



Session 7.5

Novel cell culture techniques

Poster

Evaluation of perfusion culture for investigating the toxic effects of cadmium chloride to two renal epithelial cell lines

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The limitations of conventional static cell culture systems prevent a continuous exposure of cells to toxin. In order to investigate the toxic effects of continuous exposure of the nephrotoxin cadmium chloride (CdCl_2), we utilised a perfusion cell culture apparatus (EpiFlow®).

The porcine proximal tubule cell line LLC-PK₁ and the human proximal tubule cell line HK-2 were exposed to CdCl_2 for up to 72 h as a single static dose or under continuous perfusion. Lactate dehydrogenase (LDH), adenylate kinase (AK), glucose consumption and lactate production were assayed in the supernatants. Cell viability was assayed by resazurin reduction.

Perfusion experiments with control, NOEL (1 μM for the HK-2, 3 μM for the LLC-PK₁) and EC_{50} (5 μM and 15 μM , respectively), as detected after 72 h under static conditions, were performed for 4 days.

Under perfusion conditions, the 5 μM and 15 μM concentrations increased enzyme release strongly; a marked peak was observed at 12 h for the LLC-PK₁ and at 25 h for the HK-2 cells.

The 15 μM concentration resulted in 100% lethality to the LLC-PK₁ and the 5 μM concentration resulted in 70% lethality to the HK-2 cells.

The results indicate a greater susceptibility of renal epithelial cell lines to CdCl_2 under continuous exposure in perfusion culture than compared to a single dose in static culture.

In conclusion, the EpiFlow system is well suited for long-term toxicity testing under continuous perfusion as well as for testing of acute toxicity.



Poster

The effect of tetrabromobisphenol A at a low concentration on rat embryos in culture

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We examine the alternative method *in vitro* procedure of the developmental toxicology test of chemicals. Although the effects of bisphenol A have been extensively investigated, there is little information concerning the effects of tetrabromobisphenol A (TBrBPA). TBrBPA, which is widely used as a flame retardant in the building industry in USA. We have reported that TBrBPA had similar embryological toxicity on cultured rat embryos to that of bisphenol A at the concentration (1 ppm). At higher concentration bisphenol A had a severe toxicity on rat embryo in culture. In the present experiments we examined the embryological toxicity of TBrBPA at a lower concentration.

Rat embryos on day 11.5 of gestation were removed from the uterus and cultured as whole embryo for 48 hours in normal

medium. Two hours after incubation in normal medium, TBrBPA or its solvent DMSO, was added to the medium. The rat embryos were further cultured for 46 hours.

In embryos cultured in the medium containing TBrBPA at the concentration of 1ppm a clear decrease in heart rate and blood circulation in yolk sac vessels were observed at 24 and 48 hours in culture. In rat embryos cultured in medium containing vehicle only cacogenesis was observed at the corresponding times. No clear abnormality was observed in cultured embryos at the concentration below 1 ppm of TBrBPA. It was found that TBrBPA had the toxicity on the circulation of rat embryos at concentration up to 1 ppm.

Lecture

Human hepatoma HepaRG cells: a reliable surrogate to primary human hepatocytes for xenobiotic metabolism and toxicity studies

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Most human hepatoma cell lines are deficient in the major CYP-related enzyme activities, making them unsuitable for xenobiotic metabolism and toxicity studies. The aim of this work was to analyse the xenobiotic metabolism capacity of HepaRG cells derived from a human hepatocellular carcinoma, that exhibit a highly differentiated pattern after 2-3 weeks at confluence in the presence of DMSO (Gripon et al., PNAS, 2002, 99, 15655). mRNAs encoding various nuclear factors (AhR, PXR, CAR, PPAR α), CYPs (1A2, 2C9, 2D6, 3A4) and phase 2 enzymes (GSTA1, A4, M1 and GT1A1) were measured by qRT-PCR in HepaRG cells at their optimum level of differentiation and were found to be expressed for most genes, at lev-

els close to those estimated in primary human hepatocyte cultures and equal to or much higher than those in HepG2 cells. Similarly, values of basal CYP activities and their response to prototypical inducers as well as metabolic profiles of the compounds studied were comparable to those obtained with cultured human hepatocytes. Moreover, the hepatotoxicants tested were more cytotoxic to HepaRG than to HepG2 cells. In conclusion HepaRG cells represent the first human hepatoma cell line expressing the major CYPs involved in xenobiotic metabolism and appear to be a unique tool for investigating xenobiotic metabolism and toxicity in human liver.

**Poster****The use of the modified CULTEX[®] system for the direct exposure of bacteria to mainstream cigarette smoke***Michaela Aufderheide and Ulrich Mohr*

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The evaluation of the mutagenic activity of mainstream cigarette smoke is mostly based on studies with condensates or extracts in the standard Ames assay. Due to the methodological difficulties of testing air contaminants in their natural gaseous or aerosolised state, there are no accepted concepts and techniques for effective exposure of bacteria under such conditions. Therefore, we established a novel approach using an exposure device based on the cell exposure system CULTEX[®], which was connected to a smoking machine (smoking robot VC10). This allows us the investigation of chemically and physically unchanged mainstream cigarette smoke by exposing bacteria of *Salmonella typhimurium* strains directly to diluted mainstream

smoke of the research cigarette K2R4F. In preliminary experiments the treatment of strain TA98 to whole smoke resulted in the induction of revertants dependent on dilution and the number of cigarettes smoked, whereas the gas phase induced no mutagenic signal. In comparison to studies with condensates by using the plate incorporation assay, the exposure of the bacteria directly to native cigarette mainstream smoke seems to enhance the susceptibility of the bacteria to mutation. The introduction of our exposure device in the field of inhalation genotoxicology offers new possibilities in the evaluation of genotoxicity in the Ames assay by taking in consideration not only hydrophilic but also hydrophobic substances in the particulate and gas phase.

