Linz 2007
Abstracts of All Lectures and Posters

In alphabetical order of the first authors – alphabetisch nach Erstautor/inn/en geordnet

Lecture: free communications

In vitro alternatives to animal models for screening and evaluation of anticonvulsive compounds

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In vitro models of pharmacosensitive and pharmacoresistant epileptiform activity have been developed on the basis of organotypic slice cultures of hippocampus. Slice cultures (on average 25/animal) are prepared from 6 to 10-day old rats and cultured under 5% CO_2. Single neuron and neuron population activities as well as extracellular K^+ concentrations are recorded with microelectrodes and ion sensitive electrodes, respectively. The drugs tested were carbamazepine, phenytoin, sodium valproate, phenobarbital sodium, diazepam, clonazepam, gabapentin and ethosuximide. Drug concentrations *in vitro* corresponded to upper therapeutic-low toxic ranges in the brain extracellular fluid of patients treated with the respective antiepileptic drugs. High frequency stimulation of Schaffer collaterals in CA1 of hippocampus induces a primary afterdischarge (PAD) in the hippocampus. The PAD consists of a tonic and clonic component, lasts between 10–30 s and can be interpreted as an electrographic correlate of the maximal electroshock seizure (MES) test in animals. The pharmacosensitivity of the PAD is similar to that of the MES-Test: the PAD is inhibited by phenytoin and carbamazepin and not by ethosuximide. 1,4-benzodiazepines and sodium valproate cause a partial suppression of the PAD only. Low magnesium or blockade of K^+-channels reliably induces recurrent tonic-clonic seizure like activity in the hippocampus and the dentate gyrus. In more than 94% of 120 slice cultures investigated so far the studied antiepileptic drugs failed to block induction of epileptiform activities even when toxic concentrations were applied. The pharmacoresistance persisted over the time *in vitro* explored so far (2 months). We present here two simple to establish *in vitro* models, one of pharmacosensitive, the other of pharmacoresistant epileptiform activity of which the latter in comparison to respective animal models is a priori pharmacoresistent. After further validation the *in vitro* models might be well suited for inclusion in the panel of tests used for the identification of new antiepileptic compounds thus reducing the number of animals currently used in anticonvulsive drug screening and testing and eventually replacing the respective animal models.

Supported by set (Stiftung zur Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen).

Keywords: hippocampal slice culture, seizure-like activity, antiepileptic drugs, pharmacosensitive, pharmacoresistant

Lecture: computer assisted procedures

Knowledge based search – a new search machine for knowledge intense domains

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The next generation search engines are intelligent assistants. With the help of background knowledge Transinsight's search technologies speeds up the search (for answers) significantly and gives an overview over large query results. When people search, they have questions in mind. For example if writing a report on 1) chemical testing, 2) animal welfare or 3) animal use alternatives, relevant information about the keywords in the right context are needed in order to compile a comprehensive report. Today's search technologies are by fare not elaborated enough to cope with the complexity of such topics. Although texts hold answers to most questions it is very difficult to obtain them with classical search engines, as they merely present possibly long lists of search results and leave it up to the user to find the answer to his/her question. To find answers rather than to just search for them, our next-generation search engines are intelligent and use background knowledge and boost search to a next level of intelligence. The biomedical Web 2.0 search engine www.GoPubMed.org is now online. In the talk we give examples of how knowledge can improve searching significantly.

Keywords: animal use alternatives, search engines, GoPubMed ontology, semantic web, background knowledge, mesh, gene ontology

Poster: free communications

Fabrication and validation of corneal holders for porcine corneal opacity and permeability assay

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The bovine corneal opacity and permeability assay (BCOP) has been endorsed by the ECVAM Scientific Advisory Committee as an alternative method to *in vivo* rabbit eye irritation test. Since porcine corneas are more similar to humans than those of bovine and fresh porcine corneas are available locally, this study aims at using porcine corneas for conducting the porcine corneal opacity and permeability assay (PCOP). As porcine eyes have different size and shape from bovine eyes and porcine corneal holders are not available commercially, a set of new corneal holders is needed to hold the porcine cornea in a natural manner during experiment. New corneal holders were constructed with information from non-contact laser scanning of the anterior surface of porcine eyes. Modifications were made until the holders could hold the porcine cornea without wrinkles and there was no leakage of culture medium from inside. The holders also fit in a stand suitable for measurement with a UV-visible spectrophotometer in order to measure the opacity of the corneas. A set of glass windows located at both ends of the holders was checked to ensure minimal absorbance at the wavelength used. The suitability of these corneal holders for eye irritation testing was validated by studies regarding corneal morphology and histology after mounting in the holder with and without irritants (absolute ethanol, acetone and 1% benzalkonium chloride). The holders were utilized in PCOP experiments with a few irritants using the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) protocol for BCOP. The *in vitro* irritation scores of negative control (normal saline solution), positive control (absolute ethanol) and irritants (acetone and 1% benzalkonium chloride) were 0.5, 29.4, 39.7 and 28.9, respectively. Therefore, preliminary PCOP experiments using these corneal holders could distinguish the non-irritant from the irritants. The new corneal holders could retain fresh porcine corneas in natural manner during experiments without damaging corneal tissue and were suitable to use in PCOP assay.

Keywords: PCOP, corneal holders, eye irritation testing alternative, porcine

Poster: 7th cosmetics amendment

Allergenicity testing using plasmacytoid dendritic cells

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An *in vitro* predictive test system for assessing the allergenicity potential of substances will have utility throughout industry to monitor products for contact allergenicity. Development of such non-animal alternative assay systems for skin sensitization hazard assessment is within the provisions of the European Union chemicals policy known as REACH (Registration, Evaluation, and Authorization of Chemicals). We investigated whether phenotypic and functional changes to subset of dendritic cells (DC), plasmacytoid DC (pDC), could be used to identify allergens. To achieve this goal, normal human DC were generated from CD34+ progenitor cells and cryopreserved. Frozen DC were thawed and the pDC fraction (CD123+/CD11c-) was harvested using FACS sorting. The pDC were cultured, expanded, and pulsed with chemical allergens (n=13) or irritants (n=7). Sub-toxic concentrations of each chemical were determined using FACS analysis of propidium iodide stained cells. Results showed that exposure of pDC (n=2-5 donors) to allergens induced a 1.5 fold expression of CD86 for 12 of 13 allergens tested. On the other hand, 7 of 7 non-allergens did not result in increased CD86 expression. Based on these results, a preliminary prediction model was developed to identify chemical allergens (sensitivity = 91-93% and specificity = 93-100%). In conclusion, CD86 expression in pDC appears to be a sensitive and specific predictor of allergenicity of chemicals. When compared with existing animal models, the assay is advantageous because high throughput screening of chemicals using cells of human origin is possible at low cost.

Keywords: plasmacytoid dendritic cells, skin sensitization and allergy, CD86 expression, REACH

Poster: free communications

Irritation screening of vaginal-care products using EpiVaginal™, a human vaginal-ectocervical tissue model

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The utility of an organotypic vaginal-ectocervical (VEC) tissue model, composed on normal human cells, to test the irritation potential of vaginally-applied chemicals and their formulations was examined. Histologically, the VEC tissues have nucleated basal and parabasal cell layer and glycogenated intermediate and superficial layers. To insure tissue reproducibility, standardized quality control (QC) tests utilized the MTT assay to determine the exposure time necessary to decrease the tissue viability to 50% (ET_{50}). ET_{50} results for the positive control (1% Triton X-100) showed the tissues to be highly reproducible; the average intra-lot coefficient of variation (CV) was less than 10% and ET_{50}s averaged 1.40 h ± 0.26 (n=55 lots). Endpoints including MTT ET_{50}, histology, RT-PCR, and cytokine release patterns were used to evaluate 20 commercially available test articles. Upon exposure to test articles, the tissue model was able to discriminate between the mildness of test articles. The ET_{50} values ranged between 3.5-7.0 h for contraceptives, between 6.9- >18 h for anti-itch creams, and between 1.7-2.7 h for feminine washes. Released cytokines and gene expression levels showed that IL-1α, IL-1β, IL-6, and IL-8 were associated with toxicity of test materials. In conclusion, the VEC tissue model will serve as a useful, highly reproducible, non-animal test method to assess the irritation potential of vaginally applied chemicals and their formulations. Models such as the epivaginal tissue which mimic the vaginal microenvironment could provide essential data set for early identification of the biological/toxicological property of chemicals/products intended for vaginal use. Development of such *in vitro* test model for risk-assessment and management of chemicals is in line with the new European Union chemicals policy known as REACH.

Keywords: EpiVaginal, organotypic vaginal tissue model, irritation, women's care products

Poster: free communications

Alternative *in vitro* method for detection of pertussis toxin in vaccines to replace the mandatory animal test

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To guarantee non-hazardous application, the detoxification of vaccine against Whooping Cough (Pertussis) has to be monitored for residual Pertussis Toxin (PT) activity under standardized conditions. The traditional procedure to determine toxicity is done by animal testing, with the involvement of high resource (at least 15 mice per test) and personnel costs. Due to that an alternative testing method has to be developed. The literature describes the activation of monocytes by PT. This effect was attempted to utilise by applying the Monocyte Activation Test (MAT). For this reason, human whole blood was incubated with PT and monocyte activation is measured by ELISA. PT is produced by the Gram-negative bacterium *Bordetella pertussis* and, therefore, it is naturally contaminated with Endotoxin (Lipopolysaccharide, LPS). The latter represents the most potent monocyte stimulator. We could detect LPS in each PT preparation tested without having any chance to remove it due to the high affinity of LPS to PT. Furthermore, blocking of CD14 as the LPS receptor on monocyte's surface by monoclonal antibodies abolished monocyte activation by PT. In consequence, MAT is not suited for the estimation of PT activity. Therefore, a new principle of detection has to be found. To achieve this, the fluorescence characteristics of etheno-NAD (1,N6-Ethenonicotinamide adenine dinucleotide) are thought to be exploited. Etheno-NAD represents the fluorescence analogon of the natural substrate NAD (Nicotinamide adenine dinucleotide) of PT. In our new test, PT hydrolyses etheno-NAD into Nicotinamide and etheno-ADP-Ribose (1,N6-Ethenoadenosine 5'-diphosphoribose). The cleavage of etheno-NAD leads to an increase of fluorescence. An alternative approach to assess PT activity is to measure these increasing fluorescence signals. The data obtained with this new alternative test principle for the estimation of active PT are discussed in the poster.

Keywords: drug safety, alternative methods, pertussis toxin, monocyte activation test, fluorescence assay

Lecture: good cell culture practice

Establishment of realistic *in vitro* models of angiogenesis: How to make the right choice

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Angiogenesis, the sprouting of new vessels from pre-existing ones and its inhibition, so-called anti-angiogenesis, are in the focus of modern medicine. In particular, a promising strategy of cancer treatment combines chemo-, radio- and anti-angiogenic therapy. Identification and evaluation of angiogenic and anti-angiogenic factors are still carried out in animal models like the cornea model or dorsal skinfold chamber where substances are tested in rabbits, mice and hamsters. The aim of our studies is the replacement of these animal models by *in vitro* models of angiogenesis and anti-angiogenesis. We have established *in vitro* models of angiogenesis based on cultivated microvascular endothelial cells of different species and tissues. In the bovine model a method for quantitation of angiogenesis and anti-angiogenesis was developed which should be adapted to different human endothelial cell cultures (from heart, lung and two foreskins). This method is based on the staging of the angiogenic cascade in strictly defined stages and thus allows quantitation of all phases of angiogenesis up to the development of capillary-like structures. Stimulated by a selective medium the bovine cultures run through all defined stages of angiogenesis within 60 days. A similar run of angiogenesis could be observed in one of the foreskin and the heart derived human cultures. In the other foreskin derived cells angiogenesis remained at a certain stage after 30 days of culturing. In endothelial cells of the lung angiogenesis began running backwards from day 30 onward. Immunolocalization of endothelial expression of collagen type IV, an essential component of the basal lamina, showed a slow but continuous expression in the bovine cultures over the entire cultiva-

tion period. These observations were confirmed in the "angiogenic" heart and foreskin derived cultures which also ran through all stages of angiogenesis. In opposite, in the "non angiogenic" cultures of lung and foreskin collagen type IV was expressed very moderately resulting in only a fragmentary extracellular collagenous network. Our results show that only particular microvascular endothelial cells can be stimulated to run through all stages of angiogenesis *in vitro*. Therefore only specific cultures may be used for the establishment of realistic *in vitro* models of angiogenesis. One potential reason for this may be the ability of the cells to express collagen type IV. Further investigations are on the way to identify the expression profile of endothelial cells to be chosen for *in vitro* models of angiogenesis.

Keywords: in vitro model, angiogenesis, endothelial cells, collagen type IV

Lecture: ethical and legal aspects in animal experimentation

Trial for personhood of a chimp

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Matthew "Hiasl" Pan is a chimp, who was abducted from West Africa in 1982 to Vienna in Austria to be used for experiments on AIDS and Hepatitis. Since his abduction was illegal, he was taken in by customs and handed over to an animal shelter. A caretaker took him home and raised him like a child in his family. The company Immuno, who had instigated Matthew's abduction, won the legal procedures to get him back. However, activists prevented the chimp being handed over. Later, chimp experiments were ended and Matthew was formally sold to the animal shelter. However, in 2007 the shelter went bankrupt. That meant Matthew could be sold and deported. As someone, who was abducted as a small child, with most likely his mother being killed in the process in front of his eyes, who was brought to an alien environment and who spent most of his life locked up, and hence cannot look after himself and safeguard his interests, and as someone, who is in imminent danger of being deported, he is the paradigmatic example of a person needing a legal guardian. If, indeed, he is a person. Hence, in February 2007, I did an application to the district court in Mödling (Austria) for a legal guardian for Matthew Pan. We argued that he is a person according to Austrian law. Firstly, that is because the law says that every human is a person. The only possible definition of a human within the law is the biological definition of genus. Scientists argue biologically that chimps belong to the genus homo and hence Matthew Pan is a human and a person according to the law. Secondly, the law clearly differentiates between human and person. All humans are persons but not all persons are humans. The definition of personhood must be sought in the philosophy of the enlightening era and is best scientifically described by what is referred to as "theory of mind", the ability to recognise intentional states – and hence personhood – in others. Persons are all those beings, who recognise personhood in each other. Scientists say that chimps have a "theory of mind", Matthew has himself passed the mirror self-recognition MSR test. Hence he is a person and deserves a legal guardian. In practice, that would mean he is not someone else's property but belongs to himself. He can receive his own donations and safeguard his own future by legally fighting deportation procedures. Only as a person represented through a legal guardian will his interests be recognised and represented in a court of law. Only then is justice possible. Eventually, that means he could sue those responsible for his abduction and his misery for damages.

Keywords: personhood, animal rights, chimp, legal guardian

Lecture: free communications

3Rs in the field of radiopharmaceutical research and development

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The aim of the present publication is to collect possible alternative methods in the field of radiopharmaceutical research and development that could serve the idea of the 3Rs, namely replacing the animal tests or, if this is not possible, reducing the number of them and refining the techniques. *In vitro* cell-radiopharmaceutical binding assays (receptor-binding assay, immune-binding assay and non-specific binding), the superfine, specific and sensitive nanoSPECT/CT (Bioscan/Mediso Ltd) imaging method and the possible use of spontaneously occurring veterinary patients will be considered as available alternative methods in the field. *In vitro* cell binding assays proved to be replacing the *in vivo* rodent xenograft models in the preselection of possible radiopharmaceutical candidates. The nanoSPECT/CT hybrid (fusion) imaging method allows high-resolution pictures including correct anatomical structures and quantified functional content at different times after radiopharmaceutical application using a single, anaesthetized animal that need not be sacrificed at the end of the process. Re-use of laboratory animals allowed us to compare the characteristics of different agents on the very same biological model. Spontaneously occurring canine, feline and egzotic animal diseases are a constant source of animal models for radiopharmacists as well. Osteosarcoma, mammary gland carcinoma, thyroid carcinoma, brain tumours and a lot of others in dogs and cats might be the best known animal models of the appropriate human diseases. Diagnosing and then treating them with the most promising human methods provides a chance for the suffering animals and produces useful data for human oncologists and biomedical researchers. This work was conducted with the financial support of Mediso and Bioscan Ltd, Medi-Radiopharma Ltd, and under the umbrella of different international scientific programmes (EMIL-NoE 503569-2.2, IAEA CRP No E1.30.33 and Indo-Hungarian Intergovernmental Programme No 2003/07).

