical entities). We are applying these techniques to reference cell lines namely Hep G2 and HL-1 but these techniques are applicable to more human relevant cells that is the human embryonic stem cell derived hepatocytes and cardiomyocytes within our European projects namely; Vitrocellomics and Invitroheart.

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Keywords: preclinical drug development, toxicity screening, kinetic studies, metabolome, flux analysis

Poster: skin models as alternatives to animal testing

***In vitro* micronucleus assay using a co-culture system: towards new tools for *in vitro* risk assessment of dermally applied compounds?**

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In vitro reconstructed skin models such as Episkin® (reconstructed epidermis) and Realskin (reconstructed full thickness: epidermis + living dermis) are biological models mimicking human skin. They are of growing interest in safety or efficacy pre-screening tests and for regulatory purposes as alternatives to animal testing (7th amendment to the European Cosmetic directive, REACH). The reduction and eventually the replacement of *in vivo* toxicity testing require the development of new complementary biological models and methods with improved ability to predict the genotoxic or other endpoint risk with *in vitro* data. This can be achieved if these new assays take into account the exposure conditions in a more relevant way than the current ones. To that end, new approaches using human reconstructed skin models for *in vitro* toxicology assessment are proposed.

The skin is the target organ for dermally exposed compounds or environmental stressors. A co-culture system using Episkin® or RealSkin and target cells to perform a regular micronucleus assay is used with six different compounds. This way of using human reconstructed skin for genotoxicity testing aims at improving the relevance of exposure conditions in *in vitro* genotoxicity assays for dermally applied compounds. The skin is indeed a biologically active barrier driving the exposure to compounds and their possible metabolites. The exposure of the target cells to a given substance can be assessed after topical application as was the case here. Episkin® and Realskin were used as a metabolically active tissue and a physiologic barrier. The test compound can be metabolized by the skin and/or by the target cells ($\pm$ S9 if needed).

Metabolism is an important event to consider in genotoxicity and skin sensitization evaluation. Compared to cell models, a broad variety of chemicals with different physico-chemical features can be evaluated in this system (after topical or systemic application): compounds with different pH, physical state (liquids, gels, solids, formulations).

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Keywords: *reconstructed skin models, micronucleus assay, genotoxicity, co-culture, topical route*

Lecture: 7th cosmetics amendment – can all goals be achieved in time?

The incorporation of 3R alternatives in the safety evaluation of cosmetic ingredients by the SCC(NF)P

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Although animal tests are commonly used by regulatory bodies as the basis for hazard and safety assessments of all types of chemicals, the upcoming testing and marketing ban in the cosmetic field legally imposes urgent substitution for these animal studies by replacement alternatives. At the EU level, the Scientific Committee on Consumer Products or SCCP (formerly called SCCNFP) assesses on a regular basis colourants, preservatives, UV filters, hair dyes and other specific cosmetic ingredients for which suspicion of potential toxicity exists. Therefore it appeared useful to investigate the incorporation of 3R alternatives in the dossiers submitted to this European scientific committee.

To achieve this, we programmed a database in which the contents of 185 SCC(NF)P opinions were systematically loaded and which allowed to search for the occurrence of alternative methods and to perform animal counts.

A first set of results reveals that, when validated, 3R alternative methods appear to smoothly find their way in the submissions to the SCC(NF)P. Examples are the gradual incorporation of the fixed dose procedure, the toxic class method and the up and down procedure in the field of acute toxicity, the introduction of reduction and refinement measures in existing animal protocols in the areas of skin and eye irritation, the transition to the local lymph node assay for skin sensitisation, the standard use of the in vitro mutagenicity/genotoxicity testing battery and the regular occurrence of the 3T3 neutral red uptake phototoxicity test. A subsequent post-validation study on the available data sets, however, showed that several methods still required optimization and further development (e.g. mutagenicity/genotoxicity, skin irritation).

For the major animal-consuming endpoints, namely repeated dose toxicity and reproductive toxicity, as well as for toxicokinetics and carcinogenicity, the studies encountered in the SCCP dossiers appeared to be the classical animal assays as performed for decades. Unfortunately, no replacement alternatives are in the pipeline for these endpoints.

In a separate step, the database was used to compute the numbers of animals that were involved in the data generation for the dossiers submitted to the SCC(NF)P. In a worst case calculation, 21,000 animals are estimated to be used per year.

It is clear that the deadlines of 2009 and 2013 will not be met with regard to the timely development of replacement alternatives for all toxicological endpoints. The SCCP, the Scientific Committee dealing with the safety assessment of cosmetic ingredients at the European level, to date still mainly uses results from animal studies for its safety assessments, although it clearly welcomes the use of alternatives once validated (see also SCCP/1111/07).

Therefore further efforts need to be focused on development, validation, post-validation and more than anything on trust-building in alternative methods. Meanwhile the cosmetic world remains caught in a deadlock created by political influences and unsolvable by the scientific community.

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Keywords: cosmetics, alternative methods, SCCP, SCCNFP, risk assessment, safety evaluation

Poster: Ethical and legal aspects in animal experimentation

The Mahatma Gandhi Centre for Alternatives to the use of animals in life sciences

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The Mahatma Gandhi Centre for Alternatives to the Use of Animals in Life Sciences has been proposed as a national center for alternatives to the Government of India, with the mandate to promote the quality and excellence in life science education/research by way of introducing value based learning systems that will promote “humane science”. The centre will endeavor to blend the Gandhian philosophy of Ahimsa in life science education/research and promote the use of alternatives to the use of animals in research and education. This center will be a unique insignia of the Gandhism where in the psycho-social concept of Ahimsa will be introduced to redefine, reform and revolutionize the teaching/research in life sciences in India. As a national body, it will promote this philosophy by promoting the “The 3Rs”- in the use of animals in education, research and testing and it will also promote the concept of the 4Th R – “rehabilitation” of laboratory animals.

Central to Mahatma Gandhiji's vision was an impassioned conviction that at the heart of all life there is ‘Truth’ which sustains all creation; a ‘Truth’ which demands a personal response from each individual. He saw this ‘Truth’ as a truth present in every person. In particular, he held nonviolence as a basic tenet of this ‘truth’, a positive force that can bring about fundamental change at all levels. For Gandhiji ‘Nonviolence’ was the discovery of a new kind of power and taught this philosophy which has universal applicability. The core of that philosophy is the search for truth through nonviolence or Ahimsa. Gandhiji taught respect for animals as well as humans, a non-exploitative relationship to the environment and nonviolence applied at all levels of relationships, be it man to man, man and animal or be it man and environment.

It has been recognized that the teaching of the philosophy of non-violence is an imperative a psycho-social need of the hour and the proposal has been accepted in principle by the University Grants Commission, Government of India for implementation.

Keywords: Mahatma Gandhi, National Center, Ahimsa, India

Lecture: nanotoxicology / nanobiotechnology

Nanotoxicology and alternative testing methods – development, chances and needs on OECD and EU level

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Manufactured nanomaterials are intentionally produced to have specific properties or specific composition, a size range typically between 1nm and 100 nm and material which is either a nano-object or is nanostructured (OECD, 2008a). They are to a great extent products of the chemicals industry which by definition should be regulated by chemicals legislation. Chemicals legislation, however, was originally developed and designed to regulate macroscopic substances (Fischer and Hirmann, 2007). The current developments in nano-technology and insights in related hazard effects may cause the need for additional information on the substances in the future. The current state of development is not mature enough to include guidance on the identification of substances in the nanoform in the Technical Guidance Documents (TGD) for REACH (European Chemicals Agency, 2007). Toxic effects of nanomaterials tested to date show that they may be more toxic than micron sized particles of the same material (Hallock et al., 2008).

In order to address the specific properties, hazards and risks associated with nanomaterials, additional testing or information may be required. To determine specific hazards associated with nanomaterials, current test guidelines may need to be modified. Until specific test guidelines for nanomaterials exist, testing will have to be carried out according to already existing guidelines (SEC 2008 2036). Currently, testing of a set of 14 nanomaterials is being planned by the OECD in order to develop appropriate testing guidelines, taking into account the specific properties of nanomaterials. A steering group has been formed to work on alternative testing methods, evaluating *in vitro* and other methods, or validating them. It will identify additional endpoints that should be considered by the testing programme, and will give consideration to other alternative approaches and the broader issue of integrated testing strategies (OECD, 2008b).

This lecture will give an overview of the activities of the Working Party on Manufactured Nanomaterials (WPMN) of the OECD, as well as activities on EU level, and introduce the OECD Database on Safety Research and call for data entry. The role of the Umweltbundesamt will also be explained briefly.

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Keywords: *manufactured nanomaterials, regulatory testing, specific properties, hazard assessment, OECD, EU, Umweltbundesamt*

Poster: free communications

The use of histopathology to improve the predictive capacity of the Bovine Corneal Opacity and Permeability (BCOP) assay

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Introduction

The BCOP assay has gained widespread usage since it was first described in its current form by Gautheron, *et al.* in 1992. The assay combines two mechanistic endpoints – corneal opacity and epithelial permeability – to create an In Vitro Score that is then used to predict ocular irritation potential. However not all eye irritants are detected by these two endpoints, e.g. several strongly irritating materials used in the European Commission/British Home Office study (EC/HO study) did not induce a commensurately high In Vitro Score in the BCOP. Subsequently we were able to show that these underpredicted materials did induce damage that was detectable using histopathology.

Rationale

Although the BCOP assay utilizes “living” (freshly excised; still viable) corneas, we hypothesized that some of the damage seen in *in vivo* treatments could be a consequence of inflammation subsequent to the initial injury. This type of irritation would most likely not be seen in an excised tissue since there would be no opportunity for the recruitment of inflammatory cells from outside the cornea. Jester *et al.* (1998) showed that the depth and area of injury occurring immediately after the initial ocular exposure to irritants was generally predictive of the extent of the final injury and especially of the potential recovery. Therefore we began studies to determine if there was damage to the epithelial, stromal, or endothelial layers of the excised cornea which were not being expressed either in the opacity or permeability scores.

Results

Initially we assessed the results of the EC/HO study on ocular irritation. Although the BCOP assay was arguably the best performing of all the *in vitro* assays, there were still several materials that were under predicted, e.g. parafluoroaniline, quinacrine, and sodium oxalate. When we retested these materials and added a histopathological examination of the corneas, we found that all three induced damage that could be observed microscopically.

Further studies over the next five years revealed additional cases where histopathology of the bovine corneas was important in predicting eye irritation. As a result, the ICCVAM review of “In Vitro Test Methods for Identifying Ocular Corrosives and Severe Irritants”, suggested creating 1) a reference atlas of histopathology for chemically-induced ocular lesions and 2) a standardized scoring scheme to be used in conjunction with the histopathology atlas to identify decision criteria that could be used in hazard classification.

We have now developed an atlas of ocular lesions observed *in vitro* and have convened a meeting of ocular histopathology experts to standardize the evaluation and lesion nomenclature for bovine corneas. Examples from this atlas and a summary of the histopathology meeting will be presented.