Poster**Harvest, proliferation, and functional testing of human dendritic cells***Seyoum Ayeahunie, Mitchell Klausner* and Sarah Lamore*

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Difficulty in harvesting large numbers of cells, short survival time, and rapid phenotypic changes in culture have prevented the widespread use of human dendritic cells (DC) for many fundamental studies applicable to the initial stages of pharmaceutical discovery and development. To develop a commercial source of DC, precursor cells were harvested from umbilical cord blood samples, proliferated, and induced to differentiate into DC with a newly developed medium. Use of the novel culture medium allowed increases which averaged >400 fold in DC number. FACS analysis showed that the DC expressed CD1a, HLA-DR, CD11c, CD40, CD80, CD83, and CD86 for up to 35 days in culture; Birbeck granules were also observed over this culture

period by TEM. Upon stimulation, the DC showed gene and protein responsiveness in terms of IL-12, MIP-1 α , MIP-3 α , IL-6, and TNF- α expression and DC were able to stimulate allogeneic TC. In addition, DC exposed to allergens enhanced both primary and secondary TC responses and the DC were infectible with HIV-1. Thus, improved culture conditions have allowed the harvest and expansion of functional DC. MatTek's new DC product (DC-100) will be useful in: 1) allergenicity, 2) viral infection, 3) antigen presentation, 4) immuno-therapeutic, and numerous other studies related to the development of prophylactic and therapeutic pharmaceuticals.

**Poster****Preliminary characterisation of an *in vitro* model of blood brain barrier useful for toxicological studies**

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The blood-brain barrier (BBB) plays a pivotal role in maintenance of brain homeostasis and is crucial for neuronal functions. *In vivo*, enzymatic activities of cerebrovascular endothelial cells against endogenous molecules or xenobiotics are important to protect brain from toxic damage.

In animals, measurement of BBB functions is both difficult and costly to perform, therefore its reliable *in vitro* model would be of great value to study transport, absorption and toxicity mechanisms in consequence of chemicals exposure.

We have developed an *in vitro* BBB system, in which an immortalised rat brain endothelial cell line (GPNT) is co-cultured, on microporous membrane filter, with primary rat cortical astrocytes (either fetal or neonatal) or retinal Muller glia, seeded in the opposite side of the filter. That is, the direct interaction between astrocytes and endothelial cells, crucial for the induc-

tion of *in vivo* BBB properties, is maintained. The choice to use at least one continuous cell line, has been taken to reduce animal numbers and to simplify experimental procedures.

We have characterised our BBB model, and preliminary results show that it matches some *in vivo* BBB features, such as: cell morphology, revealed by SEM analysis, presence of tight-junctional complexes, analysed by ZO-1 and occludin immunostaining, P-gp expression and activity, determined by cytofluorimetric assay, low paracellular permeability to (H)-mannitol and slight increases of Trans-Endothelial Electrical Resistance.

Further studies are in progress to elucidate metabolic properties of our BBB model, in order to better characterise it as a possible useful tool for toxicological evaluation.

Poster**Impact of excipients and solubilisers on the *in vitro* gastrointestinal permeation of marker molecules**

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The aim of the present work was to determine the impact of excipients used in oral dosage forms and solubilisers on the transport of drugs across Caco-2 monolayers without damaging membrane integrity or influencing permeability. For this purpose we tested seven excipients/solubiliser: PG, PEG 400, Tween 80, Cremophor EL, TPGS 1000, HP- β -CD and BSA. For the permeation studies the low permeability marker mannitol, digoxin (substrate for Pgp), phenylalanine as example for an uptake-transport and propranolol as high permeability marker were chosen.

Permeation studies on Caco-2 cell monolayers were carried out on Transwell® clear filters with an area of 1.13 cm² and 0.4 μ m pore size. The transport (21-30 days after seeding) was performed in triplicate. The solutions of excipients/solubilisers

with radiolabelled test substances was added to the donor compartment and KRB Buffer (pH 7.4) with 2% BSA to the receiver compartment.

The ratio of the Papp values from the basolateral side to the apical cell side depends on the transport mechanism. For mannitol the ratio of permeability coefficient is ≈ 1 , for digoxin $\gg 1$ and for phenylalanine $\ll 1$.

The highest influence on mannitol transport was established for PEG 400 and PG. Cremophor EL, Tween 80 and TPGS 1000 inhibit the efflux-pump Pgp. The other solubilisers show different effects on digoxin transport. For all tested solubilisers only low effects on phenylalanine transport were observed. Therefore the individual interactions between the test substances and the excipients/solubilisers play a key role in oral absorption process.



Poster

Organotypic hippocampal slice cultures: A novel *in vitro* assay system for simultaneous analysis of neurotoxicity, proliferation and neurogenesis in the same sample

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A major step in drug development is the assessment of the inherent potential risk that the respective compound might bear when used in man. Neurotoxicity testing poses considerable difficulties to realistic *in vitro* modelling. The currently used *in vivo* methods are expensive and time consuming. Besides the toxicity of the compound one has also to consider other side effects that might not be directly toxic but could have deleterious consequences. These side effects include impact on cell proliferation and differentiation.

Organotypic hippocampal slice cultures (OHC) combine the accessibility of an *in vitro* system and the retained 3D structure of the respective tissue found *in vivo*. OHC also greatly reduce the number of experimental animals and time needed.

We have established two injury models of neurotoxicity: Excitotoxic injury induced by glutamate and ischemic injury

induced by oxygen-glucose-deprivation. Quantification of pyramidal neuronal death is performed by densitometric assessment of propidium iodide (PI) incorporation. Proliferating cells are labelled with BrdU and subsequently detected by immunohistochemistry and confocal laser microscopy. Neurogenesis is identified by immuno-histochemical double-labelling (BrdU/neuronal marker: Doublecortin, beta-III tubulin, NeuN). Neuronal cell death and neurogenesis was characterised under normal and injury conditions. Our data demonstrate the existence of neurogenic zones in this rodent brain tissue culture. Thus, this OHC-based *in vitro* assay represents an excellent model system for simultaneous (one sample-three endpoints) detection of compound effects on neuronal viability, proliferation and differentiation in the CNS.