Keywords: radiopharmaceuticals, nanoSPECT/CT, spontaneous diseases

Poster: free communications

Hungarian consensus platform on alternatives – here we go!

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A novel organization would like to introduce itself for the international partners working in the same field. The Hungarian Consensus Platform on Alternatives (hucopa) was grounded by 12 founding members in Budapest and has been starting to work. Hucopa is a full-right member of the European mother organization (European Consensus Platform on Alternatives – ecopa) and similarly consists of the 4 relevant platforms as: the Academy (research and education), the Industry (pharmaceutical, chemical and other companies), the Animal Welfare (animal welfare and right organizations) and the Authority (government people). The main goal of hucopa is to increase the knowledge about alternative methods in the public and specialists by the theory of 3Rs recommendations. Performing the above listed activities hucopa organizes 2 meetings in a year (one for the 4 platform specialists and another one for the public), platform-leaders and other key-personals participate as many as possible other national and international meetings, where they provide information also about the activities of hucopa, take a close co-operation with the European mother organization (ecopa) and constantly communicate with the prominents of the 4 platforms and the medias. Hucopa wants to open and work-together also with other national 3Rs organizations. This work was conducted by the financial support of Mediso and Bioscan Ltd, Medi-Radiopharma Ltd, and under the umbrella of different international scientific programmes (EMIL-NoE 503569-2.2, IAEA CRP No E1.30.33 and Indo-Hungarian Intergovernmental Programme No 2003/07) (www.hucopa.com).

Keywords: new organization for alternative methods

Poster: free communications

Use of skin derived bovine dendritic cells to discriminate between potential haptens and irritants

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Animal experiments are often used to determine allergenic or irritating potential of substances. To reduce, refine and replace *in vivo* systems, new methods such as characterisations of surface antigen expression are currently under development. In the present study, we evaluated the maturation of bovine skin-derived dendritic cells (DC) to given hapten/irritants by means of surface antigen expression. In contrast to *in vitro* analysis of DC derived by other methods, our approach takes advantage of a physiological skin barrier, which has to be penetrated by a given substance before a reaction occurs, thus more likely mimicking the *in vivo* situation. To analyse the expression of different surface markers, dermatomized bovine udder skin (thickness 100 µm) was topically treated with known haptens and irritants. Skin samples were cultured in medium, incubated for 48 hours, emigrated DC were harvested and analysed by flow-cytometry for expression of CD14, CD80, CD86 und MHC II. TDI and DNCB in concentrations of 0.05% and 0.5% were used as strong sensitizing haptens, whereas SDS in concentrations of 0.1%, 0.5% and 1.0% was used as a strong irritant. These concentrations were chosen based on our unpublished observation that higher concentrations induced cell-death. Exposure of DC to TDI and DNCB in tested concentrations led to an up-regulation of the activation markers CD80, CD86 and MHC II, while CD14 (a monocyte marker) was dose dependently down-regulated. In contrast, exposure of DC to SDS up-regulated CD80/86 expression but down-regulated MHC II expression. Our results indicate the potential use of flow cytometric analysis to discriminate between cellular reactions to allergens/haptens and irritants. As the bovine udder has no commercial value and is accessible in large amounts, the proposed system may provide a useful approach to test such substances.

This study is supported by ZEBET (WK 3-1328-175).

Keywords: dendritic cells, cell culture, contact sensitivty, activation makers

Lecture: nanotoxicology

A newly developed *in vitro* model of the human epithelial airway barrier to study the toxic potential of nanoparticles

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With the advent of nanotechnology, the prospects of manufactured nanomaterials in many applications have progressed rapidly. The potential health effects of these nanoparticles (diameter less than 0.1 µm) associated with human exposure are unknown. In order to avoid and to replace toxicity studies with animals which are time consuming and stressful to the animals we have established a triple cell co-culture system composed of epithelial cells, macrophages and dendritic cells which simulates the most important barrier functions of the epithelial airway: the surface active phospholipids, a tight epithelium, and cells of the defence system. The exposition of cell cultures at the air-liquid interface with polystyrene nanoparticles (0.05 µm) was done using a hand-held microsprayer. Quantification of particles and intracellular localisation of the particles was studied by laser scanning and transmission electron microscopy. The visualization and the quantification of the polystyrene particles by laser scanning microscopy revealed that the particles entered all cell types, however, to a different extent. Using transmission electron microscopy we could show that the particles were

within the cells either in membrane-bound agglomerates or as single particles free in the cytoplasm, hence having direct access to cytoplasmic proteins. Penetration of nanoparticles into mitochondria and the nucleus could be shown. Our *in vitro* triple cell co-culture model of the epithelial airway barrier offers a great tool to study particle-lung cell interactions at the nanostructural level. Using the model and advanced microscopic techniques we have shown that nanoparticles (i.e. polystyrene particles) entered the epithelial cells as well as the cells of the defence system. Nanoparticles were found intracellularly membrane-bound or free in the cytoplasm as well as in organelles, like in mitochondria and in the nucleus. This points to their enormous toxic and carcinogenic potential by interfering with the respiratory chain in the mitochondria and with the DNA in the nucleus and perhaps also in the mitochondria.

Keywords: epithelial airway model, nanoparticles, toxicity, laser scanning microscopy, transmission electron microscopy

Poster: free communications

In vitro testing of the biocompatibility of degradable magnesium alloys for use in biomedical technology

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Magnesium alloys are a promising new material for the production of biodegradable implants in biomedical technology. While their technical properties are well characterised, there is only limited information available concerning their degradation behaviour in biological matrices and their effect on human or animal organisms. In the present studies a variety of *in vitro* examinations were conducted to establish a test facility that enables the pre-selection of suitable candidates for further alloy development.

In cell culture investigations alloy degradation was examined by scanning electron microscopy (SEM) and inductively coupled plasma (ICP) measurements. The influence of the alloy elements on the viability and proliferation of murine keratinocytes (MSC-P5) and murine fibroblasts (L929) was examined by supplementing the culture medium with salts and by co-cultivation of the cells with alloy samples using membrane inserts. Using the *in vitro* model of the isolated perfused bovine udder, acute effects of alloys on skin, connective tissue and teat mucosa were investigated after subcutaneous and intracisternal (teat) incubation of samples. Degradation and tissue distribution of the alloy elements were investigated by microdialysis and microscopy. As parameters for tissue irritation the viability and the release of pro-inflammatory mediators (prostaglandin E2, tumour necrosis factor alpha) was determined.

As a great variety of chemical elements may be alloyed with magnesium, a high number of different materials have to be evaluated concerning their biocompatibility. By using the presented *in vitro* models to pre-select promising candidates for *in vivo* trials, the use of laboratory animals can be significantly reduced.

These studies are supported by the Deutsche Forschungsgemeinschaft (DFG) as part of the collaborative research centre (SFB) 599: "Biomedical Technology".

Keywords: biocompatibility, implants

Poster: nanotoxicology

In vitro three dimensional trachea model

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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 as for example nanoparticles. 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. This model could be used as a test system for the analysis of different substances and it could be also used as a biological graft. For the construction of a three-dimensional trachea model, we isolate porcine cells with an enzymatic treatment and seed them in preliminary tests on 24-well inserts with a pore size of 1 µm. For further tests, the colonisation also occurs in an air liquid interface in a special biomembrane reactor with a pulsatile flow of the optimized cell culture medium and regulate breathing rate. The colonisation occurs with Airway Epithelial Cell Growth Medium and by way of comparison in DMEM Medium with different supplements. The cell characterization occurs *via* histological and immuno-histological methods and measurement of the ciliary beat frequency. In preliminary tests porcine respiratory cells were cultivated on inserts and they show a longterm but no metachrone ciliary activity. The cells were also cultivated on an acellularized scaffold in the bioreactor. Most of these cells are vital and show a high rate of proliferation and also longterm ciliary activity. There are ongoing experiments with a co-culture of fibroblast cells and ciliated epithelium cells and tests to initiate the metachrone ciliary beat in the bioreactor system. Our aim is to develop a functional tubular trachea test system in a bioreactor in which the different cells of the trachea are cultivated as a co-culture under physiological conditions and simulation of the respiration.

Keywords: trachea, three-dimensional test system, primary cells, nanotechnology

Poster: EU-chemicals policy (REACH)

Bio-analytic silicon chips for the detection of developmental-neurotoxic effects of chemicals and drugs in the context of the European REACH program

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The measurement of cellular reactions under controlled *in vitro* conditions in cell monitoring systems (CMS®) has been established in our group in 1992. CMS® permit for non-invasive measurements of different parameters such as acidification, respiration and adhesion by the use of micro-sensors. Multi-electrode arrays (MEAs) can be applied to detect the electric activity in neuronal networks. Both features are combined on a silicon neuro-chip allowing for parallel measurements of metabolic and electrical network parameters. The chip allows for multi-parametric monitoring of neurotoxic effects in cellular systems for hours and up to days. These properties are key features in drug development, high-content screening, and quantitative safety classifications in neurotoxicity as well as developmental-neurotoxicity studies. This may also be important for the industry when approximately 30,000 existing chemicals will be evaluated for their toxicological properties under the REACH program of the European Commission. We work on the reduction of primary cell use by establishing differentiated stem cell lines on our chips. Appropriate culture protocols for the promising D3-cell line (ATCC), guaranteeing the viability and the differentiation of the cells into neuronal networks are currently developed. New protocols aim at the development of spontaneous electrical activity in the network. The metabolic activity of the differentiating cells is detected by the Bionas® 2500 analyzing system with six parallel SC1000-chip modules that were solely designed for metabolic measurements. This system allows for the evaluation of three additional potential end-points (cell adhesion, acidification and oxygen consumption) in addition to the electrical network activity. The dose-dependent responses for different test substances were analyzed with special attention to effects at the different developmental stages of the neuronal cell systems. In addition, the prototype of a new glass neuro-chip has been developed, that is compatible to our silicon-chip system (see poster of Koester et al.). The glass neuro-chip ensures microscopic observability in future experiments.

Keywords: lab-on-chip, biochip, REACH, multi-electrode array, neuro-sensorchip, glass chip, neuronal networks, developmental neurotoxicology, DNT, neurotoxicity, action potential detection

Lecture: free communications

Pulmonary cell culture models to study safety and efficacy of innovative aerosol medicines

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The determination of drug absorption after aerosol deposition is an important step in the development of new aerosol medicines. Because of the complexity of the inhalation process mainly animal experiments are used to predict the *in vivo* drug absorption rate. Even if different cell lines like Calu-3 and A549 are widely used, an *in vivo* realistic deposition of medical aerosol particles on cell models is not yet realized. In our study we investigated the influence of deposition conditions like deposition device, fluid volume, particle size, and particle wettability on the absorption rate of pharmaceutical aerosols across cell models. We applied drug solutions/suspension via pipetting, or dry aerosol particles via an insufflator syringe or with the aid of a multi stage cascade impinger on dry cell monolayers and measured subsequently the drug absorption rate. Commercial available salbutamol and budesonide aerosols with different solubility and particle sizes were used. In summary, air interface deposition on dry epithelial surface resulted in increased absorption rates compared with liquid interface application. The fluid volume in the apical compartment controls at first the drug release rate from particles. Thereafter, the fluid volume influences the concentration gradient, which is according to Fick's first law the motor of passive transport processes. The limitations of the air interface deposition with the relatively simple insufflator syringe, however, become apparent in cases of drug formulations where aerodynamic properties of the aerosolized powder particles get critical. To address also aerodynamic properties more sophisticated setups like some modified multi stage liquid impinger can be used. Air interface deposition on pulmonary cell culture models offers a way to simulate the most important peculiarity of aerosol drug delivery: absorption of a relatively high metered dose after deposition on a slightly wetted epithelial surface. Therefore, this approach is an attractive alternative to inhalation experiments on laboratory animals and may provide important additional information about pulmonary permeability of active pharmacological ingredients, as well as about safety and efficacy of new excipients (e.g. polymers, surfactants etc.) and formulations (micro- and nanoparticles, liposomes etc.).

Keywords: alveolar epithelium, drug absorption, mucus, in vitro

Poster: ecotoxicology

Development of a screening assay for teratogenicity: combination of DarT with a mammalian metabolic activation system

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The assessment of teratogenic effects of chemicals is generally performed using *in vivo* assays in rats and rabbits. Following the 3R concept and public expectations, the use of alternative methods is promoted to reduce the number of animal tests. From this perspective, we have developed an *in vitro* test with the zebrafish *Danio rerio* embryo test (DarT) combined with an exogenous mammalian metabolic activation system (MAS), able to biotransform proteratogenic compounds. Cyclophosphamide and Benzo[a]pyrene were used as proteratogens to test the efficiency of this assay. Briefly, the zebrafish embryos were co-cultured at 2 hpf (hours post fecundation) with varying concentrations of the test materials, induced male rat liver microsomes and NADPH for 60 min at 32°C under moderate agitation in Tris-buffer. The negative control (test material alone) and the vehicle control (MAS alone) were incubated in parallel. For each parameter, 20 eggs were used for statistical robustness. Afterwards fish embryos were transferred individually into 24-well plates filled with fish medium for the next 48 hours at 26°C with a 12 hour-light cycle. Terato-

genicity was scored using morphological endpoints. No teratogenic effects were observed in fish embryos exposed to the proteratogens alone, i.e. without metabolic activation. In contrast, Cyclophosphamide and Benzo[a]pyrene induced teratogenic effects in fish embryos in the presence of the metabolic activation system. The severity of malformations increased with the proteratogen concentration. We conclude that the application of the MAS will improve and refine DarT as a predictive and valuable alternative method to screen teratogenic compounds.

Keywords: zebrafish, embryo, MAS, cyclophosphamide, benzo[a]pyrene

Poster: free communications

Toxicity of TBTC and Arsenic on adult and fetal blood progenitors

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Many environmental pollutants target the hematopoietic system, aging directly on adult tissue (bone marrow) or, indirectly, on the fetal progenitors (umbilical cord blood) by crossing the placental barrier. Tributyltin Chloride (TBTC) and Arsenic (Sodium Arsenite) are widely present in the environment as agricultural pesticides or contaminants, and in fewer amounts, in plastic, glass or ceramic industry. Both the compounds are toxic *in vivo* on different hematopoietic compartments, indeed TBTC causes thymus atrophy in rodents, depletion of lymphocytes in spleen and lymph nodes and alteration of serum immunoglobulin levels, while Arsenic induces immunotoxicity, in addition to skin and lung cancer and cardiovascular lesions. The aim of this study was to evaluate the *in vitro* toxicity of these compounds on human blood progenitors, comparing their effect on the capability to clone (GM-CFU) of bone marrow (BMC) and cord blood mononuclear cells (CBC). Our results indicated that both the compounds have a relevant toxic effect both on adult and fetal tissue, being TBTC much more toxic than Arsenic. BM resulted more sensitive than CBC, being IC_{50} of TBTC in this tissue (0.03 µM) two times lower than in CBC (0.07 µM), and IC_{50} of Arsenic (0.34 µM) even 10 times lower than in CBC (1.22 µM). Moreover, at very low concentrations (0.006 µM), TBCT induced a strong significant increase of clonogenicity of BM progenitors, differently than in CBC. Our data indicate that human hematopoietic progenitors are strongly affected by the toxic effect of Arsenic and TBTC. Bone marrow GM-CFU resulted more sensitive than cord blood, in particular after Arsenic treatment. Most colony forming cells are found in $CD34^+$ fraction, which is higher in BM than CB. Although CB contains a lower number of $CD34^+$, they have a higher capacity to form colonies. This could explain CB less sensitivity to the toxic effect of these chemicals. Further investigations will be needed to check this hypothesis or to identify different mechanisms of action exerted on the two cell populations. Taking into account that these xenobiotics pass through the placental barrier, the effects of their exposure are of great concern for health on both adults and next generations.