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Keywords: eye irritation, ocular irritation, BCOP, *in vitro* testing

Lecture: good cell culture practice

Alternatives to the use of fetal bovine serum: platelet lysates as serum replacement in cell and tissue culture

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Fetal bovine serum (FBS) is commonly used as essential supplement to cell culture media. FBS is a cocktail of most of the factors required for cell attachment, growth, and proliferation *in vitro* and is thus used as an almost universal growth supplement effective with most types of human and animal cells. However, the use of animal serum also bears a number of disadvantages. These can either be seen from (1) a theoretical, cell biological point of view, since serum in general is an ill-defined, heterogeneous component in culture media, (2) from ethical perspectives in terms of animal protection arguments about harvest and collection of FBS from bovine fetuses, and (3) in terms of recent concerns about the global supply vs. demand of FBS. It is estimated that about 500,000 litres FBS are produced per year for the world market. This means, that more than 1,000,000 bovine fetuses have to be harvested, and it is expected, that these numbers will continue to increase annually. As a consequence, a number of strategies have been developed to reduce or replace the requirement for FBS in cell culture media. It is a well known fact, that natural clot serum rather than plasma promotes the growth and proliferation of cultured cells. This appears to be due to the release of VEGF, β various mitogenic growth factors (GR), like EGF, IGF-1, PDGF, FGF, TGF- etc., from activated platelets. We recently explored the growth promoting and mitogenic potential of human platelet lysates (PL) on cultured mammalian cells, and we have shown, that PL can serve as valuable alternatives to the use of FBS in cell and tissue culture (Rauch et al., 2007). In the present study, the repertoire of adherent epithelial cell lines tested was extended to anchorage-independent Raji human lymphoma cells. PL fully supported growth and proliferation of Raji cells in suspension. We further determined the actual amount of GR in different PL batches by ELISA and ascertained the optimal steps in GR enrichment during the PL extraction process. In addition to GR, the amount of hydrocortison and of prostaglandin E₂, acting as synergistic/additive catabolic stimulators in chemically-defined culture media, was found to be in sufficient quantities. In order to biochemically determine the proliferative potential of PL, the stimulation of extracellular signal-regulated MAP kinase (ERK1/2) was determined. Activation of the MAP kinase signaling pathway by GR results in specific phosphorylation of downstream kinases, like ERK1/2. Addition of PL to quiescent LLC-PK1 cultures resulted in specific phosphorylation, and thus activation, of ERK1/2 within minutes. This time course is identical with ERK1/2 activation upon addition of FBS. The data further confirm the high potential of PL as valuable substitute of FBS in mammalian cell and tissue culture.

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Rauch et al. (2007). *ALTEX* 24, 353.

Keywords: fetal bovine serum, human platelets, thrombocytes, cell and tissue culture, alternatives, 3R

Poster: 7th cosmetics amendment – can all goals be achieved in time?

The comet assay in the Phenion® full thickness model as a putative tool for improved *in vitro* genotoxicity testing

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Genotoxicity is one of the first toxicological endpoints to be tested within the course of safety assessment e.g. for cosmetic ingredients, pharmaceuticals, or agrochemicals. Regulation requires that cosmetic ingredients are subjected to an *in vitro* test battery first. The currently accepted *in vitro* tests exhibit high sensitivity but unfortunately low specificity. Therefore, *in vivo* tests are often needed to clarify the *in vitro* data. However, due to the 7th Amendment to the European Cosmetics Directive *in vivo* tests for several toxicological endpoints, e.g. genotoxicity, will be banned starting in 2009. In addition, the new EU regulation on chemicals REACH requires safety tests for nearly 30,000 cosmetic or other raw materials during the next decades which is expected to trigger high numbers of animal testing.

Therefore, it is a challenging task to develop new alternative methods which have a higher biological relevance than the already validated genotoxicity methods in order to supplement existing test batteries and to finally replace current *in vivo* genotoxicity assays.

The high rate of false positive results of the currently used *in vitro* tests might be caused by a wide-spread use of cell lines often of non-human origin which are partly transformed and which lack normal metabolism. To overcome these limitations we established a Comet Assay with the Phenion® Full Thickness Skin Model. Thus, the skin as the first site of contact for most cosmetics and many environmental stimuli and as the organ with maximum exposure is introduced into *in vitro* genotoxicity testing. The tissue enables realistic application of test substances and by the use of primary human cells an organ- and species-specific metabolism is provided.

The Comet Assay as a widely used and scientifically well accepted method was chosen because it detects several types of permanent or transient DNA damages and allows the analysis of both keratinocytes and fibroblast. Recommendations for methodological standards of international expert groups are available and adopted for the presented protocol.

Several direct acting mutagens (Methylmethane Sulfonate, 4-Nitroquinolineoxide) or pro-mutagens (e.g. Benzopyrene) in different concentrations were applied topically to the tissues. After defined time points keratinocytes or fibroblast respectively were isolated and analyzed separately regarding the fluorescence intensity in the tail. The tested compounds induced a dose-dependent increase of %tail DNA.

In conclusion, this model represents a system close to the *in vivo* situation and allows analyzing the *in vivo* relevance of *in vitro* positive substances in more detail.

Keywords: comet-assay, genotoxicity, 3D skin model

Poster: computer assisted procedures

Implementation of *in silico* models in the cosmetic industry to face the 7th Amendment of the European Cosmetics Directive

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The 7th Amendment to the EU Cosmetics Directive has made *in vitro* and *in silico* approaches to warrant the safety of products a key issue to support regulatory dossiers and future innovation. A ban on animal testing for chemicals to be used in cosmetics comes into effect in the EU in March 2009 for acute toxicity, genotoxicity, and skin or eye irritation. For repeat-dose (or systemic exposure) toxicity, the EU ban is subject to the 2013 deadline.

Over the past years there have been considerable efforts to develop alternative methods, including *in silico* methods that would comply with regulatory constraints (OECD principles). In this context, transparent and mechanism-based approaches are needed. Progress in the area of computer science and information technology has triggered the creation of curated databases and the development of predictive models for various toxicological endpoints.

A number of commercial or free software integrate global models targeting the 2009 endpoints. From the cosmetic industry perspective, the predictive performance of such models has to be evaluated on chemical series of interest for:

- Chemical prioritization
- Mechanistic understanding
- Elaboration of regulatory dossiers by providing additional information

The purpose of the present study was to use data generated on “real-life” chemicals from the cosmetic industry to challenge some of the global models available today.

Historical in-house data obtained from the *in vivo* skin irritation, bacterial mutagenicity and *in vivo* oral acute toxicity tests, have been curated in collaboration with in-house experts for this purpose. Global models available in the Derek, MC4PC and TIMES software were evaluated for mutagenicity (Derek, MC4PC and TIMES), acute toxicity (Derek, MC4PC) and skin irritation (only Derek since ToxTree will be evaluated in the next phase of this project). Applicability domains and predictive performance were compared in order to identify potential gaps and assess the complementarities of these tools and their relevance in Integrated Testing Strategy (ITS) workflows.

Keywords: silico, model, prediction, human health, cosmetic

Lecture: alternative testing methods for toxicity to reproduction

The three dimensional human neurosphere model identifies developmental neurotoxicants

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Current developmental neurotoxicity (DNT) testing guidelines propose investigations in rodents, which require huge amounts of animals. With regard to the 3Rs and the European Regulation of Chemicals (REACH), alternative testing strategies are needed, which refine and reduce animal experiments by allowing faster and cheaper screening.

We have established a 3D test system for DNT screening based on primary human fetal neural progenitor cells which is now embedded in the German BMBF joint project “Development of predictive *in vitro* test for developmental neurotoxicity testing”.

Within this project, different cell models are compared with regard to their DNT pre-dictability employing a set of reference compounds with different DNT potentials.

In our system first results indicate that the well known developmental neurotoxicant methylmercury effects proliferation, migration and differentiation of neurospheres in a nanomolar range, while a negative test substance, the liver toxicant paracetamol, showed interference with these processes in millimolar concentrations.

More test compounds are investigated momentarily and will give us information on the validity of the system. To allow high-throughput screening for measuring proliferation and differentiation, we have developed automated computational systems for image analyses. Macros written in the program Metamorph (Universal Imaging Corp.) allow automated analyses of gain in neurosphere size over time as one measure for proliferation, as well as pixel recognition for nuclei, neuron, astrocyte and oligodendrocyte immunocytochemical stainings as measures for differentiation.

Taken together, we have established the human neurosphere model as a system-based *in vitro* test method for elucidating the potential of chemicals to disturb human brain development. Testing more chemicals will give us an answer on the predictability of our test system.

Keywords: neurosphere, methylmercury, proliferation, differentiation, migration

Lecture: strategies to reduce animal numbers for testing biologicals

Analysis of potential toxicity caused by genetically modified plants: a combination of *in vitro* methods as an alternative to feeding studies

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The approval of genetically modified plants (GMP) as food or animal feed is a major issue especially with regard to potential effects on human and animal health. While the admittance of drugs has to follow a strongly regulated process involving a variety of preclinical and clinical assays and tests, the rules for the approval of GMPs are in part still to be determined. In this study we have fed GMPs to rats according to OECD guideline 407. In addition, we have established a combination of *in vitro* assays using *ussing* chambers equipped with small intestine of pigs (slaughterhouse material) to simulate intestinal transport from mucosal to serosal side and hepatocyte cultures to assay for potential effects on hepatocellular metabolism and toxicity. We analyzed the synthesis of albumin as a marker of blood synthesis and anabolic function of the liver as well as the synthesis of urea as an example for a catabolic process in the liver. Several different GMPs were assayed *in vivo* and *in vitro*. For the *in vitro* assay, lyophilized potato tubers were dissolved in phosphate buffered saline and applied to the mucosal side of the transporting tissue. After 6 hours transport processes were stopped and probes from the serosal as well as the mucosal site were assayed in hepatocyte cultures derived from human, monkey, dog and rat. Dog and monkey hepatocytes were isolated from animals that either served as controls in animal experiments or were mercy killed due to terminal illness.

There was no indication, that the GMPs used in feeding studies influence the health or behaviour of the animals. Moreover, despite the fact that recombinant expressed protein was transported from the mucosal to the serosal side none of the GMPs did have any toxic effect on hepatocytes of the different species and, in addition, hepatocellular function as detected by albumin and urea release was not altered by GMPs in any of the species tested. The similarity of the *in-vivo* and the *in-vitro* results strengthens the assumption that, the combination of these two *in vitro* methods can be used as an alternative to animal feeding studies in order to determine potential toxicity of GMPs and their effect on liver metabolism. Of course further evaluation of the *in-vitro* systems by substances able to serve as positive controls has to be performed.

Keywords: *hepatocyte cultures, ussing chamber, genetically modified plants, toxicity, metabolism, transport*

Lecture: nanotoxicology / nanobiotechnology

Animal experiments and non-animal methods in nanomedicine and nanotechnology – the results of a critical literature survey from the point of view of animal welfare

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In the last years, nanotechnology and nanomedicine (the application of nanotechnology in the health care area) have evolved as new, important areas of research receiving increasingly more political attention and public funding. Nanotechnology involves investigations and technological developments on the scale of 1-100 nanometres, with one nanometre being one millionth of a millimetre. Due to their specific physical and chemical characteristics, substances and products with nanoscale sizes or nanoscale surface structures are being developed and produced for innovations in energy, environmental, or information technologies and in the healthcare area. Thus nanomedicine and the safety testing of nanomaterials and nanoproductions are new scientific areas potentially involving animal experiments. Considering the significance assigned to nanotechnology, a continuous increase in animal experiments in these areas over the coming years seems likely unless effective preventive measures are put in force.

It is against this background that the literature survey, funded by the Swiss Foundation Animalfree Research, provides an overview on animal experiments performed in nanotechnology aiming at addressing main areas of concern from the point of view of animal welfare, such as scientific areas using especially large numbers of animals or specific especially distressful animal experiments.

Scientific articles from Germany, France, the United Kingdom, Italy, the Netherlands, and Switzerland published between 2004 and 2007 form the basis of the literature survey. These are the European Countries with both the highest public expenditure in nanotechnology and high numbers of animals used in scientific research as such. In nanomedicine and nanotechnology, animal experiments were performed in pursuance of scientific goals relating to nanoparticle-based targeted drug, vaccine or gene delivery, nanoscale imaging technologies, magnetic-nanoparticle induced tumour thermotherapy, tissue engineering, and the toxicity of nanomaterials.

Examples for such experiments shall be presented, the harm inflicted upon the animals classified, and the scientific outcome of the experiments evaluated. Thereupon, a harm-benefit analysis of the experiments shall be performed. Likewise, a brief overview will reveal how the application of nanotechnology in cell culture technology provides new options and new realms for *in vitro* research. The indispensability and ethical acceptability of animal use in nanomedicine and nanotechnology will be discussed.

Subsequently, concrete recommendations for necessary political action shall be made addressed at the European Commission and at European Countries, aiming at avoiding an increase in animal use for nanotechnology and nanomedicine and at achieving a replacement of the animal tests depicted, in accordance with legal provisions, such as the Animal Welfare Protocol of the European Treaty and Directive 86/609/EEC on the protection of laboratory animals.