Lecture

Modelling long-term repeat-dose toxicity: Challenges faced

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The Long term culture of tissues and cells, presents a number of specific problems. The main change in characteristics stems from the *in vitro* status whereby differentiated cells with specific functions have a slow division rate, whilst for *in vitro* culture increases in cell numbers is desirable. Changes in tissue culture components has allowed for the controlled differentiation of the desired tissue specific function allow for the expansion of cultures by growth, and then differentiation by medium signals/factors.

This has enhanced *in vitro* toxics logical assessment, in that the effects of chemicals and formulation via basal cytotoxicity on the rapidly growing relatively undifferentiated cultures can be compared with the effects on the control of the differentiation process and on the fully differentiated cultures. There by

corresponding more closely to the effects that could occur in susceptible humans.

Not only does this facilitate the evaluation of single acute accidental exposure but also the more widespread problems associated with chronic and or repeat exposure.

Developments in our understanding of the different organs control of differentiation and function need to be addressed, but also wound healing.

The understanding of recovery from insult and injury by tissues of the human body will be required to enhance the models of chronic and repeat exposure.

Additionally endpoint assays which are non invasive or non-disruptive to cultures or function are also required.

**Poster**

Evaluation of chemically induced neurotoxicity in primary culture of rat Cerebellum Granule Cells (CGCs) using specific neuronal and cytotoxic endpoints

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In vitro systems have been extensively used to study the mechanisms of neurotoxicity at the cellular and molecular level, however, their application in hazard and human health risk assessment has not been explored to any great extent. The challenge to use *in vitro* systems for neurotoxicity screening is to differentiate cytotoxicants from neurotoxicants.

In these studies, we have exposed primary neuronal-glial culture of rat cerebellar granule cells (CGCs) and the non-neuronal cell line of the mouse fibroblast (BALB/3T3) to the neurotoxic (trimethyltin, aluminium chloride, methylmercury, acrylamide, colchicine, paraquat, parathion and chloroquine) and non-neurotoxic compounds (triethylenemelamine, paracetamol, cycloheximide) for 72 hrs. To evaluate whether tested chemicals had different potency of toxicity in neuronal *versus* non-neuronal cells IC₂₀, IC₅₀ and IC₈₀ of all tested compounds was determined in culture of CGCs and compared with BALB/3T3 cell line. The

specific neuronal- and glial-endpoints (neurofilament or glial fibrillary acid protein quantification using ELISA) were applied and compared with general assays for cytotoxicity (mitochondrial membrane potential, reactive oxygen species and ATP quantification) and cell viability (alamar blue assay) to discriminate between neurotoxicity and cytotoxicity.

The results suggest that:

- 1) IC₅₀ values of chemicals tested in the culture of CGCs and BALB/3T3 correlated well with rat LD₅₀ values at the higher concentrations, whereas at the lower concentrations IC₅₀ of CGCs correlated better than those of BALB/3T3.
- 2) Neuronal specific endpoints for neurotoxic chemicals in primary culture of CGCs were more sensitive than cytotoxic assays.
- 3) The neurotoxic compounds produced characteristic dose-response profiles that differed from cytotoxic chemicals.

Poster

Alternatives to animal serum for cell culture – 2005

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Background: Aim of our work was an up to date study on aspects of animal serum usage for cell cultivation tasks and alternative approaches of nutrition medium supplementation. The final report and up to date product guide should support researchers in making their best choices for specific *in vitro* studies.

Methods: Data sheets, product descriptions, cell culture manuals and published papers on the subject were gathered/analysed by literature survey and contacting scientists working in the field at universities and industry research centres.

Results: A growing number of alternatives exists for cell lines and primary cultures derived from a broad range of tissues: e.g. chemically defined media, complementations of non-serum origin, non-animal derived proteins and also optimised

sampling/processing protocols and production systems/bioreactors for serum-free usage of all scales. A literature search structured for cell type/field of application was performed and paper abstract/citation-shortcuts were collected. The final document including the newly updated product guide 01/2005 is available at <http://www.zet.or.at>. Further updates are scheduled every 6 months.

Conclusion: In recent years various easily available alternatives to animal sera have been reported and are on sale. For optimal cell performance *in vitro*, to limit costs and, last but not least, following animal welfare considerations, the authors advise the gathering of exact informations to make qualified choices possible.

**Poster****Sheep's blood clot cell-growth promoting fractions in combination with porcine ocular fluid**

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A natural blood clot contains 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. Several components in the blood clot were recognised being a part of the natural healing process if added to the wounded tissues or surgical sites and have the potential to accelerate the healing. It was also shown to increase the bone formed from 19 to 25% when measured at 4 and 6 months. The specific components of the PRP are the platelets derived growth factor (PDGF) and the Transforming growth factor b (TGF b). Both of them are contained in the a 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 sheep's blood clot. The sheep's blood was collected and allowed to form the clot. Afterward the whole content was centrifuged at 2500 RPM for 20 minutes and the supernatant was aspirated off. The sediment ("clot") was quickly washed with the sterile PBS (Phosphate buffer saline) pH=5.8 for 10 minutes, and centrifuged 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) and for 5 days (fraction IV). All the fractions were sterilised by 0.2 membrane filtration. The content was analysed by PAG-SDS electrophoresis. The porcine ocular fluid was obtained from the whole eye's fluid content filtered through the gauze and centrifuged at 3000 RPM/30 minutes at +4°C. The clear supernatant was collected, filtered through the 0.44 mesh filter and stored at -30°C. The cell growth promotion/inhibition activity in the comparison to the SR-2.055P (serum replacement based on porcine ocular fluid) and FCS (foetal calf serum) was tested on the chicken embryonal fibroblasts, WISH, HAC-3/T2 (human amniotic cell lines), PLA-2 (adult pig kidney cell line), IPEC-J2 (porcine intestinal endothelial cell line) and WiREF (Wistar rat embryonal fibroblastoid cell line). Fraction I shows the growth inhibition and toxicity, while other fractions show the growth promotion, but different according to the group of cells used in the test. The optimal content was 5-8% in Eagle's medium. In this range up to 90% value of the SR-2.55P could be obtained.