Keywords: TBTC, arsenic, blood progenitors

Poster: nanotoxicology

Toxicological investigation of nanoparticles – effects on human cells

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The aim of the present study was the establishment of an *in vitro* test system to reveal the potential risk to human health of nanoparticles at the workplace. The essential advantage of *in vitro* investigations is to be non-invasive, the employees don't have to be bothered and the work routine doesn't have to be intercepted. At occupational settings test cells on Transwell® inserts were exposed to the workplace atmosphere or to particle filtered air for 1 to 3 hrs using a CULTEX®

System. Two types of co-cultures were tested: In the first type differentiated macrophages were exposed and post-incubated with human lung epithelial cells. In the second type differentiated macrophages were seeded on human lung epithelial cells and the co-culture was exposed. As endpoints for particle exposure cell viability (WST-1 assay), oxidative stress (DHR-Assay) and pro-inflammatory cytokines (BDTM CBA-Assay) were evaluated. Cell viability testing showed a negative effect at high exposure. In cells exposed to the workplace atmosphere an increased oxidative burst was detected compared to cells exposed to particle filtered air. Exposure of co-cultures resulted in significantly enhanced TNF-α, IL-6, IL-1β and IL-8 levels. We could show that our *in vitro* exposure system is very well adapted for the assessment of adverse effects of nanoparticles at the workplace. Our results indicate that nanoparticles involve an occupational risk and further experiments will be performed to analyse additional endpoints.

Keywords: in vitro test, co-culture, nanoparticles, workplace

Poster: good cell culture practice

Possibility to assess the harmfulness of minerals using cell culture

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The usefulness of cell cultures in trace elements research will be illustrated by the presented experiences. The cell types used in the present study were supplied by ICN Flow (UK): NBL-1 (MDBK)-bovine kidney; HEp-2-human carcinoma of larynx; J-111-human monocytic leukaemia; Chang liver, human liver; HeLaS3-clone of the human HeLa; MMT 060562-mouse mammary tumour; MG-63-human osteogenic sarcoma and A-549-human lung carcinoma. The culture medium was supplemented with the salts dissolved in PBS. After 72-hour incubation the proportion of live cells was determined. The principle of the measurement is the biological oxidation of cells: the assay contains tetrasolium salt (MTS) and electron uncoupling compound (PMS). The MTS is reduced to formazan by the live cells. The coloured formazan produced dissolves in the culture medium and can be measured directly. The quantity of arising formazan is proportional to the living cell count. The 50% cell multiplication inhibitory concentration (IC_{50}) can be calculated by regression analysis. Low cadmium sulphate concentrations abruptly inhibit cell multiplication without any transition. The IC_{50}-values of the different cell lines are in the same range (0.025 to 0.082 mmol/l). Based on these data, cadmium can be treated as a general cell toxicant. The IC_{50}-value of ammonium molibdate was between 0.21 and 1.03, for nickel ammonium sulphate between 0.09 and 0.42 and for cobalt sulphate between 0.17 and 0.53 mmol/l. Thus the inhibitory concentrations of these three nutritive compounds are in the same range. Based on this series of trials, and comparing the IC_{50}-values of Cd and the Ni, Mo, and Co salts, one can state that the toxic or nutritive effect of a given trace element can be evaluated only in comparison with the IC_{50} of a biologically known chemical compound. Since the inhibitory concentrations of cadmium sulphate are ten- to fifty-fold (in the range of 0.025 to 0.082 mmol/l) lower than those of the essential trace elements (in the range of 0.09 to 1.03 mmol/l), cadmium sulphate can be used as a reference compound in this kind of testing.

Keywords: cell lines, cadmium, nickel, cobalt, molybdenum, IC_{50}, reference compound

Poster: free communications

In tube evaluation of the effect of cobalt, nickel and albendazol upon rumen fermentation

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In vivo investigations have been carried out in order to determine the effect of albendazol, cobalt and molybdenum on microbial fermentation in the rumen. Four adult sheep were used in the tests. The rumen cannulated wethers had an average weight of 75 kg. Their basal diet consisted of 2 kg meadow hay and 0.3 kg corn meal. In the *in vitro* trial (Phase 1) one experi-

mental group was formed by 4 tubes for each treatment. One of them served as the positive control, containing only untreated ruminal fluid from the sheep (but receiving added starch and fibre as energy source), the others were supplemented with albendazol, cobalt + molybdenum or albendazol + cobalt + molybdenum containing salt solutions. The concentrations used were as follows: albendazol 10, cobalt (as $CoCl_2$) 15 and molybdenum (as ammonium molybdenate) 18 mg/litre. *In vivo* trial (Phase 1): During 14 days after the first sampling (to obtain ruminal fluid for the *in vitro* study), the animals received salt solution containing albendazol, cobalt + molybdenum and albendazol + cobalt + molybdenum, daily into their rumen through the cannula. The quantities of salts effected the same concentrations as in case of the *in vitro* trial. On day 14, "treated" (adapted) rumen fluid was collected from each animal, and the *in vitro* trial (Phase 2) was carried out in the same way as described previously. When albendazol was given to the untreated rumen fluid, the degradation of organic matter increased by 25 to 30% and that of protein by 40 to 100%, in case of both the alfalfa and corn substrate. In contrast, when salts were given to an adapted rumen fluid *in vitro*, the degradation of alfalfa protein decreased. Simultaneous application of the albendazol and the two salts to untreated rumen fluid increased the degradation of corn protein. In contrast, the same substances, given to an adapted rumen fluid *in vitro* diminished organic matter degradation both in the alfalfa and the corn substrate. Data suggest that the anthelminthic albendazol may enhance protein degradation, while the cobalt + molybdenum or their combinations with the albendazol tend to decrease it.

Keywords: in vitro, rumen fluid, cobalt, nickel, molybdenum, fermentation, degradation, protein, organic matter

Poster: free communications

Cell-growth promoting fractions originated from calf blood clot

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Sera are obtained from whole blood from adult, calf or foetal animals. After the natural clotting process, which may take several hours at 4°C, the blood consists of serum and a blood clot containing of 95% red blood cells, 5% platelets and less than 1% fibrin strands. In comparison when a PRP (Platelet-rich plasma) clots, the clot contains 4% red blood cells, 95% platelets and 1% white cells. The cell growth promoting components are the platelet derived growth factor (PDGF) and mostly the transforming growth factor Beta (TGF β). Fibronectin and vitronectin are also present. They are the cell adhesion molecules found in plasma and fibrin itself. The experiments presented were aimed to isolate, characterise and test *in vitro* on different cell cultures the growth promoting material from the calf blood clot. The blood was collected and allowed to clot. The whole content was centrifuged at 2500 rpm for 20 minutes and the supernatant (=serum) was aspirated off. The clot was quickly washed with the physiological solution (20 minutes); the content was centrifuged at 2500 rpm for 20 minutes. The supernatant was collected and stored at -20°C (Fraction I). To the remaining clot sterile 0.4 M PBS, pH=5.8, was added for 2 hours. After centrifugation at 2500 rpm for 20 minutes, the supernatant was collected and stored at -20°C (Fraction II). To the remaining clot PBS-Glucose, pH=7.3, was added for 24 hours. After centrifugation at 2500 rpm for 20 minutes, the supernatant was stored at -20°C (Fraction III) All fractions were sterilised by 0.2 membrane filtration, and analysed by SDS-PAGE. The cell growth promotion/inhibition activity in comparison to SR-2.0552P (Serum replacement based on porcine ocular fluid) and FCS (foetal calf serum) was tested on: chicken intestinal epithelial cell line, WISH, PLA-2 and Caco-2 cell line. The results of the experiments were: (1) Fractions I - III showed growth promoting activity that differed according to the cells tested. (2) The activity was strongest on the transformed cells (Chicken intestinal epithelial cell line, CaCo-2). (3) The optimal concentration was 6-8% in Eagle's medium. (4) In this range up to 80% of SR-2.0552P or FCS activity could be obtained.

Keywords: calf, blood clot, growth factors, cell growth in vitro

Poster: free communications

HuIFN-Alpha N3 and PoIFN-Beta affect the replication of Avian Influenza (H5N2) virus in the embryonated chicken egg

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Avian influenza (AI) H5N1 virus, the extremely contagious strain, can be deadly to domestic poultry. Without "jumping" the species barrier it can seldom cause infections in humans. Such an infection coincided with devastating epidemics in poultry farms in Asian countries, with the reported mortality approaching 100%. Closely related to this highly pathogenic avian influenza (HPAI) is strain H5N2, being antigenically similar to H5N1 although distinguishable from an influenza H5N1 virus isolated from a human in Hong Kong. Nevertheless the phylogenetic analysis of the haemagglutinin (HA) genes showed that the highly pathogenic H5N2 clustered with the human H5N1 virus isolated in Hong Kong, even though up to now there are no reports of the transmission of this virus from bird to human. The presented experiments were aimed to find out if low doses of HuIFN-Alpha N3 and PoIFN-Beta affect the replication of the AI (H5N2) virus in the embryonated chicken egg and through this the survival of the infected embryo. The obtained results were evaluated on the basis of the EID50 value: 10 - 4,249/0.1ml, and were the following: virus (10-4) + IFNs given simultaneously shows the effectiveness of low doses (150 I.U.) of IFN, resulting in 100% embryo survival and 100% HA inhibition. Virus (10-4) + IFNs given after 2h: only the high doses (15.000) are in some way effective (33,3 % survival). IFNs + virus (10-4) after 2 h are probably more important, because this pre-treatment with low doses and infection after 2 hours resulted in a decrease of HA as well as 66,7% survival. This is probably connected with the neuraminidase (NA) inactivation. Further experiments will show how to optimize the effectiveness of HuIFN-Alpha N3 and PoIFN-Beta toward the resistance of AI(H5N2) virus with different effectors like different l-amino acids.

Keywords: Avian Influenza (H5N1), Avian Influenza (H5N2), HuIFN-Alpha N3, PoIFN-Beta, Chicken embryo, Haemaglutination, Embryo survival

Lecture: free communications

News from the neurosphere front

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Because developmental neurotoxicity testing (DNT) requires large amounts of animals, alternative methods are needed to reduce animal consumption. Therefore, we have established a human *in vitro* model for DNT based on normal human neural progenitor (NHNP) cells, which are cultured as proliferating neurospheres. On appropriate extracellular matrices, NHNP cells migrate radially out of the sphere and thereby differentiate into the major neural cell types of the brain. During brain development, cell proliferation, apoptosis, differentiation and migration are fundamental processes. Therefore, we developed *in vitro* methods, which can detect effects of chemicals on these endpoints. NHNP cell proliferation can be measured using the Cell Titer blue assay (Promega) in a high throughput 96-well format. This test determines mitochondrial activity of living cells. The same assay is used to test for cytotoxicity of chemicals on NHNP cells and can be multiplexed with a caspase-3/-7 assay which assesses apoptosis-induced effector-caspase acitvity. In immunocytochemical investigations, cell differentiation analyses revealed that cAMP and retinoic acid induce neuronal differentiation, while methylmercury causes an increased formation of astrocytes in NHNP cells. Oligodendrocyte development was promoted by thyroid hormone and inhibited by exposure to lead in these cultures indicating that we are able to influence cell determination of NHNP cells. Measurements of the track that NHNP cells wander during their dif-

ferentiation revealed that exposure to mercury ($HgCl_2$ or CH_3HgC), ethanol or carbaryl led to an inhibition of NHNP cell migration. Investigations of the underlying mechanisms indicated that NHNP cell migration is regulated *via* the MAP kinase ERK1/2-dependent and ERK1/2-independent pathways, which are regulated by PKC, EGFR and src family kinases. Therefore, interference of chemicals with these target molecules may cause disturbed cell migration and impair brain development in humans. In summary, we set up NHNP cells as a human *in vitro* model for DNT testing by establishing endpoints which detect alterations in basic processes involved in brain development.

Keywords: DNT, neurosphere, in vitro method

Poster: evidence-based toxicology

Effects of a decoction of *Nigella sativa, Cryptolepis buchananii* and *Smilax glabra* on human hepatoma HepG2 cell integrity

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In Sri Lanka, a herbal decoction (DC) comprised of *Nigella sative* (seeds), *Hemidesmus indicus* or its very close relative *Cryptolepis buchananii* (root), and *Smilax glabra* (rhizome) has been used for many years by a particular family of indigenous medical practitioners (personal communication, Ayurvedic physician, Dr. N. Jayathilake) for the treatment of cancer, despite the lack of scientific evidence to prove or disprove its therapeutic efficacy. Recent *in vivo* investigations have demonstrated that the above decoction can offer significant protection against diethylnitrosamine induced hepatocarcinogenic changes in rats. The aim of the present study was to investigate how changes in liver cell integrity could contribute to the anti-hepatocarcinogenic actions of the decoction comprised of *Nigella sativa*, *Cryptolepis buchananii* and *Smilax glabra*. For this purpose, an *in vitro* study was conducted using human hepatoma HepG2 cells. For experimental purpose, HepG2 cells were cultured in T25 plates ($1x10^6$ cells/plate) in DMEM supplemented with 10% foetal bovine serum. Cells in test plates were exposed to different doses of the decoction (10 µg/ml, 80 µg/ml, and 160 µg/ml) for a total of 72 h. Cells in control plates were maintained in the same manner without exposure to the decoction. Cellular integrity in both test and control was assessed by (a) microscopic examination of cell morphology, (b) evaluation of lactate dehydrogenase (LDH) release from cells, cellular ATPase activity, and intracellular reduced glutathione (GSH) status, at the end of 6 h, 24 h, 48 h and 72 h incubation with or without the decoction, respectively. Results demonstrate that compared to the control the decoction even at a dose of 10 µg/ml induced a 12.99% increase in the leakage of cellular LDH along with 14.79% and -8.94% decreases in cellular ATPase and GSH, respectively at the end of 72 h incubation. Reduction in cell growth was microscopically apparent even as early as 6h post-incubation. It was interesting to note that the cellular GSH in test cells was marginally higher than the corresponding control at each time point. The overall outcome of this study indicates a potent cytotoxicity towards human liver cancer cells once exposed to the decoction of *Nigella sative* (seeds), *Cryptolepis buchananii* (root), and *Smilax glabra* (rhizome). This direct effect on cells helps confirm the results of recent *in vivo* investigations, which demonstrated a protection against chemically induced hepatocarcinogenesis in rat liver. The marginal increase in GSH subsequent to decoction exposure is supportive for a possible anti-oxidant role of the decoction, which is essential for the protection against chemically induced cancers. It may be concluded that cytotoxicity and antioxidant activity may be two mechanisms through which the DC mediates its anti-hepatocarcinogenic actions.