From the point of view of animal welfare, the time is right for a paradigm change in fundamental biomedical research, not least because of the new methodological options that nanotechnology offers in the realms of *in vitro* research, for example by enabling the evaluation of biochemical processes on the level of the single cell. It is time for a paradigm change that designs completely new research strategies that do away with animal experimentation altogether and founds scientific progress on non-animal testing strategies instead.

Keywords: nanotechnology, nanomedicine, 3Rs principle, cost-benefit analysis, bioethics

Lecture: free communication

Regulatory animal testing: factors stimulating or obstructing the adoption of 3R methods in the regulatory process

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Thirty percent of the animal tests conducted annually in Europe are performed to meet regulatory requirements. Regulatory animal testing is often repetitive in nature and more likely to cause severe suffering due to the procedures used. It proves to be a persistent element in the assessment procedures for registering a substance or product for release onto the market.

Over the last decades the heavy reliance on animal experimentation in this area meets serious objections, both ethical and economical in nature. And even though the number of test-models based on the 3R principle keeps increasing, these new methods are not automatically included in the regulatory decision process. There are several obstacles on the way from test validation to implementation, for example:

- Ongoing scientific discussions about the data coming from the validation studies
- The lengthy process in acceptance of a validated test by regulatory agencies
- Parallel existence of the validated and accepted 3Rs method and the conventional animal test in test regulations

Part of the underlying reasons are scientific of nature. However, there are several political and social reasons that hinder effective test implementation. This overview will offer some insight of the factors and actors influencing the implementation of 3Rs methods in regulatory testing. It aims at being a starting point for further discussion and focused actions to tackle the barriers on the way to regulatory acceptance of 3R methods.

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Keywords: regulatory animal testing, registration, regulatory requirements, 3Rs, validation, alternatives, stakeholders, stream

Poster: alternative testing methods for toxicity to reproduction

The human dopaminergic neuronal cell line LUHMES as *in vitro* model for Parkinson's disease

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Background: Parkinson's disease is characterized by a gradual degeneration of dopaminergic neurons in the substantia nigra. Dopaminergic neurons are continuously exposed to elevated oxidative stress conditions due to the unstable neurotransmitter dopamine that can easily undergo oxidation to form superoxide and a quinone-form capable to react with cysteine residues in proteins or with glutathione to form dopamine-conjugates. For investigations on the molecular events occurring under these conditions, as well as for the validation of potential pharmacological interventions, an experimental human *in vitro* model that closely resembles the characteristics of dopaminergic neurons *in vivo* is desired. This *in vitro* model would not only allow to significantly reduce the use of *in vivo* animal testing, but concomitantly would have the advantages to allow studies on molecular events occurring in neurodegenerative disorders in a human system.

Materials and Methods: LUHMES are human mesencephalic cells conditionally immortalized with a v-myc retroviral vector. In this system, tetracycline shuts down v-myc expression and allows differentiation into dopaminergic neurons. Tetracycline in combination with dbcAMP and GDNF (glial cell-derived neurotrophic factor) leads to a differentiation into dopaminergic cells within 3-4 days, as shown by the expression of specific markers such as tyrosine hydroxylase or dopamine transporter (DAT).

Results: LUHMES cells were validated with respect to their response toward the parkinsonian toxins MPP⁺ and methamphetamine (METH)/Fe²⁺. In both cases, a time-dependent degradation of neurites, accompanied by a loss of cellular ATP and GSH, and increased formation of radical species was observed. These effects were only detected in fully differentiated cells, whereas undifferentiated LUHMES demonstrated no significant response to the same toxic insult. The neurodegenerative effects observed were partially prevented or delayed by co-incubation with the mixed lineage kinase inhibitor CEP1347, or by inhibition of poly-ADP-ribose polymerase (PARP). The involvement of dopamine in the neurodegenerative process was further underlined by application of dopamine transporter, or tyrosine hydroxylase inhibitors that significantly protected against MPP⁺-induced degeneration.

Discussion: Parkinson's disease and other neurodegenerative disorders such as Alzheimer's disease or multiple sclerosis are one of the most challenging health issues in the aging populations of developed countries. It can therefore be assumed that efforts of the pharmaceutical industry within this field will increase dramatically within the next decades. These research projects will create a massive rise in the demand for reliable and representative test systems. The most widely used model for this purpose however is still the laboratory animal. The herein introduced human neuronal cell line closely reflects the unique properties of dopaminergic cells *in vivo*. This model can not only serve for basic research on the events occurring in neurodegenerative diseases, but can also be used as a screening system within neurotoxicological testing programs.

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Keywords: *in vitro* Parkinson model, neurodegeneration, dopamine, MPP⁺, cytotoxicity

Poster: 7th cosmetics amendment – can all goals be achieved in time?

Current activities of the foundation Animalfree Research (AfR), formerly Fonds für versuchstierfreie Forschung (FFVFF)

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The foundation Animal-Free Research (AfR) formerly known as Fonds für versuchstierfreie Forschung (FFVFF) was founded in Zurich in 1976 and has since then relentlessly pursued its activities in the spirit of the 3R's. In 2007 and 2008, AfR has partially or completely funded projects in the field of education about alternative methods, meta-analyses dealing with transgenic animals and with nanotechnology, supported the establishment of an *in vitro* co-culture model for investigating the effects of nanoparticles on lung cells and hosted and published a forum discussion on the ethical and legal implications of animal experiments. Additionally, it has repeatedly commented on the current revision of the Swiss animal welfare act.

In the field of education, AfR is a constant and regular supporter of the non-profit organization InterNiche (www.interniche.org). InterNiche (International Network for Humane Education) specializes in replacing animal experiments in higher education. It organizes info tours in universities worldwide, donates secondhand computers to universities in Latin America, Russia and Eastern Europe and provides free data on alternative methods (CDs, Videos, models) as well as technical support. In 2007 and 2008 AfR supports InterNiche's expansion to Latin America. For instance it funded the production costs of a book on alternative methods written in Portuguese ("Instrumento animal: O uso prejudicial de animais no ensino superior"). The book was released successfully and a translation into English and Spanish is planned. The book "From guinea pig to computer mouse" published by InterNiche (2nd Edition 2003) is updated regularly with the help of AfR.

AfR has funded a meta-analysis on the use of transgenic animals in high blood pressure research and its implementation into clinical therapy. The results indicate a very limited applicability of 20 years of animal experimentation in this particular field. The publication of the results in *ALTEX* is expected soon.

In 2008, AfR supports another metaanalysis aiming to provide information about number, type and severity of the animal experiments conducted in the emerging field of nanotechnology: "Comparative assessment of the scientific goals and results of medical and toxicological animal experiments with nanomaterials and of the harm inflicted upon the animals therein", which started in February 2008 and is expected to be complete in September 2008.

With the financial help of AfR, a project entitled "Development of a particle exposure system to investigate the inflammation and toxicity potential of nanoparticles in an epithelial airway barrier model" was able to develop a very sensitive triple-cell co-culture system employing epithelial cells and immune cells in order to study the interactions of synthetic nanoparticles with the lung and to better understand the potential risk for human health.

In November 2007, AfR hosted a discussion forum focussing on the ethical and legal aspects of the conflict of interests between human health and animal wellbeing. A reader of the contributions has been edited (*Gesundheit und Tierschutz – Güterabwägung bei Tierversuchen*) and can be ordered at info@animalfree-research.org.

AfR would like to introduce new members in its team: Sabine Umbricht, business executive, Dr. Stefanie Schindler, research assistant, and secretary Daniela Imhasly.

Keywords: animal-free research foundation, project funding, education, public discussion

Poster: free communications

Development of an *in vitro* cultivation system for the haemotrophic bacterium *Mycoplasma suis*

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Mycoplasma suis, the causative agent of infectious anaemia in pigs (IAP), belongs to the group of haemotrophic mycoplasmas (haemoplasmas, HM). They colonize erythrocytes of a wide range of domestic and wild animals causing acute haemolytic anaemia or chronic anaemia with immune suppression. Hitherto, HM are regarded as uncultivable *in vitro*. Thus, research still relies on the propagation of HM in splenectomized animals, a method connected with serious ethical concerns. In regard to replace these animal experiments, this research project is aimed to establish an *in vitro* cultivation system by using *M. suis* as a model organism for all HM. It is based on the hypothesis that HM can be grown in classical *Mycoplasma*-specific media supplemented with appropriate nutrients as they are available in their natural environment, i.e. the mammalian blood. In our approach, all different culture systems are inoculated with anti-coagulated blood from experimentally infected pigs. The bacterial load of the *M. suis* cultures is periodically controlled and quantified by a *M. suis*-specific quantitative real-time PCR assay. Subcultivation is carried out in both liquid and softagar media and on *Mycoplasma*-standardized agar plates.

After the first cultivation period, a successful *in vitro* maintenance of *M. suis* has already been achieved, the most important step towards continuous growth. Moreover, after a long incubation time remarkable star-like shaped colony structures became visible on agar plates growing into the agar surface. In a next step, the switch to successful subcultivation in liquid media to get bigger quantities of *M. suis* will be our focus. In conclusion, these findings demonstrate for the first time that investigation and optimization of the *in vitro* requirements of *M. suis* growth could lead to the continuous *in vitro* propagation of *M. suis* and subsequent establishment of an appropriate culture system for all HM.

Keywords: *Mycoplasma suis*, haemoplasmas, *in vitro* cultivation, IAP, pigs

Lecture: skin models as alternatives to animal testing

The application of the Phenion® Full Thickness Skin Model as an alternative test to predict toxic effects

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It is more than 20 years ago that epithelial equivalents have been successfully produced for clinical studies. Since then reconstructed tissue equivalents of different complexity have been established for scientific investigation as well as commercial use.

While less complex systems, like monolayers, have been extensively analysed in the nineties and the beginning of this century for their applicability in product development and the use as alternatives to animal testing, more sophisticated systems are now available for academia and industry. The Phenion® Full Thickness Skin Model is a skin equivalent comprising an epidermis and dermis that resembles native skin in terms of differentiation and protein production.

This talk will address the application of the Phenion® Full Thickness Skin Model to predict toxic effects. It has been characterised for its competency to metabolise xenobiotic compounds. Several phase I enzymes (CYP450) as well as phase II enzymes are expressed. The distribution pattern of these enzymes reflects the native situation. Therefore the Phenion® Full Thickness Skin Model seems to be a useful tool to show toxic effects to skin mediated by the xenobiotic metabolism.

In the field of genotoxicity the Phenion® Full Thickness Skin Model allows to determine transient as well as clastogenic effects, by analysing COMET induction and micronucleus formation, respectively. For the detection of micronuclei a proliferating tissue is required. Thus the detection of clastogenic effects is limited to the epidermis of the Phenion® Full Thickness Skin Model. Transient effects can be analysed in the epidermis as well as the dermal compartment.

The sensitising properties of compounds have been assessed *in vitro* in the past using immunocompetent cell lines or cells deriving from human peripheral blood. Coming into contact with test compounds these cells change their composition in cell surface proteins. A metabolic competent skin as first site of contact has not been considered in this context. A coculture system of the Phenion® Full Thickness Skin Model and immunocompetent cells might overcome the failure in predicting prohapten correctly.

The close resemblance of the Phenion® Full Thickness Skin Model to native skin recommends it as a promising tool to analyse different toxic effect, such as genotoxicity and sensitisation, directed to human skin.