Poster

High-dose atrazine affects ovarian steroidogenesis and oocyte maturation but not pre-implantation embryo development in mouse studies

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Introduction: Studies suggest that the herbicide atrazine disturbs fertility by altering the pituitary secretion of luteinising hormone and prolactin. We used a follicle bioassay to investigate whether atrazine affects the ovary directly. Embryotoxicity of atrazine is observed in animal models. We evaluated its specific effect on pre-implantation embryo development by the mouse embryo assay (MEA).

Materials and methods: Preantral mouse ovarian follicles were cultured during 13 days, up to ovulation and were chronically exposed to 1, 10 or 100 µM atrazine (60 follicles/dose). One-cell mouse embryos were cultured under oil in the presence of the same doses (40 embryos/dose).

Results: No effects were observed on folliculogenesis, but 100 µM atrazine affected oocyte maturation: only 26±18% of the oocytes was able to extrude a polar body (versus 90±9% in

the control). Estradiol and testosterone production were increased at the 3 dose levels, but only estradiol was significantly different from the control on day 12 (70.1±31.5 µg/l versus 23.0±10.3 µg/l). The 100 µM dose increased pre-ovulatory progesterone significantly on day 12 (38.1±17.6 µg/l versus 5.2±1.6 µg/l in the control), whereas hCG-induced progesterone was elevated at the 3 dose levels (significant when exposed to 1 and 100 µM: 451±43 µg/l and 494±76 µg/l versus 311±71 µg/l in the control). In the MEA blastocyst formation and hatching capacity were normal irrespective of the atrazine dose level.

Conclusion: Atrazine seems to affect the ovarian function only directly at high dose levels, as was evidenced by disturbed oocyte maturation and steroidogenesis, whereas pre-implantation embryo development was not influenced.

Poster

Adaptation of cell lines to chemically defined minimal (cdm) media and evaluation of a protocol for cryo-preservation of cells cultured in absence of animal derived components

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Our research attempts to replace foetal bovine serum (FBS) by supplementing the cell culture media with alternative additives. Removing FBS from cell culture media will have broad benefit to both scientist and to animal welfare groups. Researchers will benefit by the down stream processing and registration of cell products for pharmaceutical use. Eliminating the brutal practice of collecting blood from bovine foetus will reduce animal cruelty.

In the present project we adapted several cell lines frequently used by biotechnologists and molecular biologists to cdm-media. We followed the growth behaviour before and during the adaptation phase. After a stringent quality control and authentication, the cell lines are now available for the scientific community by the European Collection of Cell Cultures (ECACC).

Cells cultured without FBS are in general more sensitive to

chemical agents and mechanical stress. The proteins in FBS, consisting mainly of albumins, create a balanced viscosity in the cells' environment and bind added reagents, as well as toxic metabolites. These facts are especially important in the process of cryo-conservation as it is often done using a mixture of culture medium, FBS and DMSO as anti-freeze agent.

The main part of our presentation deals with the evaluation of a freezing protocol designed for cells cultured in absence of FBS. The mechanism of the freezing process and the results obtained by varying the cooling rate using different freezing media are discussed. We compare simple "home-made" media with commercially available freezing media.

This project was sponsored by FFVFF, Zurich, Switzerland (www.ffvff.ch).



Poster

Modelling the human bronchi and its responses *in vitro*

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Technological developments in cell culture techniques have lead to the production of increasingly organotypic organ cultures. MatTek currently produce 3-dimensional, multi-differentiated of a variety of organs, including the human bronchi. The EpiAirway® bronchial cultures not only present an organotypic phenotype, but also provide the opportunity for representative aerosol exposure as they are grown at the air:liquid interface. These are currently utilised for a variety of purposes, but the aim of this study was to investigate their efficacy as a toxicological tool.

EpiAirway® cultures were exposed to a range of known respiratory toxins and a variety of their responses assessed. These included TEER, MTT, protein exudation, histopathology and cytokine analysis. From this a toxicological response profile was created and compared previous studies into alternative lung models. Morphological analysis showed that the cultures

were representative of bronchial epithelium, and that damage occurred indiscriminately across cell types. Biochemical results showed immediate, transient release of pro-inflammatory cytokines concurrent with a similarly transient loss of TEER. This was followed by dose-dependent change in TEER and a dose- and time-dependant decrease of cellular viability. The toxicological profile was significantly attenuated following co-exposure to antioxidant-rich, surrogate, epithelial lining fluid (sELF).

Overall, the cultures displayed significant morphological and biochemical correlation with both *in vitro* and *in vivo* models, including primary Type II cell monocultures (Richards et al., 1990). This study has served to iterate the potential value of air-liquid interface cultures in modelling the human respiratory tract *in vitro*.