Keywords: Nigella sativa, Cryptolepis buchananii, Smilax glabra, cytotoxicity, HepG2 cell line, hepatoma

Poster: computer assisted procedures

Bounded animal use through *in vivo* body fat screening with the laboratory CT LaTheta™

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Current methods of body fat evaluation use either isolated tissue samples or *in vivo* techniques using instruments. However, only a few *in vivo* instruments can distinguish between different body fat tissues, yet produce only numerical data. Consequently, determination of the ratio of subcutaneous (SCAT) and visceral fat (VAT) becomes a manual process. Visualisation of the shape and site of adipose tissues inside a living animal was also limited. In this presentation, we focus on an alternative approach, which overcomes these issues in rodents. The Computed Tomography Scanner LaTheta™, Aloka Inc., Japan, includes a fast and exact *in vivo* body fat evaluation method for rodents with a visual display. We show an example of an easy to use screening procedure for body fat measurement in mice: Image data giving a visual impression is captured in less than a minute per scan. SCAT and VAT ratio or weights are evaluated immediately with just one mouse click. This method has been developed especially for long-term studies in mice and other small laboratory animals. CT is also suitable for other complementary methods such as fast tumour monitoring or bone parameter evaluation, saving animal use within these research areas.

Keywords: small animals, rodent, mouse, rat, in vivo, body fat, screening, long-term study, CT, functional imaging

Poster: free communications

Best search practice for animal alternatives – the ECVAM guide for untrained database users

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Scientists are obliged by animal welfare legislation not to conduct animal experiments, if alternative methods are reasonably and practicably available to obtain the information. Scientists should consult literature and other relevant information sources on alternatives prior to any experimental study using laboratory animals. Over the years scientists have pointed out the difficulties in identifying information on alternatives among the increasing amount of scientific publications stored in electronic-data retrieval systems. The comprehensive search guide, initiated and financed by ECVAM, aims at providing a step-by-step approach for data-retrieval procedures for untrained database users. The project has been started in 2007 and will be finished in 2008. The guide will consist of five sections: (1) Legislation Requirements of the EU, (2) Main Problems during Data Retrieval, (3) Information Retrieval Systems, (4) Basic Search Principles, and (4) Search Terms, Key Words and their Use. Each section will be represented by data sheets that inform about its main characteristics. The data sheet's format is the very reason to create a kind of a "file-card box". The "ECVAM Guide" will be made available via the Internet by the ECVAM Database service on Alternative Methods to animal experimentation (DB-ALM) currently accessible at the address: http://ecvam-dbalm.jrc.ec.europa.eu.

Keywords: animal welfare, animal testing alternatives, legislation requirements, information retrieval, searching strategies, search principles, search terms

Poster: computer assisted procedures

Partition and diffusion – the experimental basis for *in silico* modeling of skin absorption

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Pharmaceutical and cosmetic industries as well as governmental institutions have diverse interest in *in vitro* skin investigation such as bioavailability studies, risk assessment and consumer protection (EU-chemicals policy REACH). The high demand conflicts with an insufficient availability of human skin. Animal and bioengineered skin is used alternatively however; large interspecies variabilities and insufficient barrier formation limit their significance for the situation in man. In addition the use of animal skin for cosmetic applications will largely be restricted due to the 7th cosmetics amendment from March 2009. Therefore computer simulations may be a suitable alternative. Skin absorption may be described as partition between donor and superficial stratum corneum lipids, intercellular lipids and corneocytes, stratum corneum and viable epidermis and diffusion through lipids, corneocytes and viable skin layers. Based on these partition and diffusion coefficients Heisig et al. (1996) developed a mathematical model that is able to follow non-steady-state drug penetration into the stratum corneum. Our aims were first to determine these partition and diffusion coefficients experimentally in order to limit the number of unknowns and thus increase the predictive power of the model. Second, concentration-depth profiles and permeability data were needed to validate predictions. Experimental and simulated data will be presented for two exemplary compounds: flufenamic acid (lipophilic, ionizable) and caffeine (hydrophilic, non-ionizable).

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Keywords: in silico modeling, skin absorption, diffusion, partition

Poster: EU-chemicals policy (REACH)

The use of a human *in vitro* airway epithelium tissue model (EpiAirway) for metabolism, toxicity screening and drug delivery applications

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In vitro airway models are urgently needed as alternatives to animal testing for compliance with REACH legislation. The current poster describes a highly differentiated *in vitro* model of human tracheal/bronchial epithelium (EpiAirway). The model is produced by culturing normal human tracheal/bronchial epithelial cells on microporous membrane inserts at the air-liquid interface. Here we present physical and biochemical characterization of EpiAirway morphology, barrier function and drug metabolizing capability. Histological cross-sections of EpiAirway cultures show an *in vivo*-like, pseudo-stratified structure with numerous apical cilia. The useful lifespan of the cultures is at least 1 month. Dot blot analysis demonstrates apical mucin secretion. Transmission electron microscopy reveals ultrastructural detail of cilia and tight junctions between cells. Trans-epithelial electrical resistance (TEER) of 300-500 ohms x cm^2 demonstrates functionality of tight junctions. RT-PCR gene expression experiments were conducted to evaluate baseline and inducible expression of CYP isoforms in EpiAirway cultures derived from 4 individual donors. CYP1A1 (weak), CYP1B1, CYP2A6, CYP2B6 (weak), CYP2C8 (weak), CYP2C19, CYP2D6, CYP2E1 and CYP3A5 are constitutively expressed,

while CYP3A4 and CYP3A7 were not detected. 3-Methylcholanthrene (3MC) strongly increased expression of CYP1A1 and slightly increased CYP2B6 and CYP2C8 expression. Thus, CYP expression in EpiAirway shows a high concordance with CYP expression of *in vivo* human bronchial epithelium. Total GST activity in EpiAirway was demonstrated by measuring conjugation of glutathione with 1-chloro-2,4-dinitrobenzene. The results demonstrate that the EpiAirway model possesses *in vivo*-like physical and biochemical attributes for evaluating airway irritation, toxicity potential, metabolism and delivery of drugs and environmental/occupational chemicals.

Keywords: EpiAirway, REACH, CYP expression, TEER, metabolism, delivery of drugs

Lecture: free communications

When *in vitro* toxicology meets a global player: research and development of Degussa's super absorbing polymers are accompanied by alternative methods in toxicology

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In 1986 Degussa's Krefeld site became the world's first large-scale manufacturer of super absorbing polymers (SAP). The product FAVOR® is a cross linked, partially neutralized sodium polyacrylate and its primary function is to absorb liquids and keep them retained, even under pressure. Depending on the type of liquid, FAVOR® can absorb 30–500 times its own volume. SAP is an essential part of modern hygiene articles like baby diapers, and adult care specialities. To meet todays and future needs of those hygiene articles, FAVOR® is continuously improved by our research and development. We demand high safety requirements from all improved FAVOR® products. Despite strict control of the raw materials we additionally use a battery of *in vitro* screening tests to guarantee the safety of FAVOR®.

Here we demonstrate how *in vitro* toxicological test methods are capable to meet the strict demands of the world's leading supplier of SAPs. The first safety assessments of FAVOR® were performed on the basis of the required *in vivo* test methods according to international guidelines. However, results from valid *in vitro* test methods have always been generated in parallel and today these *in vitro* data serve as our standard. Using the respective *in vitro* test battery, each modification and improvement of the product now has to comply with our well established reference *in vitro* data. In the present study extracts and gels derived from modified SAPs have been tested to detect possible effects on the human skin using *in vitro* reconstructed human skin models. Additionally, the data have been amended by BALB/c 3T3 NRU-Assays and HET-CAM Tests, all performed under GLP conditions. From our results we conclude that the battery of these *in vitro* tests is suitable to confirm a consistent and sustainable cutaneous tolerance towards each of the chemically modified SAPs.

Keywords: in vitro screening, safety assessment, HET-CAM, BALB/c 3T3 NRU, reconstructed skin, cutaneous tolerance

Poster: nanotoxicology

In vitro toxicity of polyethyleneimine nanoparticles on human distal lung cells in mono- and co-culture

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Pulmonary administration of novel micro- and nano-scaled drug and gene delivery systems is a suitable alternative to injection, even though the drugs must overcome numerous barriers in the lung to reach the target site. Our aim was to investigate the *in vitro* toxicity of low molecular weight polyethylenimine (PEI) towards specific cells of the human distal

lung barrier. Therefore, we studied the uptake and influence of PEI on a human lung epithelial cell line with characteristics of alveolar type II and Clara cells (NCI H441) and on the microvascular endothelial cell line (ISO-HAS-1) in an *in vitro* co-culture model of the distal lung. This co-culture model mimics the two barriers a therapeutic drug has to pass to reach the systemic circulation. Different concentrations of unlabelled and Oregon Green 488 (OG)-conjugated 25 kDa PEI (0.14–2.23 µg/cm^2) in serum-free medium were used for cellular uptake studies over a 2 hr incubation period by fluorescent reading and confocal laser scanning microscopy (CLSM). Possible mitochondrial damage after exposure to PEI was studied using the MTS assay. In addition, viability of the cells was assayed by staining for Ki67. In the *in vitro* co-culture model, the influence of PEI-nanoparticles on barrier properties was investigated by measuring trans-bilayer electrical resistance (TER). The uptake of OG-conjugated PEI in the serum-free medium depended on the incubation time and on the nanoparticle concentration. PEI nanoparticles were taken up by both microvascular endothelial cells and distal lung epithelial cells, whereas the latter take up OG-conjugated PEI much faster. TER-values of the PEI exposed co-cultures stayed similar compared to the controls without PEI during incubation up to 2 hrs. Additionally, the expression of Ki67 remained similar compared to cells without PEI. A concentration of 1.12 µg/cm^2 OG-conjugated and unloaded 25 kDa PEI was biocompatible with the cells, as it did neither affect the mitochondrial activity (MTS assay) nor the cell viability in a 24 hr mitogenic assay. Incubation with 1.12 µg/cm^2 OG-conjugated PEI nanoparticles did not affect the TER-values of the co-culture model resulting in a sustained functional barrier after 24 hrs. Our system will therefore be a suitable model to investigate the targeting of drugs or genes to lung epithelium and endothelium *via* coupling to OG-conjugated PEI-nanoparticles. The *in vitro* co-culture model will be useful to further systematically study the uptake and trafficking of new drug carrier systems, delivering drugs to deep lung epithelial cells and across a distal lung barrier.

Keywords: pulmonary drug delivery, nanoparticles, distal lung, alveolar type II, Clara cells, microvascular endothelial cells

Poster: evidence-based toxicology

Determination of skin phototoxicity properties of different compounds using epidermal skin test 1000 (EST-1000)

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Modern risk assessment, pharmacotoxicology and dermatology research implies the development of *in vitro* models with high reproducibility and confidentiality to the native situation. In this study we present data obtained by the use of "Epidermal Skin Test 1000" (EST-1000, CellSystems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany). EST-1000 is a reconstructed human epidermis which shows a high comparability to normal human *in vivo* skin and which has already shown its abilities in the field of *in vitro* skin corrosion and irritation testing. The major focus of this work was to compare the toxicity properties of several substances, e.g. nerol and chlorpromazin, in the absence or presence of UV radiation (UVR) light. Without UVR most of the tested substances have provoked none or weak effects to EST-1000 while in the presence of UVR EST-1000 they have shown increased histological damages (H & E staining) and a significant reduce of cell viability (conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)). Furthermore the tissue reacts with an increased release of Lactate Dehydrogenase (LDH) as determined by standard ELISA assay. We conclude from these results, that EST-1000 is a useful tool for a correct classification of substances concerning their phototoxic properties.

Keywords: reconstructed skin, epidermis, phototoxicology, evidence-based toxicology

Lecture: evidence-based toxicology

Toward an evidence-based toxicology

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Increasingly concerns about limitations of regulatory toxicological methodologies, such as an insufficient test method assessment or the lack of transparency and consistency in data integration and interpretation, are expressed. We identified the concept of evidence-based medicine (EBM) as a promising starting point to remedy these issues as clinical medicine faces a similar problem of combining state-of-the-art scientific and traditional approaches. Evidence-based medicine strives for making decisions regarding patients, e.g. by providing sound scientific bases *via* systematic reviews of available evidence. Systematic reviews, being scarce in toxicology, could be a valuable tool for mapping the state of today's toxicology in a consistent manner. Moreover, obvious fundamental parallels between diagnostic and toxicological tests suggest that the available methodology for an evidence-based diagnostic test assessment might be transferable to toxicology. In addition, quality assurance aspects, quality assessment tools and elaborated experimental designs of EBM might constitute approaches relevant for toxicology. Along with Guzelian et al. (2005), we propose an evidence-based toxicology (EBT) (2006). To discuss this proposal with toxicologist and other interested scientists, the first international forum toward an evidence-based toxicology will be held in Como, Italy, October, 15th-18th, 2007 (www.ebtox.org).

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Keywords: evidence-based, transparency, test assessment, decision making

Poster: evidence-based toxicology

Online monitoring of suspension cells in the focus of *in vitro* immunotoxicity-studies

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The Bionas®2500 analyzing system provides a cell based *in vitro* monitoring of dynamic cell behaviour influenced by test compounds. It is a very useful tool for the analysis of cytotoxic and dose dependent effects as well as for the investigation of regeneration effects after treatment and removal of test compounds. Investigations of adherent cells are already established. Currently in focus of European Medicines Agency (EMEA) is the study of immunotoxicity, defined as unintended immunosuppression or enhancement. EMEA recommends the evaluation of potential adverse effects of pharmaceuticals on the immune system. On cellular level this could be achieved by examination of dynamic immune cell behaviour in reliance on potential immunotoxic compounds. One prerequisite for such studies is the analysis of suspension cells because immune cells commonly grow non-adherent. By the Bionas®2500 analyzing system a continuous monitoring of physiological parameters of suspension cells is now possible. Our silicon chip based analyzing system is able to observe 2 different physiological parameters (acidification, respiration) per chip. Six chips can be measured in parallel. Changes of acidification activity and respiration rates caused by test compound are hints of the effect on the cell physiology and presumably the cytotoxic property of the compound used. Usually changes in cell physiology, especially on immune cell physiology are indices for immunotoxicity. Generally we have successful established the using of suspension cell for the online monitoring of oxygen consumption and acidification behaviour. An important advantage of our Bionas®2500 is the continuous detection of all parameters from a few hours to several days contrary to the so called end point test like MTT or LDH release. Our analyzing system is able to record in real time all compound actions on the living cells until the endpoint of treatment and moreover subsequently the recovering effects after removing the compounds. First experiments revealed effects of doxorubicin on T-lymphocyte Jurkat cells. Doxorubicin (Dox) is known as an anticancer drug and also as an immunosuppressive agent. Different concentrations of Dox were applied and a dose-dependent decrease of acidification activity and respiration rates could be observed. Regeneration of Jurkat cells after removing Doxorubicin from the medium could not be observed.

Keywords: cell metabolism, suspension cells, immunotoxicity, in vitro method, label-free, non-invasive

Lecture: good cell culture practice

Standardisation of cell culture techniques: a prerequisite to effective multi laboratory comparison of microarray data

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As technology develops and we enter the realm of omics, standardisation of *in vitro* cell culture techniques has become more important than ever. In the case of DNA microarrays where it is possible to measure the transcriptional activity of over 47,000 transcripts, every effort must be taken to limit the effect of unknowns in a given experiment. Although this technology is still relatively expensive the cost in time for analysis is the major factor. Therefore, engaging this technology with *in vitro* cell culture requires a considerable amount of foresight in an attempt to pre-empt possible costly (financial and time) experimental design errors. In the 6th Framework project, PREDICTOMICS "Short-term *in vitro* assays for long-term toxicity", we attempted to perform an interlaboratory comparison of microarray data from four European laboratories. In each laboratory, the human proximal tubule cell line HK-2, was treated with Cyclosporine A and RNA was isolated. At three different centres microarray processing was conducted using Affymetrix HGU-133 Plus 2 gene chips. The approach, experiences, results and whether or not we were successful will be discussed.