Keywords: skin equivalent, full thickness, genotoxicity, sensitisation, xenobiotic metabolism

Poster: current activities regarding the EU-chemicals policy (REACH)

Bringing toxicology into the 21st century: a global call to action

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Conventional toxicological testing methods are often decades old, costly and low-throughput, with questionable relevance to the human condition. Several of these factors have contributed to a daunting backlog of tens of thousands of chemicals and mixtures that have been inadequately assessed for their effects on human health. Some authorities have responded to this challenge by implementing complex and burdensome data collection and testing programmes, such as the Registration, Evaluation and Authorisation of Chemicals (REACH) regulation in the European Union. Others have concluded that nothing less than a fundamental paradigm shift in regulatory toxicology is warranted, given the tremendous advances that have taken place in biology, technology, and bioinformatics in recent decades. One such call came in 2007 from the United States National Research Council, which articulated a landmark vision of “twenty-first century toxicology” based predominantly on cutting-edge, non-animal techniques. The cornerstone of the NRC vision is a systems biology approach, integrating high-throughput, human cell-based *in vitro*, ‘omic and computational models to develop a robust understanding of xenobiotic effects on fundamental biological pathways. Potential advantages of such an approach include the capacity to examine a far greater number of chemicals, mixtures and biological outcomes at more relevant exposure levels; a substantial reduction in testing costs, time and animal use; and the grounding of regulatory decisions on human rather than rodent biology. In order for the NRC’s and similar calls to action to make a significant impact on regulatory toxicology in the foreseeable future, they must be translated into sustained multidisciplinary research programmes that are well co-ordinated and funded on an international level. The Humane Society is calling for a “big biology” project to meet this challenge, akin to the Human Genome Project of the 1990s. We are in the process of forging a multi-stakeholder consortium dedicated to implementing the NRC vision. Discussion will include implementation activities to date by government agencies in the US and elsewhere, as well as projected funding and infrastructure needs for the research effort that will be critical to the realisation of the NRC vision.

Keywords: animal replacement, high throughput, integrated testing, human cells, systems biology

Lecture: 7th cosmetics amendment – can all goals be achieved in time?

Respiratory toxicology and immunotoxicology in precision cut lung slices (PCLS)

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Introduction: There is a clear evidence that a variety of chemicals cause allergic sensitization of the respiratory tract. PCLS offer the opportunity to gain insight into lung morphology and physiology after *in vitro* exposure to chemicals. Aim of this study is the evaluation of PCLS as an *ex vivo* model to test for chemical-induced sensitization. This work is part of the EU project Sens-it-iv. Furthermore, air/lifted exposure of PCLS is under development to establish a method for the future application of insoluble respirable chemicals in a reproducible and *in vivo* relevant way.

Method: Lung tissue (mouse, human) was cut with a microtome. Firstly, PCLS were cultivated submersed and exposed to lipopolysaccharides (LPS) and allergens. Secondly, tissue was air/lifted exposed to synthetic air using a method under development at Fraunhofer ITEM. Vitality was controlled by measurement of LDH activity and live/dead fluorescence staining and expressed as EC₅₀. Cytokines and chemokines were detected with Luminex technology and ELISA. Proteomic DIGE was performed.

Results: LPS induced profound pro-inflammatory effects on cytokines such as IL-1 alpha, TNF alpha, and Rantes in PCLS. This shows that PCLS provide *per se* a suitable *in vitro* model to predict immune modulating potencies of substances. Chemical-induced local respiratory irritation and inflammation were characterized in PCLS. *Ex vivo* EC₅₀ values were determined and induced cytokines were quantified. There is a clear evidence that chemical-induced loss of cell viability is accompanied by the production of pro-inflammatory cytokines TNF alpha, IL-1 alpha, and MIP-1 alpha. But, for some allergens we also found an induction of cytokines at subtoxic concentrations. For example, respiratory allergen AHCP increased TNF alpha, IL-1 alpha and IL-8 while the contact allergen cinnamaldehyde was not able to induce the same cytokines. It, therefore, still needs to be elucidated to which extent a concurrent irritation influences the inflammation processes induced by allergens. Gel-based proteome analysis revealed an up-regulation of enzymes involved in the production of ROS, sensing of reactive xenobiotics and energetic metabolism. Preliminary experiments with PCLS exposed air/lifted to synthetic air using varying flows of up to 30 ml/min/PCLS did not induce irritation.

Conclusion: PCLS provide a suitable *in vitro* model to predict immune modulating potencies of substances and to characterize local respiratory irritation and inflammation induced by chemicals. Future exposure of PCLS to gaseous compounds will offer the possibility to investigate *ex vivo* parameters for a wide repertoire of chemicals in a complex biological test system.

Keywords: allergens, sensitizers, lipopolysaccharides, pro-inflammatory cytokines, air/lifted culture

Poster: free communications

A simple and inexpensive *in vitro* model to study the effects of hypoxia on pulmonary epithelial and microvascular endothelial cells

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Hypoxia is associated with a number of chronic lung diseases, a major cause of mortality in western populations. Exposure to chronic hypoxia results in pulmonary hypertension and changes in the structure of pulmonary arteries in animal models. *In vivo* test methods are expensive, animal intensive and time consuming. In addition, animal models may not be readily suited to detailed investigations at a cellular and molecular level. Therefore, we have developed a simple and inexpensive *in vitro* system which can mimic the lung environment and allow us to investigate the responses of alveolar epithelial cells (A549) and microvascular endothelial cells (HMEC1) exposed to hypoxia, as these cell types are a major contributors to chronic pulmonary diseases. A549 cells and HMEC-1 cells were exposed to 15%, 7% and 1% oxygen for 24 hours and the effects of hypoxia on gene expression, protein secretion and transcription factor activation were investigated. Specific gene expression was assayed by real time PCR. Transcription factor activation and protein secretion were determined by Enzyme Immuno Assays (EIA). Hypoxia resulted in an induction of cAMP Response Element Binding Protein (CREB) in both cell types. CREB responsive genes were induced under hypoxia conditions in HMEC-1 cells but not in A549 cells. Both cell types demonstrated hypoxia induced secretion of Endothelin-1 (ET-1), VEGF and IL-6. These results demonstrate that both epithelial and endothelial cells can contribute to hypoxia pulmonary hypertension, vascular remodeling and inflammation. Furthermore, these findings suggest a key role of CREB in hypoxia cell specific responses and provide important information for the further elucidation of the molecular mechanisms involved in hypoxia-associated pulmonary diseases. In conclusion, the developed system allows us to study *in vitro* the effect of different oxygen concentrations on the regulation of intracellular signaling pathway in specific cell types.

Keywords: hypoxia, pulmonary hypertension, CREB, ET-1, VEGF, IL-6

Lecture: 7th cosmetics amendment – can all goals be achieved in time?

Use of computer-assisted models for the prediction of toxic effects of chemical substances

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For the evaluation of existing and new chemicals as well as for the development of pharmaceutical and agrochemical compounds toxicological data are required. For animal welfare reasons and to save time and costs, computer assisted prediction models are more and more often used to gain knowledge about the toxicity of a compound. The currently mostly used models in chemical and pharmaceutical industry are combinations of different commercial software packages and in conjunction with in house developments.

The use of *in silico* tools in pharmaceutical development is more advanced than their use in the evaluation of chemicals. *In silico* tools have become standard in the selection of pharmaceutical lead candidates as well as for identification of critical metabolites and potentially toxic impurities.

Before *in silico* models can be used with confidence the applicability domain and validity of the predictions have to be assessed with in house experimental data and their limitations have to be identified. Several validation studies published in literature show that models suitable for certain pharmaceutical classes may not be appropriate for other classes or industrial chemicals.

In addition, the quality of the models is very much dependent on the size and quality of the underlying database. The larger the training set, which was used for the model development, the larger is the chemical domain of the model. The more specific information on the mechanisms of action is available for model development, the better will the resulting model perform. Toxicological model development is hindered for certain endpoints due to lack of sufficient experimental data for modelling and lack of information on the mechanism of action. Therefore, for the more complex endpoints, like reproduction toxicity, chronic toxicity, carcinogenicity involving ADME, species specificities, etc. model performance is still not sufficient for general use. However, useful information may nevertheless be gained from these models.

The level of uncertainty of the prediction is decisive for their use. For screening and prioritization purposes, a higher level of uncertainty may be acceptable than for their use in a regulatory setting. For a regulatory use, the prediction models need to be validated in order to meet acceptance by regulatory authorities and to replace current *in vitro* and *in vivo* test systems.

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Keywords: in silico toxicology, (Q)SAR, use of prediction models, validity, quality

Roundtable: Embryonic or adult stem cells: scientific and ethical considerations

The use of stem cells in toxicology

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Embryonic and adult stem cells (eSC & aSC) have the potential to differentiate in culture into many different cells and tissues depending on the conditions of culture. For ethical and scientific reasons predominantly culture conditions for rodent SC have been established. However, as outlined by other participants in the round table discussion, increasingly interest is focusing on human stem cells (hSC), consequently methods for applying hSC to safety studies are emerging.

SC are capable of renewing themselves; that is, they can be continuously cultured in an undifferentiated state, giving rise to more specialized cells of the human body such as heart, liver, bone marrow, blood vessel, pancreatic islet, and nerve cells.

Therefore, SC are an important new tool for developing unique, *in vitro* model systems to test drugs and chemicals and a potential to predict or anticipate toxicity in humans. An overview of the applications of SC technology will be given in the area of toxicology. Specifically, core technologies will be addressed that are emerging in the field and how they could fulfill critical safety issues such as QT prolongation and hepatotoxicity, two leading causes of failures in preclinical development of new therapeutic drugs. aSC derived from various sources, such as human bone marrow and placenta, can potentially generate suitable models for cardiotoxicity, hepatotoxicity, genotoxicity/epigenetic and reproductive toxicology screens.

For embryotoxicity testing the embryonic stem cell test (EST) has been developed in our lab during the past 20 years applying mouse ESC. The meSt have successfully been validated in an ECVAM validation study and molecular endpoints have been established in addition to morphological ones as well as reporter gene assays. Currently several groups are implementing the basic principles of the EST to hESC and the first encouraging results have been published on applying the heSt.

From a more general perspective, the most likely immediate uses of hESC will be *in vitro* test systems and disease models. Major and minor pharmaceutical companies have entered this field, and the European Union is sponsoring academic research into hESC-based innovative test systems. This is perfectly in line with approaches on both sides of the Atlantic, to use human cell-based *in vitro* systems for safety evaluations, and to shift the focus of toxicology away from classical animal experiments and more towards a mechanistic understanding.

Keywords: stem cells, embryonic stem cells, adult stem cells, human stem cells, mouse stem cells, toxicity testing, safety testing, embryonic stem cell test, EST

Lecture: nanotoxicology / nanobiotechnology

Toxicological evaluation of nanomaterials in cosmetic products

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Nanoparticle are particles with one or more dimensions at the nanoscale (at least one dimension <100nm). A nanomaterial is a material with one or more external dimensions, or an internal structure, on the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features. Nanoparticles can be divided into two groups: i) soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into their molecular components (e.g. liposomes, microemulsions, nanoemulsions), and ii) insoluble particles (e.g. TiO₂, fullerenes, quantum dots).

For the soluble and/or biodegradable group, conventional risk assessment methodologies based on mass metrics may be adequate, whereas for the insoluble particles other metrics, such as the number of particles, and their surface area as well as their distribution are also required. It is crucial when assessing possible risks associated with nanoparticles to consider their uptake. It is primarily for the insoluble particles that health concerns related to possible uptake may arise. Should they become systemically available, translocation/transportation and eventual accumulation in secondary target organs may occur.

At present, there is inadequate information on: i) hazard identification, ii) exposure assessment, iii) uptake (including physiologically normal and compromised human skin), iv) the role of physico-chemical parameters of nanoparticles determining absorption and trans-port across membranes in the gut and lungs, v) the role of physico-chemical parameters of nanoparticles in systemic circulation determining biokinetics and accumulation in secondary target organs, vi) possible health effects (including susceptible individuals), vii) translocation of nanoparticles via the placenta to the foetus.

For the safety assessment of cosmetics, the 7th Amendment imposes animal testing and marketing bans, which prohibit *in vivo* testing of finished cosmetics now and their ingredients in the near future. All *in vivo* and *in vitro* risk assessment methods for nanomaterials are still under development. Although some validated *in vitro* methods do exist they have not yet been validated and/or optimized with nanoparticles as reference compounds. This implies that for safety assessment of cosmetic ingredients, there are no validated *in vitro* methods available for nanoparticles.