Poster

A high-throughput *in vitro* model of human tracheal/bronchial epithelium (EpiAirway™) for preclinical safety and efficacy testing of pharmaceuticals

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A growing need exists for high throughput *in vitro* models that can provide rapid, reliable safety and efficacy screening in preclinical drug development. MatTek has recently adapted EpiAirway, an *in vitro* model of human tracheal/bronchial epithelium, to a 96-well high-throughput screening (HTS) format compatible with robotic manipulation. HTS EpiAirway (AIR-196) is derived from normal human cells cultured at the air/liquid interface in 96-well microporous membrane plates to produce three-dimensional organotypic cultures. AIR-196 exhibits a pseudostratified structure and displays a differentiated mucociliary phenotype with barrier properties similar to native tracheal/bronchial or nasal epithelium, including development of transepithelial electrical resistance (TEER), conferred by functional tight junctions. Over the course of several consecutive culture lots, the intraplate and interlot barrier func-

tion reproducibility of the AIR-196 cultures was determined by measuring TEER. Average baseline TEER readings of all wells in a given 96-well plate (intraplate average) ranged from 388.6 to 445.0 Ohm * cm². The average coefficient of variation between wells on the same plate (intraplate variability) was 19.5 %, while the variability between plates (interlot variability) was 6.8 %. The utility of AIR-196 for drug formulation developed was demonstrated by looking at the peptide induced permeation enhancement of FITC-dextran (MW=4000) through the tissue. Optimal peptides reversibly decreased TEER values to <15% of controls, did not induce cytotoxicity (viability >95%), and increased permeation by 3 fold. Thus, the HTS EpiAirway will find utility for drug permeation studies as well as *in vitro* irritation/toxicity screening, high content cell testing, and molecular biology assays.



Poster

***In vitro* culture of *Echinococcus multilocularis* metacestodes as alternative to animal use**

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Infection in humans with the larval stage (metacestode) of fox tapeworm *Echinococcus multilocularis* causes alveolar echinococcosis (AE). The disease is fatal if not treated appropriately. The *in vitro* culture model for the continuous proliferation of *Echinococcus multilocularis* metacestodes was established, which has made this parasitic stage experimentally accessible, and has since then provided a unique model to study a number of aspects of the parasite biology and its interaction with the host. This model has served as an unlimited resource of antigen for both diagnosis as well as experimental studies, and at the same time, most importantly, it has enabled

us to avoid the extensive use of laboratory animals for a number of studies. We have shown, that this model is highly suitable for first-round *in vitro* drug screening assays, and we developed an easy-to-use and reliable test to determine parasite viability, which allows to investigate large numbers of potentially interesting compounds. During such studies, we identified the 5-nitrothiazole analogue nitazoxanide as a novel potential drug for anti-echinococcal chemotherapy. Following very limited experimentation in laboratory mice, this drug will be evaluated in a clinical study involving human AE patients, starting towards the end of this year.

Poster

***In vitro* development of tissue cysts of *Neospora caninum* and use of *in vitro* culture in structure-function analysis of anti-parasitic drugs**

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Neospora caninum is a protozoan parasite, belonging to the *apicomplexa*, and is a pathogen of considerable veterinary importance. It causes neuromuscular disease in dogs and represents the most important cause of abortion in cattle. Infection with the proliferative and disease-causing stage of this intracellular parasite, the *tachyzoites*, leads to destruction of tissues and immunopathological events. *Tachyzoites* can be cultured *in vitro* in the presence of host cells. The cystic stage, the *bradyzoites*, will form tissue cysts that can persist within an infected animal for many years, leading to chronic infection. These tissue cysts have long been experimentally accessible only through extensive and time consuming animal experimentation, with a large number of laboratory mice being required for these studies. We

have now succeeded to develop an *in vitro* culture model for the production of *N. caninum* tissue cysts. We have applied this model to study the cell biology of stage conversion, and to characterise the *bradyzoite* stage and its interaction with the host. More recently, we have adapted the *N. caninum tachyzoite* culture model to screen for anti-parasitic drugs. Parasite proliferation is easily measured through a quantitative real time PCR assay developed in our group, and these assessments are complemented by light- and electron-microscopical observations. Drug efficacy studies using a series of selected compound, nitazoxanide, have been carried out, and allowed to us to determine the structure-function relationship of defined alterations of this molecule.



Poster

Cytomic assays for *in vitro* toxicity assessment

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Classical *in vitro* toxicity assessment involves the measurement of either cell proliferation or gross cell death. With the dawn of genomics and the rising of proteomics these classical assays seem antiquated. However, newer safer more reliable cell death and cell proliferation assays are available. Also, fluorescent and luminescent based assays which can measure such sensitive endpoints as inner mitochondrial membrane potential and hydrogen peroxide production are now on the market. It is possible to combine many of these assays together in one experiment thereby generating as much data as possible, saving time, cost and biological material. Using the chronic nephrotoxin cyclosporine A as an example we discuss the applicability of a wide variety of assays to the determination toxicity *in vitro*.

The human proximal tubular cell line HK-2, was treated with various concentrations of CsA for 24 or 72 hours. On live cells resazurin reduction, cell cycle analysis, mitochondrial membrane potential and thymidine (BrdU) up-take were determined. In supernatants glucose, lactate, LDH, adenylate kinase and hydrogen peroxide was measured. In cell lysates caspase-3 activity was determined.

Using these assays we could determine CsA induced effects at concentrations below cell death. CsA at sub-lethal concentrations increased ROS, altered glucose metabolism, increased mitochondrial membrane potential and was anti-proliferative. Multi-parametric cytomic analysis when used appropriately can be very useful in toxicity assessment *in vitro*.