Keywords: microarray, nephrotoxicity

Poster: vocational training

InterNICHE Humane Education Award: assessing the international impact

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The InterNICHE Humane Education Award is an annual grant program established to support ethical and effective education and training within biological science, medicine and veterinary medicine. Supported by Dutch organisation Proefdiervrij, the Award has offered an annual 20,000 Euro (US$ 27,000) since 2002. The Award was initially focused on one region or country, such as India, and has been global since 2005. Applicants may be teachers, students, campaigners or other individuals committed to best practice education and training. Proposals are assessed primarily according to their potential to replace harmful animal use, based on number of animals and/or severity of procedures, potential pedagogic effectiveness, and innovation, resourcefulness, and overall ethical design. Funds are split between the successful applicants, who number on average 8 per year. Examples of projects that have been funded include the development and implementation of freeware, videos, models and mannekins; purchase and use of existing alternatives, including an advanced surgery training device; establishment of a student-based self-experimentation program to replace animals in physiology practical classes; and establishment of a client donation program to secure ethically sourced animal cadavers for replacement of animals used for anatomy and surgery training. Specific projects will be described in the presentation, and the impact of supporting multi-local humane education initiatives assessed.

Keywords: InterNICHE, Humane Education Award, funding, replacement, education, training

Poster: EU-chemicals policy (REACH)

Expanded applicability domain for assessing ocular irritation using EpiOcular™ human cornel tissue model

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The EpiOcular™ tissue model (OCL-200) is an organotypic model of the human corneal epithelium (HCE) cultured from normal human keratinocytes. Paraffin embedded, histological cross-sections show the structure of EpiOcular™ closely parallels that of the HCE. Quality control of weekly EpiOcular™ batches is performed using the MTT assay, which historically has been the *in vitro* endpoint of choice for European and US regulators. The exposure time needed to reduce the viability to 50% (ET_{50}) for 0.3% Triton X-100 is determined. Since 1997, yearly average ET_{50} values have ranged from 20.6 minutes to 25.0 minutes with average coefficients of variation under 7%. Thus, EpiOcular™ has demonstrated long term reproducibility over the past 10 years. This is important since regulators require that test methods provide consistent data during and after the validation process. EpiOcular™ has been increasingly used by many personal care and household product companies to determine the ocular irritancy of their products without using animals. In particular, EpiOcular™ has been very useful for evaluating water soluble and low irritancy materials. Currently, a new protocol to handle industrial chemicals spanning a range of chemical categories and irritancies has been developed. 41 materials including alcohols, hydrocarbons, amines, esters, and ketones have been evaluated. Using a straightforward MTT tissue viability protocol, a prediction model for discriminating between ocular irritants and non-irritants was developed which resulted in 100% sensitivity and 63% specificity. This new protocol will prove very useful in fulfilling the provisions of REACH (Registration, Evaluation, and Authorization of Chemicals) in the EU.

Keywords: EpiOcular™, ocular irritation, REACH, MTT

Lecture: 7th cosmetics amendment

Genotoxicity screening using the EpiDerm™ human 3D model skin specific micronucleus assay

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Safety assessment of new products for human use requires genotoxicity testing to insure their non-carcinogenicity. Current assays are based on non-human cell systems and result in low specificity due to the lack of human-like metabolism and low relevance to the target organs. Dermally exposed substances require a test which determines skin-specific genotoxicity. Starting in 2009, manufacturers of cosmetics products will need to assess genotoxicity using non-animal methods. Thus, industrial organizations such as COLIPA are actively pursuing development of micronucleus assay (MNA) for dermally exposed chemicals. EpiDerm™ forms a 3D skin-like tissue that is highly reproducible and contains epidermis-like barrier. Also, EpiDerm™ possesses human *in vivo*-like biotransformation capabilities including CYP450, GST, and UDP enzymatic activity. The EpiDerm™ MNA protocol utilizes two 10 µl topical doses of test material given 24 hours apart in the presence of 3 µg/ml of Cytochalasin-B in the medium. Cells are harvested from the tissue 24 hours after the last dose. This protocol results in the generation of a reproducible population of binucleated cells (43.9% +/-7.6) with a low background frequency of micronucleated cells (0.08% +/-0.08). We have shown dose-related, statistically significant increases in micronuclei induction for 3 model genotoxins and for 5 out of 6 rodent skin genotoxins. In addition, 4 non-genotoxic chemicals have been evaluated and shown to be negative. Finally, 3 genotoxins that require metabolic activation were also positive in the MNA. In conclusion, the MNA that utilizes EpiDerm™ with inherent metabolic activity holds excellent promise to predict genotoxicity while avoiding animal welfare issues.

Keywords: EpiDerm™, genotoxicity, 7th cosmetics amendment, micronucleus assay

Lecture: EU-chemicals policy (REACH)

Increased sensitivity of the EpiDerm™ skin irritation protocol evaluated in the ECVAM skin irritation validation study

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During 2001-2003, refined EPISKIN and EpiDerm™ *in vitro* skin irritation protocols were developed (Cotovio et al., 2005; Kandárová et al., 2005), which resulted good correlation between *in vivo* and *in vitro* data. Both methods were based on the idea of a common protocol, comprised of 15 min application and 42 h post-exposure period, and measuring the reduction of tissue viability using MTT endpoint. In 2004, the two skin models proceeded into the ECVAM validation study with the aim of replacing acute skin irritation tests performed in rabbits. The study revealed that the EPISKIN assay (based on MTT endpoint) showed sufficient sensitivity and specificity to be endorsed as a replacement of the *in vivo* test. The EpiDerm™ model was recognized as a validated constituent within OECD testing strategy. Faller and Bracher (2002), Schaefer-Korting et al. (2007) and others demonstrated that differences in the barrier properties of the reconstructed human epidermal models exist. We hypothesized that the "false negative" outcomes observed for EpiDerm™ using the common protocol were likely due to EpiDerm™'s enhanced barrier. Therefore, we modified the common protocol by extending the exposure time from 15 min to 60 min. Using this modified protocol, we re-tested 60 readily available chemicals from pre-validation and validation studies and obtained significant increased sensitivity, without loss of specificity, using EpiDerm™. The modified assay provided a very balanced outcome, resulting in total accuracy of 80%. An inter-laboratory study will be performed with chemicals endorsed by ESAC to evaluate the reproducibility of the improved performance of EpiDerm™ method. Taking into account evidence about the variability (Gilman et al., 1978; Weil and Scala, 1971; Worth and Cronin, 2001) and borderline predictive capacities of the Draize test in terms of human health effects (Calvin, 1992; Campbell and Bruce, 1981; Robinson et al., 2000; Basketter et al., 2004), it can be concluded that both EpiDerm™ skin irritation protocols provided sufficient levels of sensitivity and specificity. The presentation will summarize results obtained with the two EpiDerm™ protocols compared to *in vivo* rabbit data, existing and recently performed human patch study (Jírová et al., 2007).

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Keywords: EpiDerm™, skin irritation, validation, in vitro methods, ECVAM

Lecture: 7th cosmetics amendment

In vitro skin corrosion test: reproducibility over time and optimized methodology for testing chemicals interfering with the MTT endpoint

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The potential for chemicals to cause skin effects such as corrosion is a concern of industrial toxicologists in their assessments of possible worker and consumer safety issues. Moreover, the U.S. Department of Transportation (DOT), the new regulation on the Registration, Evaluation and Authorization of Chemicals (REACH), and other international and national regulatory agencies require that substances should be labeled as to skin corrosivity potential. In the past, skin corrosion assessments were based on tests involving topical application of test substances to the skin of rabbits. However, based on two ECVAM Validation studies performed during 1996-2000 (Fentem et al., 1998; Liebsch et al., 2000) with two reconstructed human skin models (EpiDerm™ and EPISKIN), the OECD approved use of skin models as regulatory accepted methods (OECD TG 431) replacing the *in vivo* test. In the present study, the EpiDerm™ skin corrosion test was repeated with the commercially available test substances previously used in both ECVAM validation studies. The aim was to demonstrate the long term reproducibility and reliability of the EpiDerm™ model and the method, as required by regulators (Rispin et al., 2006). The data obtained show very good correlation over a period of 7 years. We also conducted a formalized evaluation of a procedural modification proposed several years ago to correctly predict the corrosive potential of materials that interfere with the MTT endpoint normally used in the method. An attempt was made to optimize this method and demonstrate with several chemicals (sometimes reported to be false negatives) the predictive capacity of the EpiDerm™ corrosivity assay when this procedural modification is included. The presentation will summarize data from the above mentioned studies and describe optimized methods for assessing materials which interfere with the MTT endpoint.

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Keywords: EpiDerm™, skin corrosion, REACH, MTT endpoint, regulatory toxicology

Poster: good cell culture practice

Long term reproducibility of *in vitro* reconstructed skin models for regulatory testing purposes – Evidence of good tissue culture practice

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Validated *in vitro* methods are urgently needed as alternatives to animal testing for compliance with REACH and EU Cosmetics Directive legislation. Some of the *in vitro* assays/tissue models have been, or are being, formally validated for use in testing for corrosivity, irritation or toxicity. However, regulatory agencies and other users require assurance that models will provide consistent, quality data over time, not just during validation (Rispin et al., 2004). Recommended guidelines for good cell/tissue culture practice include e.g. full characterization of cells or tissues, sampling of each lot for performance, and regular use of controls and benchmark chemicals to provide assurance of the consistency in

performance of the models and assays (Cooper-Hannan et al., 1999; Coecke et al., 2005; Rispin et al., 2006). One demonstration of good cell/tissue culture practice for commercially manufactured skin models is their long-term reproducibility and consistence performance in validated test assays. In compliance with GMP and ISO standards, MatTek Corporation evaluates the quality of each lot of commercially manufactured product. The release criteria include: viability determination, proof of product sterility, screening of histology and evaluation of the barrier properties of the model using the MTT ET_{50} assay with the benchmark chemical Triton X-100. Acceptance and rejection criteria have been established for each *in vitro* product and are strictly adhered to. The presented poster summarizes data showing long-term reproducibility of EpiDerm™ and EpiOcular™ reconstructed tissue models. Quality controls for each tissue lot of EpiDerm™ and EpiOcular™ were performed and the yearly averages have been calculated. Since 1997, EpiOcular's yearly average ET_{50} values ranged from 20.6 minutes to 25.0 minutes. Coefficients of variation for negative control tissue have averaged under 6% for the entire historical data set. The yearly average CV for all tissues never exceeded 6.5%. For EpiDerm™, the yearly average ET_{50} ranged from 6.0 to 7.5 h. The CV for the negative control tissue averaged under 7.5% for every year since 1996. Results of quality control data for commercially available products, manufactured over the past 10 years have address regulatory concerns regarding long-term reproducibility and have fulfilled of the standards for good cell/tissue culture practice.

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Keywords: REACH, EpiDerm™, EpiOcular™, long-term reproducibility, good cell/tissue culture practice

Poster: nanotoxicology

Effect of ZnO, TiO_2 and CuO particle size on the growth of Saccharomyces cerevisiae

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Antimicrobial/biocidal properties of TiO_2, ZnO and CuO have found use in water purification, dentistry, wood preservation, antimicrobial textiles etc. Currently, nanosize formulations of TiO_2 and ZnO are growingly used in consumer products (cosmetics, sunscreens, etc.), mostly as UV-protection ingredients. However, their potential harmful properties are poorly studied. Due to their small size, nanoparticles gain new properties (optical, physical, etc.) and thus also new biological properties are expected. Thus, nanosize metal oxides (as well as all types of nanoparticles) need to be tested for toxicity even if the bulk materials (like ZnO) have been tested and allowed to used in consumer products. The aim of this study was to elucidate how the size of ZnO, CuO and TiO_2 particles influences their toxic properties. For that, nano and bulk formulations of these metal oxides were studied for their growth inhibitory effects towards *Saccharomyces cerevisiae* – a widely used eukaryotic model. All the studied metal oxides were purchased. The sizes of nanoscale metal oxides were as follows: ZnO (50-70 nm), CuO (~30 nm) and TiO_2 (25-70 nm). *S. cerevisiae* S288C was grown batch-wise on orbital shaker (200 rpm) at 30°C in malt extract broth (pH 5.4) that was supplemented with various concentrations of metal oxides. The metal oxide suspensions in growth medium were characterized for nanoparticles size distribution using Nanosight LM10™ system. The growth of yeasts was evaluated by viable cell counts at 4, 8, 24 and 48 hours. The toxic effect of metal-oxides on the growth of *S. cerevisiae* was characterized and quantified from the respective dose-effect curves as IC_{50} and IC_{20} (reduction in the specific growth rate by 50% or 20%, respectively). Our data showed that both ZnO formulations were of analogous toxicity (8-h IC_{50} about 300 mg/l). However, nano CuO (8-h IC_{50} about 60 mg/l) and nano TiO_2 (IC_{50} about 2500 mg/l) was about 10-20 fold more toxic than respective bulk formulations. This is the first comparison of the potential adverse effects of nanosize and bulk ZnO, CuO and TiO_2 on yeast *Saccharomyces cerevisiae*.

Keywords: nanoscale metal oxides, toxicity, Saccharomyces cerevisiae

Poster: free communications

In vitro assessment of cytotoxic and membrane toxic potential of dental materials

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Biocompatibility testing of dental materials is an essential requirement for their application in clinical practice. The objective of the present study is to investigate the *in vitro* cytotoxic and membrane toxic potential of six glass ionomer cements (Ionofil® Molar, Argion® Molar, KetacTM Molar Aplicap, Ketac™ Silver Aplicap, Alpha® Fil, Alpha® Silver), one silicophosphate cement (Cupro Dur® N), one compomer (F2000) and one denture resin (Kallocryl®). The test samples are prepared in practically relevant sizes according to the manufacturer's instructions. The cytotoxicity is determined using the XTT tetrazolium reduction assay which bases on the ability of living cells to reduce the colourless tetrazolium salt XTT to an orange water-soluble formazan product. The membrane toxicity is examined by means of the [3H]arachidonic acid release assay. The U937 permanent cell line is chosen because of its human origin and the ability to perform many monocytic functions. Biocompatibility is comparatively evaluated on the base of CD_{50} (dose required to kill 50% of the cells) and MTF (membrane toxicity factor) values. The results show CD_{50}s between 43 and 107 mg/0.2 ml for glass ionomer cements and 154 mg/0.2 ml for the compomer F2000 after 1-hour exposure. Kallocryl® did not exert any cytotoxicity within the concentration range tested. The cytotoxicity increases with prolonged exposure: CD_{50} values were found to be 7 to 41 mg/0.2 ml for glass ionomer cements after 24-hour and 0.23 to 31 mg/0.2 ml after 48-hour exposure. The CD_{50}s of F2000 were 73 and 52 mg/0.2 ml and of Kallocryl® 149 and 32 mg/0.2 ml after 24-hour and 48-hour exposure, respectively. The membrane toxicity of the samples proved to be generally low. After 1-hour exposure most of the tested dental materials showed no membrane toxicity (MTF).