Keywords: nanomaterials, skin, nanoparticle, cosmetics, safety, toxicology, alternative methods, skin penetration

Lecture: computer assisted procedures

A logic-based approach for quantitative toxicology prediction

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This talk presents the application of logic-based machine learning for the prediction of toxicology. Our approach starts with the use of inductive logic programming (ILP) which reasons with chemical substructures in a relational framework and learns chemically understandable rules. This is then followed by the use of a support vector machine to extend qualitative ILP-based structure-activity relationships to a quantitative predictor in an approach we term SVILP. The SVILP approach learnt predictive rules from the Distributed Structure-Searchable Toxicity (DSSTox) database from the U.S. Environmental Agency (www.epa.gov). For a diverse dataset of 576 molecules with known fathead minnow fish toxicity, the cross-validated correlation coefficient (R2CV) from SVILP is 0.66 compared to a chemical descriptor approach which yields 0.52. Our SVILP approach has a major advantage that it uses ILP automatically to learn rules, mostly novel, describing fragments that are toxicity alerts. The SVILP approach has general applicability to chemoinformatics, including *in silico* screening to identify novel hits and construction of predictors for ADME properties.

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Keywords: toxicology prediction, machine learning, logic-based rules

Lecture: alternatives & education

Novel test lung for real life supported simulation

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Ventilation methods are gaining more importance in the areas of intensive care (Oczenski et al., 1997) and veterinary medicine. In Austria for the *in vivo* implementation a variety of many engineering standards (DIN, 1997; 1998; 2004) have to be accomplished, in addition the consent of the ethics commission has to be given.

In many cases the accomplishment of these engineering standards presume animal testing. Other tests, for example the artificial application of harmful substances and toxins, are as a matter of principle not conducted *in vivo* on humans. The multifariousness in harmful substances range from air pollution and to fractionally self-applied substances. Realizing the complexity of inhaling dangerous substances on its own is often not enough to estimate the future health implications. In addition, the allocation of those substances in the lung has to be carefully examined. Hence, allocation measurements and analysis in health engineering are obligated. To realize these measurements artificial lung systems are implemented.

Artificial lung systems vary from passive to almost lifelike active ones. Passive systems usually only reflect few aspects of real lung functions. Constructive breathing bags or springs induce thermally stabilized passive lung systems with usually few lifelike characteristics.

Today new ventilation technologies, which are summarized by the umbrella term of lung protective ventilation, and rapid enhancement of computer technology enable later on substantially technological progress in design and implementation of artificial lung systems. These systems were created with the ability to measure pressure, volume and flow and then use those measurements in a mathematical lung model, calculating the output required to drive the mechanical lung.

Finally, despite the rapid enhancement of computer technology mechanical lung systems can not completely mirror *in vivo* measurements, because the biological lung structure due to its very complex construction is nearly impossible to artificially duplicate. To negotiate this serious drawback we constructed an active lung which central heart is a real pig lung.

In the area of development and research our construction and configuration can measure and analyse harmful substances and toxins *in vivo* lungs. Within those accomplishments it is possible for us to implement those essential measurements, which until now have been executed on still living animals, in a laboratory with the artificial lung. The applied pig lungs were exclusively used from animal for slaughter and afterwards for our applications prepared. There is a special focus in the close to reality simulation to create lifelike respiration procedures.

We present a novel test lung for real life supported simulation. First this special set up can perform the natural functionality of a passive lung, second under adequate respiration conditions and third in gasping mode. In order to provide various breathing patterns a pneumatics based driving system has been designed. The test lung consists of the "lung" itself (a breathing bag or a preserved pig lung) and a Perspex-lined box enabling a free view on the test-lung from outside for the purpose of demonstration in education and traineeship.

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Keywords: artificial test lung, education

Poster: alternative testing methods for toxicity to reproduction

Assessment of DNA oxidation by nitroheterocyclic compounds using cultured YAC-1 cells

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Contradictory results have been published on the DNA damaging properties of the nitroheterocyclic compounds metronidazole and nitrofurantoin, antimicrobial agents widely used in human and veterinary medicine. In order to further study whether their interaction with cellular DNA is linked to the generation of reactive oxygen species we investigated the effects of metronidazole and nitrofurantoin on UV-C induced generation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in molecular DNA in solution as well as in cultured murine hybridoma YAC-1 cells.

Calf thymus DNA (CT-DNA) dissolved in hepes-buffered saline or commercially available YAC-1 cells resuspended in hepes-buffered saline were treated either for 45 min or for 72 hours, respectively, with metronidazole, nitrofurantoin or histidine (as a nitrogenous control compound; 50 µg/ml each) before exposure to UV-C-irradiation (peak wavelength 253.7 nm; 15 min, 6 mJ/min x cm²) or sham irradiation. In some experiments, cells were incubated for a further 180 min period. In separate experiments, the free radical scavenging compound TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) was added. A HPLC system equipped with UV and electrochemical detection was employed to measure the levels of 8-OH-dG in isolated cellular DNA.

Under the conditions chosen, UV-C caused a 123% increase in 8-OH-dG in molecular CT-DNA. Preincubation of CT-DNA with metronidazole prior to UV-irradiation led to significantly higher levels of 8-OH-dG compared to irradiated CT-DNA not treated with metronidazole (172.2 ± 36.1 µmol 8-OH-dG/mol dG). Preincubation with nitrofurantoin did not influence UV-induced 8-OH-dG levels; preincubation of CT-DNA with histidine prevented UV-induced increases in 8-OH-dG.

In untreated YAC-1 cells UV-C irradiation caused an increase in 8-OH-dG concentrations by 304%. Exposure of non-irradiated cells to metronidazole or nitrofurantoin for 72 hrs did not change the basal levels of 8-OH-dG. Incubation with histidine caused a significant decrease in 8-OH-dG concentrations by 72% in non-irradiated cells, while no such effect was seen in UV irradiated cells.

Pretreatment with metronidazole did not affect the induction of 8-OH-dG by UV-C in YAC-1 cellular DNA. In contrast, preincubation with nitrofurantoin led to a 57% decrease in 8-OH-dG levels in DNA of UV-C irradiated cells. The addition of TEMPO lowered the increase in UV-C induced concentrations of 8-OH-dG by 74% in untreated cells and by 78% or 74% in cells pretreated with metronidazole or nitrofurantoin, respectively.

In contrast to the findings using molecular CT-DNA in solution, metronidazole and nitrofurantoin did not affect 8-OH-dG levels in YAC-1 cell DNA and metronidazole had no effect on UV-C induced formation of 8-OH-dG. Whether nitrofurantoin exerts a factual "protective" effect on UV-C induced oxidation of cellular DNA is presently under investigation. The observed "resistance" of CT-DNA against UV induced oxidation following exposure to histidine suggests this substance as acting as a quencher of singlet oxygen (in cell free systems), whereas TEMPO probably exerts its known effect as a spin trap of UV induced ROS.

It is concluded that YAC-1 cells represent a useful tool for the investigation of the complex DNA damaging mechanisms of nitroheterocyclic compounds and, possibly, of other classes of compounds.

Keywords: 8-OH-dG, cellular DNA damage, metronidazole, nitrofurantoin

Lecture: free communications

Influence of “danger signals” on CD54 and CD86 marker expression in THP-1 cells – improvement by HaCaT-Coculture

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The prediction of the sensitizing character of substances is subject of intensive research work, as the 7th Amendment to the European Cosmetics Directive bans the use of animal testing for cosmetic purposes beginning 2013. As a result, test systems have to be established, doing both, reliably survey sensitizing properties of substances and provide information about the expected strength of the possible allergic reaction. To date, numerous test systems have been suggested, based on the culture of primary dendritic cells (DC) or related cell lines.

The *in vivo* situation in allergic response is mediated by a variety of different cell types. Nevertheless, the currently used *in vitro* systems concentrate on systems using only a single cell type. Although some effort has been made, the predictive capacity of these test systems is limited to the properties of the cellular system used.

One important aspect, missing in these prediction systems is described in the concept of “danger signals”. According to this, DCs get primed to differentiate on external stimuli; in addition, auxiliary signals are necessary from the surrounding tissue to finally establish the sensitization. Thus, not solely the DCs but also the environmental cells must react properly on external stimuli to establish an allergic reaction. Furthermore, xenobiotic metabolism of DCs is subject of discussion, with the majority of scientists believing in limited metabolic competence of dendritic cells. Keeping this in mind, the environment additionally supports the formation of allergic reaction by metabolizing the pro-haptens to active haptens.

Here we present data, stressing the necessity of supportive signals from skin cells in the prediction of sensitizing properties.

We show the influence of the well-known “danger signals” IL1alpha, IL1beta, IL6, IL8 and TNFalpha on the CD86 and CD54 marker expression of THP1-cells and present results about the influence of toxicity. We demonstrate, that THP1-cell mature in contact with cellular lysates. Interestingly, the expression of maturation markers is dependent on the cellular origin of the lysate. Additionally we show results on toxicity-related soluble factors (“danger-signals”) like ATP, Calcium or Prostaglandin E2. Finally, we give evidence in considerably improved secretion of the “danger signal” IL1beta, a well-known marker-protein in sensitization-prediction, by combining the THP1-cells with HaCaT-cells in a coculture system, thus stressing the need of auxiliary cell types for the prediction of sensitization.

Keywords: danger signals, toxic influence in sensitization, coculture, metabolic competence

Poster: free communications

Non-animal approaches to inhalation toxicity assessment: a proposal for a way forward

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In the drive to develop alternatives for animal-based acute toxicity tests, the oral LD₅₀ has received considerable attention and investment compared to the development of alternatives for acute inhalation LC₅₀ testing. Concerted efforts at developing a strategic approach to *in vitro* acute oral toxicity testing have recently culminated in the European Union ACuTox project. Conversely, there are a plethora of respiratory-tissue specific cell or tissue models being used in individual laboratories for various environmental research purposes, and work has progressed towards surmounting air/liquid interface considerations are crucial that to correctly predicting respiratory toxicity. However, despite early efforts to develop a concerted strategy for replacement of *in vivo* acute inhalation toxicity testing, one has not emerged. In the alternatives field large amounts of resources are being invested into optimization and validation efforts to replace existing animal-based testing methods on a one-to-one basis. It has become clear that just as with acute oral toxicity, replacing acute inhalation testing in animals will require a more strategic, combinatorial approach versus this one-to-one replacement approach.

In order to identify a system-wide non-animal approach to respiratory toxicity, one must take into account potential respiratory insults that could occur (i.e. acute site-specific tissue damage, bronchial muscle effects, deep lung deposition and/or injury, respiratory sensitization, etc.), and also systemic effects as a result of the distribution and metabolism of a chemical should it be absorbed. Of course, one method may cover multiple insults; similarly, some insults may be covered by methods used in considering other exposure routes (i.e. oral exposure).

This presentation briefly summarises previous discussions and proposes several potential ways forward utilising the most promising current research methods. It provides a forward-looking analysis on the most efficient ways to move ahead, taking into account potential challenges as well as the explosion of recent work in cell and tissue models, computational toxicology, virtual lung and airway modelling, systems biology, and pathway analysis as it relates to inhalation toxicology.

Keywords: in vitro, inhalation toxicity, testing strategy

Poster: free communications

Gene expression analysis in chemical-treated dendritic cells: discriminating contact sensitisers and irritants employing the immune toxicity chip and a whole genome array

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The replacement of animal tests for the detection of the sensitising potential of chemicals is of great importance due to current legislation. In order to establish a test system for the identification of contact sensitisers, human immature dendritic cells (DCs) derived from peripheral blood monocytes were used as substitutes for Langerhans cells, the antigen presenting cells of the skin.

Concentration dependency of sensitiser-specific effects was determined employing flow cytometry by measuring dose-response curves for 7 contact sensitisers and 3 irritants. Gene expression in chemical-treated DCs was then compared to gene expression in solvent treated samples using a previously developed targeted microarray, the immune toxicity chip, and a commercially available whole genome microarray. Additionally, quantitative real-time PCR was performed in order to confirm treatment-related differences in gene expression detected on microarrays.