Lecture

Engineering human hepatoma cells with key transcription factors as a mean to generate metabolically competent human hepatic cell models

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Replacement of laboratory animals by alternative human-derived *in vitro* models is a goal in human pharmacology and toxicology. Within this context, the knowledge of the biotransformation pathways of a compound is a necessary step for the development of better and safer drugs. Primary cultured human hepatocytes, as they produce a metabolic profile of a drug very similar to that found *in vivo* and respond to inducers, have become the gold standard in *in vitro* studies. However, because of their restricted accessibility, other cell models (e.g. hepatoma cells) have been considered. Unfortunately, hepatic cell lines express only marginal levels of drug-metabolising enzymes (i.e. CYPs; cytochrome P450s) and are not a real alternative. To understand why human hepatoma cells do not express CYP genes, we undertook a detailed analysis and found evidences

showing that the levels of several key transcription factors and co-regulators clearly differed from those found in adult hepatocytes. Based on these observations we postulated that the re-expression of one (or more) of these transcription factors in hepatoma cells could lead to an efficient transcription of CYP genes. The feasibility of this hypothesis was demonstrated by genetic engineering of hepatoma HepG2 cells with different factors (e.g. C/EBP, HNF3) and strategies (chromatin remodelling), followed by the analysis of the expression of human CYPs. Tailored re-expression of activators and co-activators missing in hepatoma cells lead to the transcription of relevant CYP genes. Our results open a promising new experimental strategy to metabolically upgrade human hepatoma cells for human drug metabolism and toxicity studies.

**Poster**

A novel miniaturised perfusion bioreactor for predictive immunogenicity testing of drugs

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As evident with the recent high profile recalls, drugs entering the market are still vulnerable to variations in efficacy in patient groups and to additional side effects under a specific predisposition. This calls for further extension and higher precision of drug testing. Standardised *in vitro* systems based on miniaturised histotypic tissue cultures can substantially increase the predictive value of preclinical drug testing. A novel miniaturised modular bioreactor technology was developed on the basis of fully disposable culture ware. Heart of the culture ware is a unique micro culture cassette ensuring long term histotypic cultivation of human and rodent primary tissues. A bioreactor prototype with a cell culture volume of 0.5 ml was developed and tested specifically for survival of leucocytes. Furthermore human lymphatic

tissues generated in this type of bioreactor were subjected to treatment with different substances during culture. Microenvironment, tissue reactivity and metabolic parameters were monitored. In addition the micro culture cassettes with cultured cells were embedded into Technovit 7100 and histological staining of cross sections was used to evaluate micro architecture within the cell culture compartments. Observed results clearly indicated that the novel bioreactor platform provides a perfect basis for high content drug testing on miniaturised human lymphatic tissues *in vitro*. Thus the development may contribute to predictive immunogenicity testing of drugs avoiding both animal testing and risky early trials in man.

Poster

Human vaginal-ectocervical tissue model (EpiVaginal™) to test the irritation of contraceptive and vaginal-care products

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Vaginal-ectocervical (VEC) tissues were reconstructed using normal human VEC epithelial and VEC+dendritic cells. Both tissues mimic native *in vivo* tissue in that they have basal, parabasal, glycogenated intermediate, and the superficial cell layers. To test the utility of the tissues, contraceptives, microbicides, anti-itch agents, and other vaginal-care products (VCP) were topically applied. To mimic the heterosexual HIV infection, the tissue models were topically exposed to HIV-1 viruses.

Quality control (QC) testing on each batch of tissue utilised Triton X-100 (1%) and water as positive and negative controls, respectively. The MTT assay was used to determine the exposure time necessary to decrease the tissue viability to 50% (ET₅₀) for the positive control and 20 VCP.

QC testing showed the tissues to be highly reproducible; the average intra-lot coefficient of variation (CV) was <10% and

ET-50s averaged 1.10 hr±0.27 (n=15 lots). Also, the VEC tissue model discriminated between the mildness of VCP. ET₅₀ values ranged between 3.5-7.0 hr for contraceptives, 6.9->18 hr for anti-itch creams, and 1.7-2.7 hr for feminine washes. Released cytokines and gene expression levels showed that IL-1α, IL-1β, IL-6, and IL-8 were associated with toxicity induced by VCP. In addition, the VEC tissue was infectible with macrophage-tropic and T-cell tropic HIV-1 strains.

The VEC tissue models will serve as useful, highly reproducible, non-animal tools to assess the irritation of VCP. The *in vitro* tissue model will also enable studies pertaining to HIV infection, microbicides and drug absorption.



Poster

EpiOral™ (ORL-200) and EpiGingival™ (GIN-100) tissue models for oral irritation studies

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Three-dimensional models of the human oral epithelia, exhibiting a buccal or gingival phenotype have been developed using normal human oral epithelial cells cultured in serum free medium. The buccal tissue (ORL-200) contains 8-12 cell layers with cells becoming increasingly squamous toward the apical surface have been developed. No evidence of cornification is present in histological slides and immuno-staining shows the expression of cytokeratin K13 human beta-defensins in the suprabasal layers. These features are characteristic of buccal epithelium. The gingival tissue (GIN-100) has 9-13 layers of viable, nucleated cells and is partially cornified at the apical surface. Lipid analysis revealed that, of the ceramides important in the barrier of epidermis, only ceramide 2 in was present in ORL-

200, a result that matches human buccal tissue. GIN-100 showed the presence of the three least polar ceramides, C1, C2, C3, in a ratio of 1.0:8.2:4.5, respectively. When exposed to the surfactant Triton X-100 (1%), an exposure time of 52±20 minutes (n=31) reduces the viability of ORL-200 to 50% as determined by an MTT assay. For GIN-100, an exposure of >8 hours is required to damage the tissue to the same extent. In addition, MTT assay and cytokine release results from ORL-200 tissue have been used to provide industrial and academic researchers with a quick, reproducible method for evaluating the irritation potential of oral care excipients and products. The methodology correlates well to human irritation results, and can provide a reliable alternative to animal testing.