Keywords: dental materials, cytotoxicity, membrane toxicity, XTT tetrazolium reduction assay, [3H]arachidonic acid assay

Poster: vocational training

Educational studies establish the efficacy of humane teaching methods in veterinary education

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Animal use resulting in harm or death has historically played an integral role in veterinary education, in disciplines such as surgery, physiology, biochemistry, anatomy, pharmacology, and parasitology. However, many non-harmful alternatives now exist, including computer simulations, high quality videos, "ethically-sourced cadavers" such as from animals euthanized for medical reasons, preserved specimens, models and surgical simulators, non-invasive self-experimentation and supervised clinical experiences. Veterinary students seeking to use such methods often face strong opposition by faculty members, who usually cite concerns about their teaching efficacy. Consequently, studies of veterinary students were reviewed comparing learning outcomes generated by non-harmful teaching methods with those achieved by harmful animal use. Of eleven published from 1989 to 2006, nine assessed surgical training – historically the discipline involving greatest harmful animal use. 45.5% (5/11) demonstrated superior learning outcomes using more humane alternatives. Another 45.5% (5/11) demonstrated equivalent learning outcomes and 9.1% (1/11) demonstrated inferior learning outcomes. Twenty one studies of non-veterinary students in related academic disciplines were also published from 1968 to 2004. 38.1% (8/21) demonstrated superior, 52.4% (11/21) demonstrated equivalent, and 9.5% (2/21) demonstrated inferior learning outcomes using humane alternatives. Twenty nine papers in which comparison with harmful animal use did not occur illustrated additional benefits of humane teaching methods in veterinary education, including: time and cost savings, enhanced potential for customisation and repeatability of the learning exercise, increased student confidence and satisfaction, increased compliance with animal use legislation, elimination of objections to the use of purpose-killed animals, and integration of clinical perspectives and ethics early in the curriculum. The evidence demonstrates that veterinary educators can best serve their students and animals, while minimising financial and time burdens, by introducing well-designed teaching methods not reliant on harmful animal use.

Keywords: alternative, animal experiment, education, training, veterinarian, veterinary surgery

Lecture: ethical and legal aspects in animal experimentation

Animal experiments scrutinised: systematic reviews demonstrate poor human clinical and toxicological utility

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The assumption that animal models are reasonably predictive of human outcomes provides the basis for their widespread use in toxicity testing and in biomedical research aimed at developing cures for human diseases. To investigate the validity of this assumption, the comprehensive "Scopus" bibliographic biomedical databases were searched for published systematic reviews of the human clinical or toxicological utility of animal experiments. Of 27 reviews retrieved, the authors concluded that the animal models were substantially consistent with or useful in advancing clinical outcomes in only two cases, and the conclusion in one case was contentious. Included were reviews of the clinical utility of experiments expected by ethics committees to lead to medical advances, of highly-cited experiments published in major journals, and of chimpanzee experiments – the species most likely to be predictive of human outcomes. Seven reviews failed to clearly demonstrate utility in predicting human toxicological outcomes such as carcinogenicity and teratogenicity. Consequently, animal data may not generally be assumed to be substantially useful for these purposes. Possible causes include interspecies differences, the distortion of experimental outcomes arising from experimental environments and protocols, and the poor methodological quality of many animal experiments evident in at least 11 reviews. No reviews existed in which a majority of animal experiments were of good quality. While the latter problems might be minimised with concerted effort, given their widespread nature, the interspecies limitations are likely to be technically and theoretically impossible to overcome. Yet, unlike non-animal models, animal models are not normally subjected to formal scientific validation. Instead of simply assuming they are predictive of human outcomes, the consistent application of formal validation studies to all test models is clearly warranted, regardless of their animal, non-animal, historical, contemporary or possible future status. Expected benefits would include greater selection of models truly predictive for human outcomes, increased efficiency during the development of human pharmaceuticals, and decreased wastage of animal, personnel and financial resources. The poor human clinical and toxicological utility of most animal models for which data exists, in conjunction with their generally substantial animal welfare and economic costs, justify a ban on animal models lacking scientific data establishing their human predictivity or utility.

Keywords: animal experiment, animal study, clinical trial, human outcome, systematic review

Poster: EU-chemicals policy (REACH)

Modular glass chip system for the acquisition of the electric activity and physiological parameters of differentiated stem cells

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The acquisition of electrophysiological parameters from cultivated stem cells during and after their differentiation into nerve or heart muscle cells is a novel approach with the potential to reduce animal experiments. A new modular glass chip system (MOGS) has been developed, with a periphery compatible to our existing silicon neuro-sensorchip system. The glass chip (www.gesim.de) ensures microscopic observability. It features a multi-electrode array (MEA) with 52 electrodes for detecting the electric activity, e.g. in neuronal networks or cardiomyocytes. An integrated interdigitated electrode structure (IDES) in the cell-culture trough allows for impedance measurements to evaluate cell adhesion as an indicator for the viability of adherently growing cells. The trough also contains a temperature sensor in direct proximity to the cells. In the system, cells can be cultured for longer periods, from days up to months, as evidenced by electrically

active neural networks formed from primary cells. With approximately 250,000 primary cells needed per chip, one sacrificed laboratory mouse supplies cells for several hundred chips. This allows for a reduction in the number of animals used for certain tests. Nevertheless, our aim is to replace primary cells by stem cell lines. The new stem cell systems will be interesting for research into the therapy of neurodegenerative diseases, e.g. Morbus Parkinson as well as for the development of pharmaceutical compounds and the testing of neurotoxic and developmental neurotoxic effects. The microscopic observability of MOGS allows to correlate network structure to network activity and to test regenerative techniques in degenerative disease cell models. Other applications are in the fields of substance development for fertilizers, food, household chemicals and consumer goods or in the clinical, environment, food and military sectors. The use of MOGS as an alternative method to animal experimentation should be validated by ECVAM in the future.

Keywords: lab-on-chip, biochip, REACH, multi-electrode array, neuro-sensorchip, glass chip, neuronal networks, developmental neurotoxicology, DNT, neurotoxicity, action potential detection

Lecture: ethical and legal aspects in animal experimentation

Survey of the German Animal Welfare Federation concerning the work of ethics committees and licensing authorities for animal experiments

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In February 2006, the German Animal Welfare Federation started its 3rd survey since the establishment of ethics committees for animal experiments according to § 15 of the German Animal Welfare Act in Germany in 1987. The survey of 1988 focused on general parameters within the licensing process. In 1995, changes regarding the previous survey and also specific developments after the German reunification were analysed. As animal welfare has been included as a "state goal" in the German constitution in 2002, the present survey aims at an analysis of specific changes within the licensing process. In this context, the increase in value of animal welfare as a consequence of its inclusion into the constitution was of major interest. According to law experts the new legal situation would have to result in higher requirements concerning the ethical justifiability within applications for animal experiments. The question to which extent the above mentioned increase in value has penetrated into the work of ethics committees and licensing authorities is therefore an important aspect of the survey. Other important aspects include changes in the work of ethics committees and licensing authorities since the last survey as well as suggestions for improvement that have been regarded useful from the point of view of members of ethics committees and licensing authorities. The survey is based on questionnaires that have been sent to licensing authorities and members of ethics committees in February 2006. One of the main results is that the importance of animal welfare within the licensing process, after inclusion of animal welfare into the German constitution, has not changed or changed only to a little extent. It also becomes clear that ethical parameters are still of minor importance when animal experiments are licensed.

Keywords: animal experiments, ethical committee, authorisation procedure

Poster: free communications

Refinement of tissue biopsy for PCR based genotyping of GM mice by using hair samples

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The efficient and reliable genotyping is one of the most important aspects for the generation and breeding of GM rodents. To isolate genomic DNA from mice different techniques of invasive and non-invasive tissue biopsies are known. Tail biopsy is still the routinely used but also most invasive method. Therefore, hair follicles for the preparation of chromosomal DNA are discussed as possible source and several studies have been published to demonstrate the feasibility. The use of hair samples could reduce distress and impairment of the animal's well-being. However, efficiency and reliability of hair sampling as routine method remains to be certified. The present study was conducted to investigate several parameters that might have an effect on genotyping results, to be more confident in the use of hair follicles for DNA isolation. Tested parameters are hair colour, age of mice (hair cycle stages), sampling method as well as storage of the biopsies. Moreover, the risk of cross contamination was proved by micro satellite analyses. For DNA preparation different protocols were used to meet the requirements for DNA purification either for detecting a knockout allele by standard PCR or transgene integration by real-time PCR.

Keywords: mouse, genotyping, hair, PCR, DNA purification

Poster: free communications

Chorioallantoic membrane model system – different application methods for test substances

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The chorioallantoic membrane test system (CAM) represents a borderline system between *in vitro* and *in vivo* systems. In the last years the CAM has become the standard model system for investigations in angiogenesis. Because of the immunodeficiency of the chick embryos in the first half of breeding, the membrane can accept inoculation of various tumor cells or tissues. In the last couple of years we have established the chorioallantoic membrane system for research in tumor biology especially as a test system for tumor chemosensitivity. For this purpose we used different methods for the application of test substances. After topical application onto the membrane we could show the time-course of plasma concentrations in the embryo circulation. If the embryonic development is taken into account, the model is very reproducible. In a recent study we could show, that the effect of the substances topically applied is not caused by direct uptake of the tumor cells like in cell culture. We dropped the substance onto the membrane in a different area clearly separated from the tumor cells. For analysis of the plasma-level kinetics, blood of was collected from the chorioallantoic veins at different time points. The effects seen in these tumor cells were comparable with the effects seen in xenotransplanted mice after intraperitoneal application. In other studies, the substances were administered intravenously into the CAM vessels. Using fluorescent markers, we could show the redistribution in the membrane and the absorption in the tumor cells. Recently, Taizi et al. reported about the CAM as a novel *in vivo* system for testing therapeutics on human leukemias. In this report, three days after injection of human leukemia cells in the CAM veins a single dose of doxorubicin was administered i.v. The strengths of this model for testing new pharmacologically active substances are several-fold. First, it saves mice, further it is quick to perform, economic and it requires only very small quantities of precious novel compounds. Upon successful pretesting in this model, additional experiments are required to confirm the pharmacotherapeutical correlation between the xenotransplantation models in mice and fertilized chicken eggs. In our presentation we will give a review of the current application methods in CAM found in the literature.

Reference

Taizi, M., Deutsch, V., Leitner, A. et al. (2006). A novel and rapid in vivo system for testing therapeutics on human leukemias. *Experimental Hematology, Vol. 34*, 12, 1698-1708.

Keywords: chorioallantoic membrane, CAM, hen's egg test, application methods

Poster: revision of Directive 86/609/EEC

Next of Kin: the use of primates in experiments in the EU

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The Next of Kin project constitutes an extensive review of the public, scientific, legislative and ethical issues regarding the use of non-human primates in scientific research and testing in the EU. The final report highlights a discrepancy between public unease about the use of primates and their treatment in the laboratory. 10,392 primates were included in the EU statistics in 2002, a 14% increase on previous years, although many more are held in laboratories. The largest users of primates are France, UK and Germany. The majority are used within the pharmaceutical industry for safety and efficacy assessment (e.g. 726 primates were poisoned in LD_{50} tests). UK surveys of public concern about the use of primates in research consistently show that the majority of the public does not support their use, even for the study of serious medical problems. This may in part reflect our increased understanding of the capacity of primates to reason, use tools, communicate semantically, count, develop cultures and practise deception; suggesting that they are not only self aware but are aware of others. The potential for primates to suffer within the laboratory environment is therefore considerable and is reflected in stereotypies, suppressed immune function and physiological disturbances which may confound research findings. In particular, the transport of old world monkeys from non-EU countries (78% of these) causes significant distress. Despite the use of primates in the pharmaceutical industry, the current failure rate from entry to Phase 1 clinical trials to market is over 90%, largely attributed to inability to predict human ADME, toxicity and efficacy. Although alternative technologies, such as in vitro, are being developed, increased funding of the development of non-animal methods is urgently required and should be incorporated into the current revision of EC Directive 86/609.

Keywords: primate, alternatives, EU legislation, animal experiments

Lecture: computer assisted procedures

Derek for Windows: conforming to the OECD principles for (Q)SAR validation

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The OECD Principles for (Q)SAR Validation were established in 2004 and are intended to identify the types of information considered useful for the regulatory review of (Q)SAR models and their predictions. The five principles have been applied to Derek for Windows (DfW), a knowledge-based expert system which predicts the toxicity of a compound from its chemical structure. Principle 1 (a defined endpoint): DfW predicts for toxicological endpoints such as mutagenicity, carcinogenicity and skin sensitisation. Information on specific assays associated with an endpoint is included in the supporting evidence provided with each prediction. Principle 2 (an unambiguous algorithm): The reasoning methodology used by DfW has been described in the public domain. Predictions are based on alerts, examples and rules, which are supported by comments and references describing their basis. This supporting evidence can be explored for individual predictions within the program, or the knowledge base as a whole can be viewed in the accompanying editor. Principle 3 (a defined domain of applicability): At present, the applicability domain for an alert is defined in terms of the chemical environment it describes. Where sufficient evidence exists, this may be refined by consideration of physicochemical properties such as molecular weight and log P. Principle 4 (appropriate measures of goodness-of-fit, robustness and predictivity): Alerts in the DfW knowledge base have historically been supported by the inclusion of four or five supporting examples, with additional evidence appearing in comments and supporting references. Potential sources of unseen data suitable for the purposes of external validation include that contributed to Vitic restricted data groups. These provide an opportunity for industry to share non-commercially-sensitive data, not currently in the public domain, for mutual benefit. Principle 5 (a mechanistic interpretation, if possible): Where evidence is available, the mechanistic basis of an alert or rule is detailed in the associated comments and supporting references. Examples will be presented to illustrate the existing high degree of conformity of DfW to the OECD Principles for (Q)SAR Validation, and the potential for further enhanced compliance will be discussed.

Keywords: in silico, toxicity prediction, regulatory, quantitative structure-activity relationship

Lecture: good cell culture practice

The value of the BV-2 cell line as model for primary microglia

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Microglia cells are of large pharmacological interest as they appear to be involved in chronic pain mechanisms and are always found to be activated in chronic neurodegenerative diseases. Microglia are derived from the bone marrow and are more related to macrophages than to the other neural cells derived from neuroectoderm. They have a limited proliferative capacity in culture and require therefore continuous re-isolation form brain tissue to be available for experimentation. As an alternative to primary tissue, various microglia cell lines have been generated and one of the most-frequently used ones is the BALB/c based BV-2 cell. We present here initial experiments describing the suitability and limitations of BV-2 cells as surrogate model for primary microglia. For this approach, the inflammatory potential of the cell line was compared to that of primary microglia from mice. The transcriptional profile was compared on two different array platforms and by direct measurement of various cytokines. In addition, the *in vitro* data were compared to the *in vivo* reaction of microglia. The overall result was, that BV-2 cells do not show responses, not seen in primary cells, but that they also do not mount the full spectrum of activities observed in primary microglia. The genes induced *in vivo*, were also identified in the *in vitro* systems. However, the cell cultures showed regulations, not detected *in vivo*, which is a point requiring attention. In conclusion, the BV-2 cells appear to be a good model system for certain screen purposes, but basic mechanistic research may require the use of additional experimental systems.

Keywords: inflammation, neurodegeneration, cell line standardisation, cell line characterisation, primary cells

Lecture: free communications

A human neuronal model system with a potential to substitute primary cultures

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In the fields of neurotoxicity and developmental neurotoxicity, there is a lack of suitable human experimental systems. Most of the available cells are neuroblastoma cells with a transformed phenotype. Especially for experiments with dopaminergic neurons, mostly primary cells are required. We developed an alternative culture model based on LUHMES cells, a human neuronal precursor that was conditionally immortalized by a switchable myc gene. We established a model based on the toxicity of the endogenous dopamine of the cells. The cells died slowly upon exposure to methamphetamine and iron, and this was found to be due to oxidative stress triggered by the dopamine released from vesicles to the cytosol. As a consequence of the oxidative stress, the cytoskeleton was damaged, stress kinases were activated and apoptosis was triggered. We presume that this sequence of events reproduces those that may occur during Parkinson's disease and methamphetamine-induced neurotoxicity. Since the most frequently applied neurotoxin in primate and murine models of Parkinson's disease is 1-methyl-4-phenylpyridinium, we also examined the effect of this compound. Concentrations as low as 5 micromolar depleted glutathione, reduced the cellular energy charge, and eventually killed the cells. This is usually only observed in primary neurons, but not in a cell line. Thus the new LUHMES cell model may have the potential to substitute primary cells for many experiments.