Statistical data analysis of the experiments performed with the immune toxicity chip revealed a panel of marker genes suitable for the discrimination of strong allergens and irritants. In addition, whole genome analysis identified genes not represented on the targeted array that were significantly differentially expressed after either allergen- or irritant treatment. Graphical visualization of the data by hierarchical cluster analysis showed 2 major groups for sensitisers and irritants and smaller subsets for the different chemicals. Furthermore, the predictive value of the detected genes for determining sensitising potential was assessed by leave-one-out-crossvalidation.

The results indicate that our test system based on the immune toxicity chip allows the identification of strong sensitisers and the differentiation of irritants. Furthermore, additional promising indicator genes for sensitisers and irritants were found with whole genome experiments. Including these genes in the chip design of the targeted array might also permit the identification of weak allergens.

Keywords: dendritic cells, contact sensitisation, in vitro methods, microarrays

Poster: ethical and legal aspects in animal experimentation

Estimates for worldwide laboratory animal use

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Animal experimentation continues to generate public, scientific and political concern worldwide. Many countries conduct animal experiments but relatively few collate and publish animal use statistics; yet this is a first and essential step toward public accountability and an informed debate, as well as being important for effective policy-making and regulation.

The implementation of the Three Rs (replacement, reduction and refinement of animal experiments) should be expected to result in a decline in animal use, but without regular, accurate statistics, this cannot be monitored. Previous estimates of worldwide annual laboratory animal use are imprecise and unsubstantiated, ranging from 28-100 million.

We collated data for 38 countries that publish national statistics, and standardised these against the definitions of “animals”, “purposes” and “experiments” used in European Union Directive 86/609/EEC. We developed and applied a statistical model, based on publication rates, for a further 142 countries. This yielded a basic estimate of global animal use in 180 countries.

However, this basic figure excludes several uses and species/forms of animals that are included in the statistics of some countries. These should be included in statistics to represent more comprehensively the full use that science makes of animals. With the data available from a few countries that do include in their statistics other uses and types of animals, we also produced, by extrapolation, a range for a more comprehensive global estimate that includes animals killed for the provision of tissues, animals used to maintain genetically-modified strains, and animals bred for laboratory use but killed as surplus to requirements.

For a number of reasons that are explained, this more-comprehensive range figure of 82-154 million (mean 115.3 million) animals is still likely to be an underestimate.

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Taylor, K., Gordon, N., Langley, G., Higgins, W. (2008). Estimates for Worldwide Laboratory Animal Use. *ATLA* (in press).

Keywords: statistics, three Rs, international

Poster: 7th cosmetics amendment – can all goals be achieved in time?

The continued need for a cruelty-free standard for cosmetics

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An EU ban on animal testing for cosmetics purposes is introduced under the Seventh Amendment to the Cosmetics Directive, which comes into force on 11th March 09. From this date, animal testing is banned on all cosmetics ingredients, along with a ban on the import and sales of any animal tested cosmetics. An addition, although there is overlap between REACH and the 7th Amendment, REACH states, in effect, that the latter takes precedence. Nonetheless, animals may continue to be used for dose toxicity, reproductive toxicity, and toxicokinetics as the Amendment allows more time for alternatives to be developed for these endpoints (until 11 Mar 2013).

Currently animals (mainly rabbits) are still used for cosmetics purposes – a total of 5571 animals were used in the EU in 2005 (latest figures). A need therefore remains for an ethical standard for cosmetics that enables consumers to make ethical choices and for companies to gain significant marketing advantage until a complete ban on all cosmetics-related testing comes into effect. The British Union for the Abolition (BUAV) has been operating the Humane Cosmetics Standard (HCS) for over 10 years, exactly for this purpose. Symbolised by the Leaping Bunny, the HCS is the global standard for Cruelty Free cosmetics and operates across Europe, the US and Canada. It requires a company to apply a Fixed Cut-Off Date for cosmetic ingredients, be open to audit and no longer conduct/commission animal testing throughout their supply chain. The HHPS (Humane Household Products Standard) is a sister standard that aligns with our current efforts to ban animal testing for household products in the UK and Europe. Our organisation is also working towards an international cosmetics testing ban.

Keywords: cosmetics testing, cosmetics directive, animal testing, ethics, industry

Poster: revision of the Directive 86/609/EEC & national reference laboratories

Recommendations for the revision of Directive 86/609/EEC from the European animal protection movement

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The European Coalition to End Animal Experiments is an alliance of animal protection organisations from 15 European member states. The anticipated revision of the directive relating to animals used for experimental and other scientific purposes (Directive 86/609/EEC) is an opportunity for increased co-operation amongst European stakeholders towards the achievement of an eventual end to animal testing in the EU. A primary aim of the ECEAE in this revision is to seek an end to the licensing of experiments that use non-human primates. This is proposed on both scientific and ethical grounds, for which there is considerable support from members of the public. Secondary aims include seeking a ban on the licensing of experiments relating to warfare, xenotransplantation, education, tobacco, alcohol and household products. The scope of the Directive should also be extended to cover foetuses, cephalopods and all possible harmful uses of animals in laboratories (e.g. tissue supply, education, breeding). If animal testing is not ended by the revision, a number of improvements are supported by the ECEAE. These include the formalisation of a transparent system of ethical review, including lay and animal protection representation. Prospective and retrospective assessment by independent experts of the severity of harm caused to individual animals during their lifetime should also be mandatory. This will allow for a more accurate assessment of the harm: benefit of each research project, which currently underpins its authorisation. Targets for the replacement of animals used in scientific procedures should also be set. Finally, a responsibility on individuals as well as member states to develop non-animal methods should also be incorporated into the legislation.

Keywords: 86/609, animal testing, legislation, ethics, animal protection

Lecture: gentechonology & animal experiments / transgenic animals

Reporting 3Rs parameters in European scientific research papers

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It is 20 years since both the Council of Europe Convention ETS123 and the EU Directive 86/609 were introduced, recommending the implementation of the 3Rs (reduction, replacement and refinement) in animal experiments and providing guidance on animal housing. It might therefore be expected that this might have influenced reports of 3Rs related parameters in animal research papers over time. In order to test this hypothesis, a literature survey of animal-based research reported in 15 key journals was conducted. A randomly selected sample of original research papers arising from European institutions that involved primates or genetically modified mice were identified for the years 1985-6 and 2005-6 (N=50 each). Each paper was reviewed for reports of 10 parameters corresponding to replacement (consideration of non-animal methods), reduction (justification of number of animals required) and refinement (housing – related; size, social, object and food enrichment and procedure-related; the use of training, humane endpoints, anaesthetic and analgesia). Despite increasing reports of adherence to the legislation and codes of practice, reports of reduction and replacement considerations were extremely low (0-6%) with no significant difference between year or species. Reports of refinement related improvements were higher but still low (0-45%) with only a few significant differences between year or species. With increasing provision of supplementary information in online papers it should be more, rather than less, possible to acknowledge 3Rs related parameters within research papers. This is an important issue to be tackled by academics and journal editors if the 3Rs is to be promoted nationally and internationally.

Keywords: 3Rs, transgenic, mice, primates, research

Lecture: current activities regarding the EU-chemicals policy (REACH)

Opportunities and threats for animals during the implementation of REACH

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Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) was adopted on 18 December 2006 and came into force on 1st June 2007. REACH is important in terms of laboratory animals because many of the test methods in order to ensure the safety of existing and new chemicals continue to be *in vivo* based. Although for the animal protection movement REACH will result in the use of millions of laboratory animals over the next 10 years, a number of improvements to the draft legislation were made, namely Article 25 which states that "testing on vertebrate animals for the purposes of this Regulation shall be undertaken only as a last resort". However, there are a number of new developments relating to the implementation of REACH that continue to have implications for laboratory animals; including the publishing of the Test Methods (Council Regulation (EC) No 440/2008, published on 30 May 2008) as well as the Technical Guidance on Information Requirements, discussed under the Reach Implementation Projects. There also remains a number of outstanding issues that will also have an impact on laboratory animal use; awareness of REACH, especially amongst Small to Medium Enterprises, formation of SIEFS and the realities of data sharing, awareness of and use of alternative methods, in addition to the interplay between REACH and the cosmetic industry who also have to comply with the Cosmetics Directive and the 7th Amendment which imposes imminent animal testing bans. There are a number of opportunities over the coming years for further reductions in animal use including the 45-day scrutiny period, the outcomes of Framework Projects such as OSIRIS and further validations by ECVAM. The implications of these and how the animal protection movement can assist in the successful implementation of REACH and further reduction in animal use are presented here.

Keywords: REACH, animal testing, chemicals, implementation, policy

Lecture: revision of the Directive 86/609/EEC & national reference laboratories

Making the EU Directive 86/609/EEC work for animals

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The EU Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes is the key piece of legislation covering the scientific use of animals in the European Union. The Directive has not been changed since 1986 and both animal use and animal protection communities are in agreement that it requires revision. This is primarily because the use of animals has diversified since the creation of the Directive, in particular with a dramatic increase in genetically modified animals being used (1.4 million being used in the UK alone in 2005).

The lengthy process that the Directive has gone through since 2001, when revision was first suggested, will be described; including the outcomes of public consultations, expert impact assessments as well as reports by the Scientific Committees on the Scope of the Directive and the Use of Primates. The key area of debate appears to be on the use of primates and the extent to which this should be restricted; extending the scope of the Directive to include animals used in basic research and teaching is already hoped to have been agreed by the Commission. Subject to release by the Commission, the key components of the Draft will be discussed from the point of view of the animal protection movement. From our perspective it is imperative that this Directive doesn't just harmonise laboratory animal protection to the level of current best practice but becomes a Directive that has animal protection at its heart. One significant improvement would be to set targets for a reduction in the numbers of animals used in the European Union, currently at a record high of 12.1 million animals.

Keywords: 86/609, legislation, 3Rs, policy, animal protection

Lecture: gentechonology & animal experiments / transgenic animals

Epithelial cell lines for carcinogenesis and chemoprevention: an alternative to complement genetically engineered mouse models

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Background: Targeted organ site expression of oncogenes or repression of tumor suppressor genes have provided valuable *in vivo* animal models for genetically predisposed breast and colon cancer. Expression of clinically relevant genetic alterations in the target epithelial cells and quantifiable risk for carcinogenesis should provide an alternative approach to complement long term animal studies by refining and reducing *in vivo* experiments.

Models and Biomarkers: Mouse mammary epithelial cells stably transfected with Ras or c-myc oncogenes (MMEC-Ras, MMEC-Myc), and colon epithelial cells from Apc 1638N+/- and Mlh1+/- /1638N+/- mutant mice (1638N COL, Mlh 1 /1638N COL) represented the cell culture models for breast and colon carcinogenesis, respectively. Cell cycle progression, cellular apoptosis and anchorage independent colony formation represented the quantitative surrogate end point biomarkers for the carcinogenic risk.

Study Outcome: Relative to the respective parental MMEC or COL cells, the MMEC-Ras, MMEC-myc, 1638N COL and Mlh1/1638N COL cells exhibited loss of homeostatic growth control (aberrant cell cycle progression, down-regulated apoptosis), and gain of anchorage independent growth *in vitro* and tumorigenesis *in vivo*. Treatment of the genetically altered mammary epithelial cells with pharmacologically achievable doses of mechanistically distinct synthetic or natural chemopreventive agents resulted in reversal of loss of homeostatic growth control and reduction of carcinogenic risk. In the genetically altered colon epithelial cells, mechanistically distinct agents in low dose combinations operated via additive/synergistic interactions to establish homeostatic growth control and reduce carcinogenic risk.

Conclusion: Quantifiable expression of the risk for carcinogenesis in these models and its reduction by chemopreventive agents validates a novel mechanism based approach to prioritize efficacious preventive/therapeutic interventions for long term *in vivo* studies on genetically engineered mouse models.