Poster

Human oral keratinocytes primary culture: A comparative study between two isolation techniques

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The need to recover mucosal oral defects is routinely present in maxillofacial and oral interventions, when the surgeon frequently confronts himself with the lack of available tissue. Cultivation *in vitro* of oral mucosal keratinocytes into transplantable autologous epithelia could be a convenient source of tissue. The aims of the present work were to test and compare the efficiency of the enzymatic or the direct explant technique methods for keratinocytes isolation from oral mucosal fragments and to evaluate the feasibility of the obtaining transplantable grafts. A comparative study between the enzymatic and the direct explant techniques for mucosal keratinocytes isolation will give enough subsidies, so as to improve results *in vivo* for short and long time periods and at the same time a better quality in the obtained cells. Keratinocytes were extracted using both isolation techniques, from oral mucosal fragments donated from

healthy human subjects undergoing dental surgeries (research project approved by the Ethical Research Committee-IPEN, under Licence N° 087/CEP-IPEN/SP).

The cells were cultivated over a feeder-layer of previously irradiated murine fibroblasts in appropriate keratinocyte culture medium. Once sub confluent, the primary cultures were amplified into subsequent cultures. Thus, cell life span could be estimated.

The oral keratinocytes cultivation and their subsequent expansion and cultures were possible and successful, reaching a compatible number of duplications like human skin keratinocytes. Human oral keratinocytes are possible to be cultivated *in vitro* and these cell cultures could also be employed as an *in vitro* model for cell/drug interaction, avoiding unnecessary animal testing.



Poster

A novel technique for the continuous determination of Trans-Epithelial Electrical Resistance (TEER)

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Trans-Epithelial Electrical Resistance (TEER) measurement is an established method to quantify barrier function in epithelial and endothelial cells. TEER has been demonstrated to be a sensitive endpoint for determining the toxicity of compounds to epithelial cells *in vitro*. The aim of this study is to develop a TEER measurement technique to monitor barrier function of epithelial cells under continuous perfusion conditions.

The porcine renal epithelial cell line, LLC-PK₁ were cultivated on microporous growth supports in a perfusion apparatus (EpiFlow®). A newly designed TEER unit was implemented into the perfusion device and resistance measurements were monitored over the life of the culture. Additionally cells were

intoxicated with a previously established toxic concentration of CdCl₂ (15 µM). Lactate dehydrogenase (LDH) concentration in the out-flow medium was also measured.

During formation of the epithelial monolayer, TEER increases up to a steady state value, as expected. CdCl₂ exposure resulted in a gradual collapse of TEER, which correlated to an increase LDH release.

We have successfully developed a TEER unit which can be used for the continuous monitoring of barrier function in perfusion cell culture. This is a powerful, non-invasive online measuring tool of monolayer integrity.

Lecture

A novel perfusion cell culture system for acute, repeat dose and long term toxicity testing

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In vitro models for long-term toxicity assessment, defined as a recurring exposure to compounds over a prolonged period of time, are still underrepresented in the drug evaluation and chemical testing processes. A possible reason for this is that the classical approach to cell culture is not readily suitable to long term repetitive applications. One avenue to overcome the limitations of cell culture for long term testing is to use perfusion culture systems. Such systems allow continuous or repeat dose application of toxins to the cell or tissue culture. Also biomarkers of vitality can be measured in the out flowing medium such as enzyme release, consumption of medium components or the production of metabolites. In addition there is evidence that cells cultured under these conditions often more closely match the

phenotype of their *in vivo* counterpart. Thus perfusion culture systems are likely to be more relevant to the assessment of chemical induced toxicity than static cultures. We describe one such system which was developed primarily with epithelial cells in mind. This system provides a separate apical and basolateral compartment allowing the continuous monitoring of transepithelial electrical resistance through-out the experiment. Moreover this novel perfusion culture system enables the co-culturing of several cell types with or without contact between the different cell types. This is of importance when generating more relevant models for target organ toxicity testing over prolonged intervals, since many chronic toxic effects may be mediated by the release of inflammatory mediators from neighbouring cells.



Poster

Novel approaches for *in vitro* investigations concerning eczematous dermatitis and skin pigmentation

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During the last decade, a variety of models (2D, 3D) have been developed to provide useful methods for investigation of skin physiology and pathophysiology, respectively. The aim of this study was to develop reliable co-culture systems *in vitro*, which allow us to study questions concerning eczematous dermatitis and skin pigmentation.

To achieve a wide standardisation and a reproducibility of the results immortal human keratinocytes are used in both systems and depending on the focus these cells were co-cultured either with activated T cells or with melanocytes.

In case of eczematous dermatitis the model enables the reproduction of several clinical hallmarks of chronic inflammatory skin disease: T cell induced keratinocyte apoptosis (TUNEL-assay), reduced expression of the adhesion molecule E-cadherin, increased expression of intercellular adhesion molecule-1 and

upregulation of neutrophin-4. The protein expressions were analysed using the Western Blot technique.

Regarding the skin pigmentation the developed co-culture model enables the assessment of regulators of pigmentation by measuring the overall melanin content spectrophotometrically and observing visual changes in cell morphology. The well-known skin lighteners kojic acid and arbutin as well as the pigmentation enhancer IBMX showed the expected effects on the melanin content.

In summary, both co-cultured systems offer new and valuable tools for *in vitro* investigations of eczematous dermatitis or skin pigmentation. Besides they offer test systems for either therapeutics possibly influencing inflammatory skin diseases or formulations affecting skin pigmentation.