Keywords: Parkinson's disease, neurodegeneration, methamphetamine, neurotoxicity, 1-methyl-4-phenylpyridinium

Lecture: evidence-based toxicology

Judging the value of alternative methods

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Alternative methods (AM) and their success in many areas of research have a relatively low visibility outside the field. This is partially due to the fact, that there is no clear definition of what an AM is, and which value it generates. The latter point is of major importance, not only for the public support of this research area, but also as a judgment basis for financing and granting systems. This presentation attempts to highlight important issues linked to the visibility and judgment of AM, and points out potential ways forward. A constructive dialog on this topic can broaden the field of AM, sharpen its cutting edges, and give it a profile clearly recognizable from outside. In the core area of regulatory toxicology of chemicals, cosmetics and agricultural products, AM are relatively clearly defined. Here, some of the burning questions are, whether 1:1 substitutions are possible for safety areas different from acute toxicity, how one deals with the concept of severity of stress in view of benefit calculations, and how far AM have a value as filters, in addition to substituting animal based methods. Especially the latter point is of major importance in the field of pharmaceutical research and development. Here the use and value of AM seems to be vastly underestimated, if one judges their impact based on the available statistics and publications. Most of the AM data, e.g. the use of *in vitro* metabolism systems, Ames assays, hERG assays and acute cytotoxicity assays never appear in statistics or publications, although they are an integral part of most drug discovery screening plans. Thus, it appears that the application and impact of AM should not be measured indirectly, based on the animal numbers in the official statistics on experimental animals. More direct and active measures will be suggested.

Keywords: animal statistics, toxicological domains, severity of stress, test strategies, visibility, judgement

Lecture: nanotoxicology

Proinflammatory response in human alveolar epithelial cells exposed to ultrafine zinc oxide (ZnO) particles at the air-liquid interface (ALI)

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We have recently developed a novel exposure system which allows a dose-controlled exposure of bronchiolar or alveolar epithelial cells to airborne nanoparticles at the ALI. The system allows for a homogeneous and quantifiable deposition of nanosized particles in a stagnation point aerosol flow (Tippe et al., 2002; Bitterle et al., 2006). We intend to apply this system for exposures of cellular models to nanoparticles for dose titrations and identification of toxicological endpoints in order to reduce animal studies. Using the ALI system, we studied toxic effects of nanosized ZnO particles. Human alveolar epithelial type II cells (A549), chosen as biological target, were cultured on Anodisc membranes to confluent monolayers ($1x10^6$ cells/cm^2) and placed in specially designed chambers. Commercially available ZnO particles (0.024–0.071 µm; AlfaAesar) were dry-dispersed using a dust generator (TOPAS) and diluted with humidified filtered air to an aerosol concentration of $8.6x10^5$ particles/cm^3, with a count median particle diameter of 136 nm. Exposure of the cells for 3 h resulted in a total deposition of 393 ng/cm^2. After 3 h exposure to airborne ZnO, cell viability remained unchanged, while transcription of the proinflammatory cytokine IL-8 increased 4-fold. Subsequent incubation of the cells in medium for another 2 h led to a further increase of the IL-8 transcripts up to 23-fold over controls. In addition, ZnO exposure upregulated the expression of cyclooxygenase-2 and enhanced the release of prostaglandin E2. Analysis of IL-8 transcription assessed in submerged exposures using a particle concentration of 10 µg ZnO/106 cells/ml led to comparative results. In conclusion, airborne ZnO provoked a proinflammatory response. However, the concomitant upregulation of cyclooxygenase-2 and release of prostaglandin E2 suggests the activation of a counteractive pathway. The particle dose attained at the ALI is well defined in contrast to exposures under submerged conditions.

Keywords: epithelial cells, air-liquid interface, nanoparticles, zinc oxide

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Poster: free communications

Differentiated ARPE-19 can serve an *in vitro* model for the study of light damage to the eye

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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 by apoptosis in response to photooxidative injury. In the absence of a suitable *in vitro* model, many mechanistic examination of light induced photooxidative injury were examined by animal experiments. ARPE-19, a non-transformed human diploid retinal pigment epithelial cell line that displays many of differentiated properties typical to the RPE *in vivo*, exhibit a number of morphological and biochemical indications of differentiation but only when cultures are allowed to grow to post-confluent densities for several weeks. At this stage, ARPE-19 cells express RLBP1 and RPE65, both of which are synthesized by differentiated RPE *in vivo*. *In vitro* differentiated ARPE-19 cells may therefore be a suitable cell model for mechanistic examinations of light induced cell damages. With the aid of *in vitro* differentiated ARPE-19 cells we examined the cytoprotective role of mitogen-activated protein kinase phosphatase-1 (MKP-1) of light damaged cells. Analysis of gene expression showed, that the expression of protein phosphates 1 and 8 was significantly induced (8- and 5-fold). The results of microarray analysis were verified by western blot analysis using a light dose of 1- 4 J/cm^2. Low light doses (2 J/cm^2) induced significantly MKP-1 expression and inactivated JNK. Phosphorylation of p38 and ERK were not altered upon treatment. High light doses (3 and 4 J/cm^2) decreased the expression of MKP-1 which resulted in activation of SAPK/JNK. After 24 h recovery time the high light dose treatment induced apoptosis (PARP-cleavage) and increased cell death. By means of *in vitro* differentiated ARPE-19 cells we could demonstrate for the first time that the MAPK phosphatase MKP-1 is capable of inactivating the stress-inducible protein kinase (JNK). Inactivation of the JNK pathway inhibits apoptosis and ameliorated cell viability. The cytoprotective properties of MKP-1 do not appear to be mediated by the p38 or the ERK pathway but rather by the JNK pathway in differentiated ARPE-19 cells.

Keywords: cell line, age-related macular degeneration, light damage, cell differentiation

Poster: vocational training

Outreach, alternatives awareness and replacement in Russia

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Significant progress has been made in Russia during 2005-2007 concerning awareness and implementation of alternatives in education. Several major outreach visits involving presentations and demonstrations of alternatives have been made by a team of InterNICHE campaigners, bringing the concept of humane education to large audiences. Media interest in these new approaches has resulted in nationwide and international coverage. Outreach letters have been sent direct to teachers across the country to promote the resources that have been developed. Printed resources and on-line information have been complemented by the distribution of freeware physiology and pharmacology alternatives – all translated into Russian. A library gives hands-on access to alternatives nationally, and the donation of computers and alternatives to some institutes has established exemplary multimedia laboratories. This multi-pronged strategy has now led to agreements being made between InterNICHE and several institutes to abandon animal experiments for teaching purposes at the departmental and faculty level. Implementation of alternative tools has been achieved at several institutes, with multimedia directly replacing the annual use of several thousand animals. The sharing between teachers of the experience of implementation has now begun. There have also been isolated cases of determined student conscientious objection, and the small number of Russian campaigners is slowly growing, empowered by the successes of recent years.

Keywords: InterNICHE, Russia, replacement, education, freeware

Poster: vocational training

Ethically sourced animal cadavers and tissue: considerations for education and training

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This paper describes "ethically sourced" animal cadavers and tissue, as defined by the InterNICHE Policy, and addresses the importance of using cadavers and tissue only from these sources when material is needed for the purpose of education and training. The attitudes developed by students and trainees using ethically sourced material and conventional sources are compared and discussed. Examples are given where the use of ethically sourced cadavers and tissue has been successfully implemented in practical classes for anatomy and surgery. Potential use for research and testing purposes is also briefly evaluated. The paper outlines the potential practical problems of such cadaver use and offers examples of how they may be overcome. The impact on veterinary colleges and society of "client donation programs" for sourcing animal cadavers is also addressed.

Keywords: InterNICHE, ethically sourced, cadavers, tissue, Policy, education, training, replacement

Poster: vocational training

From Policy to Practice: illustrating the viability of full replacement

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The InterNICHE Policy on the Use of Animals and Alternatives in Education is a comprehensive document in 10 sections that addresses all aspects of work with animals and alternatives in life science education and training. The Policy presents guidelines to ensure effective and fully ethical acquisition of knowledge and skills. It includes a definition of alternatives in education and of harm, and presents individual policies on dissection, the sourcing of animal cadavers and tissue, work with live animals for clinical skills and surgery training, and field studies. As well as addressing non-animal alternatives, therefore, it has a significant focus on the ethical use of, and work with, animals and animal tissue. It also addresses the use of animals for the production of alternatives themselves. The Policy demonstrates the possibilities for full replacement of harmful animal use in education and training. Examples from across the world of practical classes that accord with the Policy will be given. Recommendations will also be made for ethics committees, for university policy towards student choice, and for legislation.

Keywords: InterNICHE, Policy, guidelines, education, training, implementation, replacement

Poster: vocational training

Development of a physiological three-dimensional intestinal test system

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The small intestine is the place of digestion. Oral applied substances are taken up over the intestine epithelium into the blood vessel system. The evaluation of different substances regarding their toxicity and absorption is mainly still made by the LD_{50} *via* the use of animal experiments. Our aim is their limitation by the development of a 3D physiological intestinal test system. The matrix used in our intestine module consists of a porcine jejunal segment with an obtained

vascular system. The vascular system is reseeded with primary microvascular endothelial cells and the lumen of the matrix with primary enterocytes or cells with similar characteristics (e.g. Caco-2 cells). The intestine model is cultivated in a bioreactor system under arterial perfusion with growth medium simulating physiological conditions. The absorption of different substances into the blood vessel system can be experimentally determined through the descending venous branch. The cell characterisation and the proof of epithelial and endothelial cell monolayers takes place by histological and immunohistological analysis. The evaluation of the epithelial barrier function and other qualification parameters are to be established in the future by the use of different substances *via* HPLC, ELISA and photometric methods. In preliminary tests Caco-2 cells and endo-thelial cells could be successfully co-cultivated during a 14-day period on the matrix. First absorption studies of peptides on a native small intestine segment were compared to our system. Our aim is the establishment of a physiological 3D intestinal *in vitro* test system. By the complexity of our test system an adjustment to the human body over pharmacokinetic models is quite conceivable. Oral bioavailability of drugs and active ingredients could be tested due to the given blood vessel structures in our test system.

Keywords: intestinal test system, drug absorption, vascularised matrices

Lecture: computer assisted procedures

New animal model for objective pain research: non-invasive functional imaging in anesthetized animals by BOLD fMRI

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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. Chronic pain is getting a health problem of increasing importance (prevalence of chronic low back pain, migraine) nowadays. Traditional behavioural pain examinations in animals are highly stressful and subjective. Contributing to the 3Rs non-invasive imaging approaches like fMRI in anesthetized animals would significantly reduce the stress for animals and simultaneously refine and improve objective measurements of pain without the need of a behavioural readout. Having established a model for acute mild pain in fully anesthetized animals we now could show, that this model can be extended to investigate repetitive measurements. Moreover, optimized data-analysis strategies help to reduce the number of experiments needed to obtain statistically significant activation maps. We could show that reliable and quantifiable BOLD responses in pain related brain areas (e.g. thalamus, somatosensory cortex, cingulate cortex, hypothalamus, entorhinal cortex, hippocampus, amygdala) nicely comparable to human studies (Valet et al., 2006) , were activated during the different sessions. In conclusion, our computer controlled topical repetitive heat stimulation of the rat hind paw is a robust stimulation paradigm leading to reliable BOLD activation of sensory and especially pain related pathways. It is well suited for repetitive stimulations. The results can be quantified with respect to brain area, size, and intensity in relation to the temperature applied. Therefore, 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.

Reference

Valet, M., Sprenger, T., Boecker, H. et al. (2006). Repetetive pain exposure: Neuronal correlates in the human brain. *Proceedings of the 11th World Congress on Pain*, 431-438.

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

Poster: free communication

In silico model of skin penetration based on experimentally determined input parameters

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Not only after the introduction of the EU chemicals policy REACH, the investigation of dermal and transdermal drug absorption is of great interest for the cosmetic and pharmaceutical industries, as well as for governmental institutions. Then again, a reduction of the number of *in vitro* and *in vivo* experiments is desirable from the point of view of animal protection and due to economic and legislative constraints. This work addresses this issue and presents a framework for the simulation of diffusion through human stratum corneum: The mathematical model is based on a brick and mortar geometry for modelling the stratum corneum, which is extended by an additional compartment representing the more permeable deeper skin layers. Transport through this skin model is described by time-dependent diffusion equations. Together with transmission conditions, the interfaces and appropriate boundary and initial conditions, this describes a partial differential equation, which is solved numerically. The second closely interwoven component is the experimental setup, which allows the determination of input parameters for the mathematical model. A database of these parameters was collected for one lipophilic and one hydrophilic substance, namely flufenamic acid and caffeine. Two different experimental setups were considered. While the first is an *a priori* approach, the latter one is based on an *a posteriori* analysis. The validation of the model is proven by tape stripping experiments, which are performed following standard procedures. This allows a resolution of the diffusion process in space and time. The study is one of the first studies to present a comparison between simulation and *in vitro* diffusion experiment. The diffusion coefficient in the corneocytes is shown to be a critical parameter in the model. The simulated concentration depth profiles obtained using experimental input data show good agreements with the experimental ones. The future work will include enlarging the set of substances as well as studying the effect of keratin binding.

Keywords: stratum corneum, skin, tape stripping, numerical simulation

Poster: free communications

Alternatives changing the face of education in India

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Since the year 2000, there has been a strong awakening to the concept of the 3Rs amongst academic and bureaucratic circles responsible for curricula preparation and revisions in secondary/graduate education in life sciences and in the fields of pharmacology, medicine and veterinary education in India. In a country as vast as India, with thousands of educational institutions, every policy change, effectively saves the lives of millions of animals. In the year 2000-01 the Indian School Certificate Board of Education and Central Board of Secondary Education, deleted all animal dissection in higher secondary education. In 2003, the Pharmacy Council of India, decided to include computer aided learning instead of all pharmacological applications using live animals in about 500 pharmacy colleges across India. In 2006, the University Grants Commission, of the Government of India, issued a directive to all universities teaching zoology in graduate and post graduate courses, to implement available alternatives and other meaningful exercises to replace animal dissection. In 2007, the Medical Council of India has proposed a complete ban in dissection in the teaching of Clinical Pharmacology in medical schools in India, which will become effective by the end of this year. The changes have been relatively slower in veterinary education. However, there have been significant changes in that the Veterinary Council Of India has directed that lesser number of animals should be used in anatomy teaching and supplemented by computer aided learning. Clinical cases now replace the use of experimental animals in veterinary surgery teaching. These changes over the years have been largely due to the efforts of People for Animals (PFA), Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA) and the International Center for Alternatives in Research & Education (ICARE).

Keywords: alternatives, education, India

Poster: ethical and legal aspects in animal experimentation

Ethical justification concerning the application under article 8 paragraph 1 of the German animal welfare act (TierSchG) after including animal welfare in the constitution

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In the application under article 8 paragraph 1 Tierschutzgesetz (TierSchG) the ethical justification must set out scientific evidently. This demand is written down in article 7 paragraph 3 TierSchG. Attempts to refuse experiments on animals on the basis of ethical reasons failed until 2002 because the responsible authority was only entitled to review the explanation of the applicant with regard to plausibility. This opinion has been declared clearly by the Administrative Court of Berlin in connection with the refused experiments on monkeys in the year 1994. The experiments had to be approved. Finally, in the year 2002 animal welfare could be established as aim of state in article 20a of the constitution. From now on parliamentary questions and inquiries of animal welfare organizations reached the competent authority at regular intervals. They want to know, why the establishment of animal welfare in the constitution does not result in dramatically reduced animal experiments or reported animals used in procedures, respectively. Other frequently asked questions are how the application procedure changed since then and whether more experiments on animals are rejected now. Seeing that especially in Berlin the yearly reported numbers of animals increase and the answers to the Parliamentary questions remained unsatisfactory the Parliament of Berlin passed the resolution to extent the commission under article 15 TierSchG (commission which assist the responsible authority whether to authorize experiments on animals) by including moral philosopher. This request was translated into action in September 2005. Three proven moral philosopher specialized in animal ethic are appointed to the commission as additional consultant on the recommendation of the animal welfare organizations. Since then the commission has seven regular members each with two deputies. The first experience has shown that the particular knowledge of the philosophers is only needed in individual cases. This could be shown exemplarily in connection with an application concerning experiments on monkeys, which was rejected on the basis of ethical reasons in 2006 proven in detail through the moral philosophers. The applicant abstained from his possibility to institute proceedings against the responsible authority. A lot of questions remained open like the ethical limits of action and the absolute limit of suffering for animals.