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Keywords: *genetically altered cells, carcinogenic risk, chemopreventive efficacy*

Poster: skin models as alternatives to animal testing

SkinEthic Reconstructed Human Epidermis (RHE) model: development of an alternative method for skin irritation testing

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Following extensive optimization and prevalidation studies, ECVAM launched a formal validation study on *in vitro* EpiSkin and EpiDerm reconstructed human epidermis models. In 2007, the EpiSkin model was scientifically validated to fully replace the regulatory Draize skin irritation test (EU B. 4 method; OECD TG 404) on rabbits for R38 classification of skin irritants according to EU classification. A performance standard document was developed by ECVAM for evaluating the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of a candidate Me-too test method.

The purpose of the present study was to develop such a test method able to discriminate skin irritancy potential of chemicals using the Reconstructed Human Epidermis (RHE) model from SkinEthic.

In Phase I, twenty reference test chemicals (10 irritants and 10 non irritants) were tested to determine whether the validated protocol (15 min chemical exposure followed by 42 hour post incubation time) was appropriate using RHE model. Results showed that both sensitivity and specificity did not exceed 70%. The predictive ability of the test method was therefore considered inadequate and test protocol was refined by extending contact time of test chemical on RHE up to 42 or 60 min. Applying those two protocols, an overall predictive accuracy of 85% was reached (specificity: 80%, sensitivity: 90%). Final evaluation was performed using "42 bis" protocol (42 min contact exposure followed by 42 hours post incubation time before endpoint measurement) leading to higher intra-laboratory reproducibility (>90%).

In Phase II, reliability of the "42 bis" protocol was assessed on 17 liquid and solid additional chemicals resulting in an overall predictive accuracy similar to that of validated, approved test models using MTT as endpoint and a threshold of 50% viability.

The RHE model was considered ready to enter formal interlaboratory validation.

Keywords: alternative, skin irritation, RHE

Poster: nanotoxicology / nanobiotechnology

Surface modification influences the *in vitro* toxicity of gold nanoparticles in human alveolar type-II cell lines

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Engineered nanoparticles (NPs) have been extensively used in different technologies during the last years. Nevertheless, many questions arise about the risk and the impact on human health following exposure to nanoparticles because their properties differ from those of their respective bulk materials. At present many of these questions are still unsolved. It was already demonstrated that inhaled NPs can rapidly be translocated to other organs, such as liver and kidney, but it was also shown that NPs can accumulate and induce inflammation in the respiratory tract, which is one of the most important routes of exposure. Current knowledge about the cytotoxicity induced by NPs in the respiratory tract is still largely incomplete. In this study, the human alveolar type-II (ATII) cell lines A549 and NCIH441, which retain many of the characteristics of the primary lung epithelial cells, were exposed for 24-72 hr to gold nanoparticles (Au-NPs). The cytotoxicity induced by Au-NPs, which differed in size (5-25 nm diameter) and surface modification (presence or absence of sodium citrate), was investigated by classical cytotoxicity assays. The gold nanoparticles presenting sodium citrate as surface modification could impair the cell viability after 24-72 hr exposure, as shown by the MTT assay, and a more severe impairment was observed in A549 cells. Following exposure to Au-NPs the proliferation of the human ATII-like cell lines A549 and NCIH441 was investigated by Ki-67 assay and crystal violet dye elution. The reduction in cell proliferation was more severe in those cells exposed to Au-NPs presenting sodium citrate on their surface, compared to the cells treated with gold nanoparticles which do not display sodium citrate as surface modification. Interestingly, the NCIH441 cell line seemed to be more resistant and showed a milder cell proliferation reduction. Using the lactate dehydrogenase (LDH) assay, a marker for cell membrane damage, the toxicity of Au-NPs was further investigated. Although the gold nanoparticles investigated here could induce a lower cytotoxicity in NCIH441 cells as shown by MTT, Ki-67 and crystal violet dye elution assays, the LDH released by NCIH441 cells was higher compared to the A549 cell line. In summary, we observed that the presence of sodium citrate on the surface of gold nanoparticles, and not the different diameter of the nanoparticles, impaired cell viability and proliferation of the ATII-like cell lines A549 and NCIH441 *in vitro*.

The authors would like to acknowledge Dr. Daniele Bonacchi for synthesizing gold nanoparticles. This study was supported by EC FP6 funding – CellNanoTox research (NMP-CT-2006-032731).

Keywords: pneumocytes, in vitro, nanotoxicology, gold nanoparticles

Lecture: computer assisted procedures

VirtualToxLab – *in silico* prediction of the toxic (endocrine-disrupting) potential of drugs and chemicals

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The VirtualToxLab is an *in silico* tool for predicting the toxic (endocrine-disrupting) potential of existing and hypothetical compounds (drugs and environmental chemicals) by simulating and quantifying their interactions with the human protein using automated, flexible docking combined with multi-dimensional QSAR. Currently, it includes 11 validated models for the androgen, aryl hydrocarbon, estrogen (α/β), glucocorticoid, mineralocorticoid, thyroid (α/β), and peroxisome proliferator-activated receptor γ as well as for the enzymes cytochrome P450 3A4 and 2A13. The models were validated using a total of 824 compounds (630 training +194 test substances). The fully automated technology is accessible through the Internet and allows to mechanistically verify a prediction by interactively inspecting the binding mode of the tested compound(s) with their target proteins in 3D.

The VirtualToxLab is already available for Universities, environmental NPOs and validations centers. By August 1, 2008 public hospitals and regulatory bodies and by October 1, 2008 the Pharmaceutical, Chemical, Biotech, Cosmetics, and Food Industry may benefit from the technology.

Details are given on our website from where the program documentation may be downloaded at <http://www.biograf.ch/downloads/VirtualToxLab.pdf> or the toxic potential of 250+ compounds can be viewed at http://www.biograf.ch/data/projects/virtualtoxlab_results.php

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Keywords: *VirtualToxLab, in silico prediction of the toxic potential, toxicity modeling, multi-dimensional QSAR*

Lecture: alternative testing methods for toxicity to reproduction

Perspectives for alternative methods to animal testing in the field of reproductive toxicology

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Reproductive toxicity testing is extremely animal consuming and expensive. Therefore all stakeholders are interested to reduce testing providing a high level of chemicals safety at the same time. For regulatory purposes two endpoints has to be covered for reproductive toxicity testing: developmental toxicity and fertility impairment. Under the new EU chemicals legislation REACH, two tests on developmental toxicity (OECD TG 414) and a 2-generation toxicity study (OECD TG 416) are required. According to this legislation, a significant increase in test animal numbers will be resulting, which is unacceptable from the scientific, animal welfare and economical perspectives.

Therefore scientists from the BfR as well as colleagues from regulatory agencies in the Netherlands (RIVM) and the USA (EPA) and also from industry are suggesting to save thousands of animals by restricting reproductive toxicity testing to one developmental test and a F1-generation test. As another step weight of evidence, grouping of substances and read across could help waiving many reproductive toxicity tests formally required by law.

A flexible strategy for reproductive toxicity testing of industrial chemicals will be presented, which will reduce animal use to a minimum while generating a maximum of information on the intrinsic properties of a chemical.

Keywords: REACH, reproductive toxicology, developmental toxicity study, generation study

Lecture: skin models as alternatives to animal testing

Quantitation of sensitizing potential by the loose-fit co-culture-based sensitization assay (LCSA) in comparison with LLNA-data

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Our new *in vitro* assay for prediction of the sensitizing potency of xenobiotics is based on a co-culture system that integrates human keratinocytes and dendritic cell-related cells (DCrc). The animal-free assay is called LCSA. As sensitization-indicating parameters serve CD86-upregulation on DCrc and increase in cytokine IL-6 and chemokine MIP-1 β concentrations in co-culture media. The murine local lymph node assay (LLNA) is a validated sensitization assay. Provided that the LLNA correctly predicts the sensitizing potential of a substance in humans, LLNA-results may be used as a standard during development and validation of a new assay. We have tested various substances which have already been grouped by the LLNA into potency categories. We found that EC3-values of the LLNA correlate closely with concentrations of halfmaximal CD86-upregulation in the LCSA. LCSA and LLNA achieve an analogous grouping of allergens into categories like weak-moderate-strong. In conclusion, the new assay provides concentration-response information, by which the relative ability of a chemical to induce sensitization can be predicted.

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Keywords: contact allergy, prediction of sensitization, in-vitro sensitization assay, LCSA, LLNA

Poster: free communications

Metabolism studies of the Phenion® full thickness skin model as compared to other *in vitro* models and human native skin

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The understanding of organ and species specific metabolism is essential for safety assessment to achieve biological relevant results. Skin metabolism can significantly alter the activity of topically administered substances, e.g., by converting them into harmless metabolites or genotoxic compounds. The biotransformation of a wide range of exogenous compounds is affected by several phase I and phase II enzymes. Similar to the situation in the liver the cytochrome p450 oxidases are the most important phase I enzymes in skin. In addition, a range of flavin-containing monooxygenases (FMOs) seem to play an important role in skin metabolism. However, much less is known about their regulation or substrate specificity in the dermal compartment.

As an alternative to animal testing we have developed a three-dimensional human skin model to investigate the xenobiotic metabolism of epidermis and dermis separately. Our Phenion® Full Thickness Skin Model consists of a dermal and an epidermal layer and thereby provides a higher physiological complexity than existing *in vitro* models. The interaction of both compartments is essential for cell differentiation and regeneration as well as xenobiotic metabolism.

To investigate the suitability of *in vitro* models with regard to human native skin we compared *in vitro* skin models with different physiological complexities, (1) the commercially available Phenion® Full Thickness Skin Model, (2) an alternative epidermis model, (3) monolayer cultures of fibroblasts, (4) monolayer cultures of keratinocytes and (5) human native skin. To exclude possible donor variabilities all four *in vitro* models were constructed with cells from the same donor. In addition we compared *in vitro* models from various donors to assess individual variabilities. Basal gene expression of phase I and phase II enzymes as well as the inducibility by typical model substances were evaluated with quantitative RT-PCR.

Further approaches will comprise the analysis of phase I and II enzymes on protein expression level and enzyme activity as measured by analyzing biotransformation reactions with known model compounds.

Our results demonstrate that *in vitro* skin models with rising physiological complexity mirror the native situation more realistically. This makes them an ideal tool to study questions of toxicology related to skin *in vitro*.

Keywords: human skin metabolism, skin model, cytochrome P450, flavin monooxygenase

Poster: 7th cosmetics amendment – can all goals be achieved in time?

Cell based assay for label-free, long-term investigation of living cells as alternative testing method for toxicity

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For monitoring of the metabolic and morphologic activity of living cells a six channel version of a cell based assay called intelligent mobile lab (IMOLA) was prepared (Wiest et al., 2006). IMOLA employs biochips which contain microsensors for measurement of cellular respiration, extracellular acidification and changes in morphology of the cells. These parameters are monitored in the microenvironment of living cells using sensors for dissolved oxygen, pH and impedance. Due to a sophisticated system set-up the cells can be monitored label-free, parallel, continuously and in real-time. The measurement is performed in a stop- and go-mode. During the stop-mode (7 min) the microsensors detect the slope of oxygen consumption, acidification and morphological changes of the cells. Afterwards during a pump cycle (go-mode) cell culture media or a drug are transported toward the cells to supply them with fresh media and to recalibrate the microsensors. These cycles are repeated throughout the measurement which may last up to two weeks. In the presented experiment the toxicity of doxorubicin toward MCF-7 cells compared to a control was investigated. The vitality of the cells is reduced for 33% compared to the control after 30 h of exposition. To ensure validity of the data the experiment was stopped by adding Triton X-100. Here the vitality signals of both groups declined immediately. With the presented IMOLA system a new tool for dynamic investigation of vitality and morphology of living cells and their interaction with drugs, toxins or metabolites is available. One advantage of the system is the label-free and long term investigation method. Due to these properties it is possible to monitor the kinetic of the interactions between cells and toxins. With this cell based assay, unique in-vitro toxicity data can be determined without the use of animal experiments.