Poster

A novel collagen vitrigel scaffold that can facilitate a three-dimensional culture for reconstructing epithelial-mesenchymal models

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Conventional three-dimensional culture systems in comparison with two-dimensional ones have some demerits in that 1) the culture process is complicated, 2) the observation of cells by a phase-contrast microscope is difficult or impossible, 3) the reproducibility of the distribution of seeded cells is not always excellent, and 4) the aseptic handling for the medium change or for the co-culture of secondary cells is also hard. Opaque egg white or fish eyeballs prepared by boiling can be converted into thin, transparent, and rigid materials like glass by evaporating the moisture and this phenomenon is known as the vitrification of denatured proteins. We applied the vitrification technology to a type-I collagen gel and converted it into a rigid material like glass. We attempted to rehydrate the glass-like material and succeeded in preparing a novel stable state of collagen gel that is a

thin and transparent membrane with excellent gel strength. We named it "collagen vitrigel". Further, the framework-embedded collagen vitrigel scaffold that can be easily reversed by forceps was prepared by inserting a nylon membrane ring in the collagen solution prior to the gelation. Anchorage-dependent cells can be cultured on the both surfaces of the scaffold by the manipulation of two-dimensional cultures and consequently it resulted in reconstructing a three-dimensional organoid. An intestinal epithelial-mesenchymal model was reconstructed by co-culturing fibroblasts on the opposite side of the monolayered Caco-2 cells on the scaffold. These results demonstrate that the framework-embedded collagen vitrigel scaffold can provide an excellent three-dimensional culture system for reconstructing epithelial-mesenchymal models.



Lecture

Development of culture technologies reflecting *in vivo* conditions

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Various scaffolds have been prepared from natural, synthetic, and hybrid materials for maintaining the activity of functional cells, for regulating cell behaviour, and for reconstructing organoids [1]. I have developed five culture technologies reflecting *in vivo* conditions by utilising unique scaffolds; 1) the preparation of a multicellular hetero-spheroid composed of mesenchymal cells and epithelial cells utilising a scaffold made of the mixture of collagen and a thermo-responsive polymer [2], 2) the preparation of a three-dimensionally reconstructed multicellular mass (3-DRMM) with a medium accumulating or circulating system utilising naturally branched scaffolds made of rice fibrous roots or cotton-gauze, respectively [3], 3) a concept for organ engineering that can remodel an organ into an organoid by a continuous three-step perfusion to convert extracellular matri-

ces (ECMs) into a collagen scaffold [4], 4) a concept for cellomics study to culture cells on a scaffold made of animal tissue/organ sections for histopathology (TOSHI) that conserves the microarchitecture and component of the original tissue *in vivo* [5], and 5) the simple reconstruction of an epithelial-mesenchymal model utilising a nylon ring membrane-embedded collagen vitrigel, a thin collagen gel membrane [6].

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Lecture

Use of chromatin remodelling as a new way to create differentiated primary hepatocyte cultures

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Background/Aims: The level of histone acetylation, determined by the balance between histone acetyltransferase activity and histone deacetylase (HDAC) activity, is crucial for gene transcription. As such, the HDAC inhibitor Trichostatin A (TSA) has been shown to interfere with proliferation, differentiation and apoptosis in tumour cells, including hepatoma cell lines. Here, we investigated the effects of TSA and its metabolically more stable hydroxamic acid derivative, 5-(4-dimethylaminobenzoyl)-aminovaleric acid hydroxamide (4-Me2N-BAVAH) on cell cycle progression and survival in EGF-stimulated cultures of primary rat hepatocytes.

Methods: DNA replication was measured using [methyl-3H]-thymidine incorporation. Cell cycle, apoptosis and differentiation markers were analysed by western blotting. Albumin secretion was determined via ELISA.

Results: It was found that 1 μ M TSA and 50 μ M 4-Me2N-BAVAH completely abolished DNA synthesis and this was accompanied by induced histone H4 acetylation. In addition, in the presence of the HDAC inhibitors, the S-phase marker cdk1

was down-regulated, together with G1 cyclin D1. However, in the case of TSA, the decrease of cyclin D1 was dependent on the time of onset of treatment. For both TSA and 4-Me2N-BAVAH, the expression of p21 was not altered. In contrast to hepatoma cells, TSA and 4-Me2N-BAVAH also reduced spontaneous apoptosis as evidenced by a reduction of procaspase-3 cleavage and a decreased expression of pro-apoptotic Bid. Moreover, especially in the case of the TSA analogue, increased albumin secretion, CYP2B and Cx32 expression, together with decreased Cx26 and Cx43 expression were observed.

Conclusion: Our results show that besides the induction of an early p21-independent cell cycle arrest, TSA and in particular the TSA analogue 4-Me2N-BAVAH potentiate the anti-apoptotic effect of EGF in primary hepatocytes, and positively affect hepatocyte functioning. Therefore, we propose HDAC inhibitors as an innovative way to create functional long-term cultures of primary hepatocytes (PCT/EP2004/0012134).



Poster

Novel cell culture techniques revise the role of the sebaceous gland in human skin – the fantastic future of a skin appendage

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Sebaceous glands produce sebum been responsible for seborrhea and acne; seemingly, a limited and less attractive field to work in. In addition, the new EU Cosmetic Directive caused great concern to the cosmetic industry because of missing adequate human models. Therefore, it is of major importance for dermatological and cosmetic research that a silent revolution occurred during the last years, which assisted the revision of sebaceous gland role in human skin: The development of sebaceous gland cell culture techniques and the establishment of human sebaceous cell lines (patent DE19903920). This skin appendage has turned to be an organelle with major involvement in skin homeostasis: Sebaceous lipid fractions are responsible for the three-dimensional organisation of skin surface lipids and the integrity of skin barrier and sebum transports antioxidants to

the skin surface. Moreover, sebaceous PAF acetylhydrolase-II was found to protect the skin against oxidative stress and, especially, epidermal keratinocytes against UVB irradiation, and the sebum-specific fatty acid C16:1 Δ 6 to exhibit innate antimicrobial activity. New fascinating data were acquired indicating that sebocytes express pro- and anti-inflammatory properties, present a regulatory program for neuropeptides, synthesise cholesterol *de novo* and use it in an own steroidogenic program, and selectively control the action of vitamins, hormones and xenobiotics on the skin. Sebaceous glands were shown to be “the brain of the skin” and to fulfil all requirements making skin an independent peripheral endocrine organ. Several further sebaceous gland functions are currently under investigation. Novel cell culture techniques made the future of sebaceous glands look fantastic!