References

Wiest, J., Stadthagen, T., Schmidhuber, M. et al. (2006). Intelligent Mobile Lab for Metabolics in Environmental Monitoring. *Analytical Letters* 39 (8), 1759-1771. DOI 10.1080/00032710600714089

Keywords: biosensors, cell based assay, label-free, living cells, toxicity

Poster: ecotoxicology – endocrine disruptors

Application of an intelligent testing strategy to the US EPA Endocrine Disruptor Screening Program

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The US EPA is initiating its Endocrine Disruptor Screening Program in the fall of 2008. The EPA has designed its program in two tiers. The purpose of the Tier 1 is to “identify substances that have the potential to interact with the EAT [estrogen/androgen/thyroid] hormonal systems...”, and the results would then be used to trigger Tier 2 testing (reproductive and developmental toxicity studies). At the time the Tier 1 battery was available for public comment (FIFRA Scientific Advisory Panel Meeting, March 25-26, 2008), it tentatively consisted of three to four *in vitro* methods, five *in vivo* methods, and four alternate methods (one *in vitro*, three *in vivo*). For the initial phase of the EDSP, every chemical that receives a Data Call-In notice from the EPA must be tested in all of the assays that will eventually comprise the Tier 1 battery. Although the specifics of the Tier 1 battery are still in flux, it appears likely to consist of approximately 10 assays that would use from 475 to more than 1,100 animals and cost between 220,000 to more than 700,000, USD per chemical (conservatively estimated with no range-finding or repeat experiments). Since not all of the methods – or the assumed battery – have been thoroughly validated, it is likely that the Tier 1 will be revised. Recognising the need for a faster, more accurate, valid screening battery, we propose an alternative tiered strategy here. The preliminary tier includes physical and chemical data, existing toxicological data, and *in vitro* and (Q)SAR methods that are either validated or nearly validated. The results of this alternative Tier 1 can be used in a weight-of-evidence approach to 1) identify priority chemicals and 2) design an intelligent, chemical-specific strategy for further screening or testing. Such a strategy would greatly reduce the use of animal testing for identification and classification of endocrine disrupting chemicals.

References

1. According to the presentation given by the EPA on March 25th, 2008 at the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel Meeting, the In utero through lactation assay is still being considered as an alternate assay, even though at a FIFRA on February 21, 2007 meeting to discuss the test was criticized as being too expensive for a Tier 1 test.
2. EDSTAC Final Report, January 2004, Table 5.6. <http://www.epa.gov/endo/pubs/edstac/chap5v14tbl.pdf>.
3. The in utero-through lactation assay itself is estimated to cost approximately 300,000 USD

Keywords: endocrine disruptor testing, alternative methods

Poster: computer assisted procedures

Progress in sharing data from repeated dose toxicity studies

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Databases have an important role to play in reducing the use of animals in toxicity testing. By incorporating legacy toxicity data into a database it becomes available for more effective internal use thereby avoiding repetition of experiments. The biological properties of established chemicals can also be compared allowing selection of less toxic alternatives. Additional benefits include being able to analyse the data for structure-activity relationships allowing read-across and the development of models for predicting toxicity of new chemicals. Organisations would also benefit from sharing this data with each other in a reciprocal manner. One such data sharing initiative involves a consortium of ten pharmaceutical companies and the UK charities RSPCA (The Royal Society for the Prevention of Cruelty to Animals) and FRAME (Fund for the Replacement of Animals in Medical Experiments) who are collecting vehicle control data from repeated dose toxicity studies.

The first stage in development of the database was the compilation of a user requirement document listing the consortiums minimum requirements for platform and security; database structure; data entry/correction/deletion; data retrieval; and reporting. A partner for development and hosting of the database was then selected. Issues to be addressed at this early stage included the level at which the data should be summarised. Repeat dose studies on pharmaceuticals generally include low, mid and high dose groups. The effects seen at these doses will be different and will also vary between the sexes. The consortium were interested in being able to identify the effects of a particular vehicle in a particular species, by a particular route and at a particular dose and therefore opted to summarise the data at each individual dose level and to combine results for both sexes. Although the consortium had defined the fields and controlled vocabulary they wished to use, these then had to be organised into a suitable schema. This process was aided greatly by the fact that the consortium had identified the ways in which they wished to query the data and indicated their top ten most important searches. A central table was used to store data on the vehicle formulation; underlying tables were then added to store information on vehicle components and important study conditions. An additional level of hierarchy was added to accommodate the detailed clinical chemistry and pathology findings which may be associated with individual studies.

A pilot database was prepared and populated with example data to allow consortium members to evaluate its suitability. The requirements were then further refined and the project has moved onto the next phase, development of a full scale database to be populated with donated data.

Development of the pilot database demonstrates that data sharing is possible even for complex endpoints such as repeated dose toxicity. As the database becomes populated with donated data all consortium members will benefit, avoiding unnecessary use of laboratory animals and facilitating selection of appropriate vehicles. In addition as the size of the database increases so will the potential to develop structure-activity models for predicting the toxicity of new chemicals.

Keywords: databases, data sharing

Lecture: computer assisted procedures

***In silico* estimation tools developed by the Joint Research Centre in support of chemicals legislation**

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To promote the availability of reliable computer-based estimation methods for the regulatory assessment of chemicals, the European Commission's Joint Research Centre (JRC) has developed a range of user-friendly software tools.

Toxtree predicts various kinds of toxic effect by applying decision tree approaches. The set of decision trees includes the Cramer classification scheme, the Verhaar scheme, the BfR rulebases for irritation and corrosion, and the Benigni-Bossa scheme for mutagenicity and carcinogenicity. Additional rulebases are under development.

Toxmatch generates quantitative measures of chemical similarity. These can be used to compare datasets and to calculate pairwise similarity between compounds. Consequently, Toxmatch can be used to compare model training and test sets, to facilitate the formation of chemical categories, and to support the application of chemical read-across.

DART (Decision Analysis by Ranking Techniques) was developed to make ranking methods available to scientific researchers. DART is designed to support the ranking of chemicals according to their environmental and toxicological concern and is based on the most recent ranking theories. Different kinds of order ranking methods, roughly classified as total and partial-order ranking methods, are implemented.

Finally, a web-based inventory of (Q)SAR models (the JRC QSAR Model Database) is under development, which will help to identify relevant (Q)SARs for use in the regulatory assessment of chemicals.

These tools can be downloaded (free of charge) from the following JRC website: <http://ecb.jrc.it/qsar/qsar-tools/>

Keywords: QSAR, read-across, in silico, REACH

Lecture: alternative testing methods for toxicity to reproduction

Development of an *in vitro* test system for developmental neurotoxicity

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Subject/Motivation/Background: Little is known about the effects on the human CNS development of most of the chemical substances we handle every day. Evidence is emerging that neurodegenerative diseases such as Alzheimer's disease might be correlated with exposure to toxicants in early life stages and early exposure e.g. to lead has affected the development of millions of children (Landrigan et al., 2005). Therefore we aim to establish a neurodevelopmental toxicity assay based on embryonic stem cells (ESC).

Material and Methods: To differentiate mESCs into neurons a protocol published by Ying and Smith in *Methods of Enzymology* in 2003 was slightly modified. Successful differentiation was checked by RT-PCR and Immunofluorescence analysis. 5-Fluorouracil, CH₃HgCl and PenicillinG were used as model substances to test the potential of our system to predict neurodevelopmental toxicity. Sublethal concentrations of these substances were applied to the culture for the last 6 days of differentiation. Effects were analyzed by RT-PCR and Western Blot.

Results: Differentiation of Tubulin, III- β mESC into neurons resulted in a percentage of 70% neurons (N-CAM), 15% nestin-positive precursor cells, and approximately 10% glial cells. The latter were located within rare and small islands within an even layer of tubulin positive cells to mature neurons was shown III- β neurons. Maturation of by the presence of several markers such as, NeuN (postmitotic), SNAP25 (pre-synaptic), PSD95 (postsynaptic), SV2 (synaptic vesicles-2), NMDAR1 (postsynaptic), tyrosin hydroxylase (dopaminergic neurons), glutamic acid decarboxylase (GABAergic neurons). The staining pattern of the analyzed markers within ESC-derived neurons was similar to that of primary neurons (CGCs) isolated from mice. The whole differentiation process was monitored by RT-PCR and Western blot to determine the time window of the onset of neuronal differentiation. Toxic substances were added at that point in time to the culture and marker expression was analyzed. RT-PCR analysis showed a significant difference in the synaptophysin, N-CAM, nestin and MAP2 mRNA content of CH₃HgCl as-treated cells compared to untreated cells. Glial markers like GFAP and S100 well as the endodermal marker GATA-4 and the mesodermal marker brachyury were not affected by the treatment with CH₃HgCl. Treatment with the known strong teratogen 5-FU significantly reduced the amount of the endodermal marker GATA-4. Viability of the cells was not affected by the treatment as confirmed by MTT assay and morphological observation.

Discussion: We show here the differentiation of mESCs into neurons. We additionally showed that our system can detect the impact of toxicants at sublethal concentrations. This effect was restricted to the neuronal lineage, as glia and markers for the other two germ layers were not affected. We therefore believe that our system can detect toxic effects on the development and functionality of neurons.

Keywords: mESC, development, neurotoxicity, methylmercury

Poster: free communications

A human corneal model for the prediction of eye irritation: progress report

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With the ban on animal testing for development of cosmetics in the EU, the demand for *in vitro* methods for safety and efficacy testing is growing. An extensive number of *in vitro* assays, many of which find applications in industry, have been developed and proposed as alternatives to the Draize eye irritation test. Unfortunately, none of the methods which were included in several validation/evaluation studies was found to meet all the formal validation requirements as prerequisite for replacing the current OECD *in vivo* assay. Hence, success in fully replacing the Draize eye test for the evaluation of acute eye irritation with *in vitro* methods has not yet been achieved. There remains a clearly identified need to define alternatives methods that are mechanistically-based, reliably predict the human eye response to chemicals exposure and which can replace the *in vivo* test.

To address development of alternative methods based on mechanistically relevant biological events, we have generated a full thickness human corneal model exclusively based on SV-40 immortalised cell lines. Recently, we have optimised serum-free culture conditions for the maintenance of functional and structural characteristics of the epithelial and the stromal part of the corneal model.

Methods: The human corneal epithelial cell line HCE (Araki-Sasaki et al., 1995) and the human corneal keratocyte cell line HCK (Zorn-Kruppa et al., 2004) were used in attempt to establish a rational basis for the development of serum-free cultivation media for the assembly and long-term tissue culture of a three-dimensional corneal model.

Briefly, we investigated the impact of serum-free cultivation on proliferation, morphology and barrier function of HCE cells: Multilayered epithelia were subjected to histology for the evaluation of tissue morphology and cell proliferation. Barrier functions were investigated by measuring transepithelial electrical resistance (TEER).

We characterised HCK cells in the presence and absence of serum supplements and studied the functional capacity of this cell line to contract a collagen matrix as a result of the myofibroblast differentiation.

Three-dimensional hemicorneal models were constructed by tissue engineering methods using microporous membrane inserts. The resulting hemicornea comprised a stroma of keratocytes embedded into a collagen matrix covered by a multilayered epithelium.

To ascertain applicable endpoints we examined cytokine secretion profiles of corneal cell lines and hemicorneas by antibody array/ ELISA technology and monitored cell/ tissue viability using an MTT-based assay.

Results: Our results revealed that both morphology and barrier function of epithelial constructs were comparable to human *in vivo* corneas under serum-free culture conditions. As well, stromal embedded keratocytes retain their specific characteristics and show an inducible myofibroblast differentiation. By using hemicorneal models the MTT end-point has been shown to be applicable for toxicity testing of relevant reference substances.

Conclusions: Our findings support the biological relevance using immortalised human cell lines for the development of a full thickness corneal model for toxicity tests. Preliminary results revealed that the hemicorneal constructs are suitable models for safety testing of topically applied ocular drugs. However, further work is required to refine these measurements.

Keywords: safety testing, eye irritation, corneal model