Keywords: animal welfare, ethical justification, application

Lecture: revision of agrochemicals directive

Animal welfare and the revision of EU legislation on plant protection products

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In 1991, the European Community developed a regulatory framework, Directive 91/414/EEC defining strict rules for the authorisation of plant protection products (PPPs). The Directive requires very extensive risk assessments for effects on health and environment to be carried out, before a PPP can be placed on the market and used. The use of pesticides is recognised as posing threats both to human health and the environment. In order to address these concerns, the European Commission adopted a new strategy aimed at improving the way pesticides are used across the EU.

On 12 July 2006 the Commission published a proposal for a Regulation on PPPs (COM(2006) 388 Final). Although the proposal requires producers to share data from animal tests in order to avoid duplicate animal testing, much more than that is necessary to ensure that animal testing under this Regulation is only undertaken as a last resort. There is no mention of a need or objective to minimise animal testing, ensuring access to relevant information to avoid duplicate animal testing, or the promotion and use of non-animal test methods and intelligent testing strategies. It is important that when the Regulation and its annexes are finally adopted that these objectives are fully integrated.

Keywords: animal welfare, plant protection products, data sharing, alternative methods

Lecture: free communications

ecopa's EU FP7 project START-UP: Scientific and technological issues in 3Rs alternatives research in the process of drug development and Union politics

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The START-UP project is concerned with the identification and proposals to abolish bottlenecks in the 3Rs approach in pharmaceutical discovery and development. The goal of the project is the organisation of 3 workshops in order to determine a) the state of the art of each of the 3Rs in the EU, b) to assess European strength and gaps in 3Rs and c) the identification of rate limiting steps on the political, scientific, technological level. As a result, a Consensus Paper containing the concepts and suggestions for a Roadmap for future research will be produced. Stakeholders (among them European Pharmaceutical Industries (EPI)) have identified bottlenecks in drug development and in the integration of *in vitro* methods. Early identification of wrong candidates for further development and avoiding efforts for under-performing candidates, are essential for the competitiveness of European Industry. Identification of bottlenecks in the implementation of reduction, refinement and replacement of animal experimentation in drug R&D, should assist in identifying the best *in vitro* and *in vivo* systems, and to speed up the drug development process. Existing hurdles in the scientific, technological, political and environmental level (including regulatory), play a substantial role and are rate-limiting in developing new drugs, including biological entities (almost 50% of the currently developed products). ecopa (the quadripartite umbrella NGO for alternatives) structures with its VUB partner this support action around 3 major workshops which will be preceded by 2 Expert Meetings redefining and prioritising current bottlenecks in 3Rs methodology; with EPI, drug discovery and development. Each phase has its own specific needs, and analysing the present limitations and gaps needs to be addressed, e.g., many cell systems do not yet have the required stability for genomics, proteomics or metabonomics analysis; many current *in vitro* cell systems lack crucial bioactivation capability. Consequently, the status of satisfactory "predictive" pharmacology and toxicology *in vitro* has not yet been reached. In terms of politics and ethical concerns, considerable differences in regard to the use and development of transgenic animals, human tissues and stem cells create an atmosphere of insecurity for an effective academia and industry cooperation. The final goal of this action is a Consensus Document that analyses present status and possible solutions. Based thereon, a Road Map to implement the strategy for a better integration of 3Rs in the EU drug discovery and development strategy will be proposed.

Keywords: 3R methology, bottlenecks, politics, ethical concerns, consensus document

Lecture: revision of directive 86/609/EEC

Revision of directive 86/609: demands from the animal welfare point of view

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Since the adoption of the Directive 86/609/EEC in 1986 important progress has been made in science and new techniques. Additionally, the demand of the public for a better protection of laboratory animals increased as shown by a recent internet consultation of the European Commission. Therefore, a revision of the legislation is urgently needed. Since 2002 the work to revise the text began and the first draft is expected in autumn 2007. To truly meet the goal to protect laboratory animals essential improvements are necessary. The scope of the Directive should be extended to cover all scientific procedures covering basic research and education as well as to include certain invertebrates and embryonic forms. Due to ethical reasons the use of non-human primates should be prohibited. An effective authorisation procedure for all procedures using animals should be established. This should include a harm/benefit assessment and a retrospective analysis of all animal experimental

projects. There should be a system of local, regional and/or national ethics committees in which animal welfare specialists and lay persons have to be incorporated. Procedures causing severe suffering to animals should not be permitted. Greater transparency on animal use is essential. A main focus should be put on the importance of implementing replacement and reduction methods to the fullest extent. Current standards of housing and care should completely and immediately be replaced with those agreed in Appendix A of the European Convention ETS 123 as legally binding minimum standards. Nevertheless, the German Animal Welfare Federation is of the opinion that a significant reduction of the numbers of animal experiments performed in the European Union can only be obtained if the revision of Directive 86/609 is taken as an opportunity to implement a paradigm shift sanctioning animal experiments. There should be a general ban on inflicting pain, suffering or damage to animals in the sector of animal experimentation. Exceptions from such a ban could then be regulated according to the specific provisions of a revised Directive 86/609.

Keywords: EU legislation, animal experiments, scope, primates, authorisation procedure, 3Rs

Lecture: evidence-based toxicology

A human hepatocyte long-term culture system serves as a drug testing alternative to animal models: results of metabolic assays and electron microscopy

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Human hepatocytes are the *in vitro* system of choice to study drug-induced processes in man. We have developed and validated HEPAC2: a serum-free long-term culture system for human hepatocytes. Cellular viability and liver functions (urea and albumin production) were monitored daily. These functions remained relatively constant for up to 3 weeks. We used HEPAC2 to study the effects of repetitive drug treatments on hepatocellular functions and morphology. Acetaminophen (APAP) was used as a model substance. Hepatocytes were exposed to 18.6 mM APAP for 24 h. Subsequently, culture medium was replaced by medium without APAP and the same exposure scenario was repeated every 4 days. During APAP treatment urea and albumin secretion were reversible reduced by 15-30% and 70-80%. Cytochrome P450 2E1 and 1A2 were active for at least 3 weeks, since cellular response to APAP did not change during the first 4-5 cycles of exposure to APAP. Electron microscopy revealed that APAP led to a complete replacement of rough ER by smooth ER and degradation of glycogen. After removal of APAP, hepatocytes refilled their glycogen stores within 1 day, while it took about 2-3 days for complete regeneration of rough ER. Following the exposure protocol, the same ultrastructural changes were also found after the 3rd and 5th treatment of human hepatocytes with 18.6 mM APAP. Metabolism of APAP via glucuronidation and sulfation was analyzed by HPLC. Our data demonstrate the suitability of HEPAC2 to serve as a tool for repetitive screening of drug-mediated changes on hepatocyte functions. Furthermore, it may help to overcome the sparse availability of human hepatocytes for testing drug-mediated responses in man.

Keywords: human hepatocytes, acetaminophen, electron microscopy, glycogen, rough ER, smooth ER

Lecture: 7th cosmetics amendment

COLIPA skin metabolism project towards the inclusion of appropriate metabolism in *in vitro* alternatives for skin sensitisation and genotoxicity

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The aim of this initiative is to understand how metabolism of ingredients in *in vitro* models compares with metabolism in human skin, in order to develop appropriate alternative testing strategies for topically applied cosmetic/toiletry ingredients. New *in vitro* models for assessing skin sensitisation and genotoxicity potential should be characterised metabolically in order that any data generated can be interpreted in the context of human risk assessment. Two key questions are addressed here: Qu 1 "Is a chemical ingredient metabolised within the skin upon exposure?" Ingredients could be applied to *ex vivo* human skin, or we could use "simpler" *in vitro* models (e.g. 3D skin model or keratinocytes) to metabolically profile the ingredient. Such approaches would provide "mechanism of action" data for risk assessment. Qu 2 "Are *in vitro* models (used in predictive assays) metabolically competent in an appropriate way to allow an accurate prediction of potential toxicity?" Here, it will be necessary to determine the metabolic capacity of *in vitro* models to assess if they are sufficiently representative of metabolism in human skin. Enzyme expression (protein) profiles and functional activity (i.e. whether enzymes actively biotransform chemicals) are being investigated in a range of models.

Work funded by the European Cosmetics, Toiletries and Perfumeries Association (COLIPA)

Keywords: skin, metabolism, in vitro, sensitisation, genotoxicity

Lecture: free communications

Development of a functional *in vitro* bioassay for the marine biotoxin azaspiracid (AZA) using human colonic epithelial cells

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There is an increased awareness that marine biotoxins can have a serious adverse effect on public health and the economy. Many of these biotoxins are produced by phytoplankton that are subsequently ingested and thus accumulated by shellfish. As a result there is a regulatory requirement for routine testing. The EU aims to replace the existing biological test method, the *in vivo* mouse bioassay, with alternative methods. Our work has focused on the marine biotoxin azaspiracid-1 (AZA-1) and the development of an *in vitro* assay to assess AZA-1 toxicity. AZA-1 is found in shellfish and is responsible for gastrointestinal disturbances in humans. Therefore, in order to mimic the *in vivo* situation the human colonic cell line Caco-2 was cultured on microporous membrane supports. This enabled us to examine the impact of AZA-1 on barrier function, as measured by transepithelial electrical resistance (TER). TER is a useful index of the function of these cells in maintaining the transport of solutes and water. Significant reductions in TER were detected at 5, 10 and 100 nM AZA-1 at all time-points examined. This decrease in TER corresponded with increased permeability of the epithelial barrier. This result prompted us to examine the components of the tight junction, namely members of the claudin family. Alterations in claudin family members have been reported in affecting the level of tight junction

integrity and thus barrier permeability. Western blotting analysis showed that AZA-1 induced a significant dose-dependent increase in claudin-2 expression in both cytosolic and membrane fractions, indicating an alteration in intracellular protein localisation. Another member of the claudin family, claudin-4, increased in the cytosolic fraction in response to AZA-1. In contrast, claudin-3 protein levels were decreased in the membrane fraction. This alteration of claudin levels was confirmed to be in part due to signalling via the MAPK pathway, ERK 1/2. The p38 and JNK/SAPK signalling pathways were also activated although no role in the regulation of barrier permeability was observed. These results suggest that AZA-1 is capable of disrupting the intestinal barrier via alterations in tight junction protein expression. This assay has proved to be sensitive for detection of AZA-1 and could prove to be a useful tool in detecting known and unknown marine biotoxins.

Keywords: azaspiracid, bioassay, claudin, tight junction

Poster: free communications

H4IIE hepatoma cells exposed to anisoosmotic media are an alternative to animal testing studying osmotically regulated hepatic gene expression

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H4IIE hepatoma cells exposed to anisoosmotic media are an alternative to animal testing studying osmotically regulated hepatic gene expression. The use of cultured H4IIE cells exposed to anisoosmotic media is an experimental alternative for animal experiments to study the dependence of hepatic gene expression and metabolism on cell hydration. The *in vitro* H4IIE model avoids confounding variables possibly caused by secondary effects of high salt animal treatment. Betaine-Homocysteine S-Methyltransferase (BHMT, EC 2.1.1.5) catalyzes a methyl transfer from betaine to homocysteine to form dimethylglycine and methionine. BHMT is expressed at high levels in livers of humans, rats and guinea pigs, and its activity is largely regulated at the expression level. BHMT activity becomes essential when folate-dependent remethylation is impaired due to nutritional deficiencies or genetic defects. In mouse models of homocystinuria, hyperhomocysteinemia and betaine depletion were associated with fatty liver, which was preventable by betaine supplementation. BHMT gene expression is severely decreased in rat liver cirrhosis, providing more evidence for an association between disrupted betaine metabolism and liver disease. Betaine also is an osmoprotectant that accumulates intracellular under conditions of high extracellular osmolarity. Cell hydration changes induced by anisoosmolarity critically affect liver metabolism and gene expression. The BHMT expression and activity were decreased in liver of NaCl-loaded guinea pigs (Delgado-Reyes, 2005). In the course of gene expression studies using nylon cDNA-arrays we found that hyperosmolarity (405 mosmol/l) suppressed the Bhmt mRNA expression in H4IIE rat hepatoma cells. This was confirmed by Northern blot analysis, which in addition unraveled a pronounced induction of Bhmt mRNA expression by hypoosmotic (205 mosmol/l) swelling. Osmotic regulation of Bhmt mRNA expression was largely paralleled at the levels of BHMT protein and enzymatic activity. It may be part of a cell volume-regulatory response and additionally lead to metabolic alterations that depend on the availability of betaine-derived methyl groups.

Reference

Delgado-Reyes, C. V., Garrow, T. A. (2005). High sodium chloride intake decreases betaine-homocysteine S-methyltransferase expression in guinea pig liver and kidney. *Am. J. Physiol. Regul. Integr. Comp. Physiol. 288(1)*, R182-187.

Keywords: BHMT, hepatoma cell, cell volume, osmoregulation

Poster: good cell culture practice

A vascularised liver cell module as an alternative to animal experiments

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The liver is the largest internal organ responsible for the metabolism. Therefore, it represents an interesting analysis system for substances and active agents in pharmacy, chemistry and cosmetics. Existing *in vitro* liver test systems are very artificial and no real alternative to animal experiments. The used cells show only a small part of their biological reactions *in vivo*. Essential for hepatocyte (HC) vitality and function in *in vitro* longterm culture are 1) a vascularisation to guarantee transport of nutrients, metabolites and gases, 2) extracellular matrix (ECM) contact and 3) the co-culture with endothelial cells (EC). ECs of the liver build up a filtration barrier between blood and HC, and are involved in the control of important metabolic processes. We created a vascularised matrix for longterm co-culture of HC and EC. Basis is a porcine jejunal segment with an obtained vascular system, which is chemically acellularised. Hepatocytes and endothelial progenitors are isolated from porcine biopsies. Firstly the vascular system of the matrix is reseeded with progenitor cells. In the second step hepatocytes in collagengel suspension are seeded on the matrix lumen. During the cultivation period the matrix is perfused with medium over the artery in a bioreactor system simulating physiological blood flow. The culture of hepatocytes on the matrix shows good results for cell growth and conservation of liver specific functions. The hepatocytes synthesise albumin and urea during the whole cultivation period. Phase I and II metabolism of dextrometorphan could be shown over 21 days. Immunohistochemical stainings demonstrate HC proliferation, receipt of differentiation and the formation of tight and adhering junctions between the cells. EC precursor cells were seeded successfully in the vascular bed of the matrix. After 3 weeks of culture the cells express endothelial specific markers. Our liver module can be used to analyse substances and active agents and to examine regeneration processes or cell to cell interactions. At the conclusion of the work, the goal is the transfer of the results to a human model.

Keywords: liver module, metabolism analyses, vascularisation

Poster:Lecture: ecotoxicology

Towards replacing or refining fish tests in chemical regulation and effluent testing – strategies to advance fish cell lines and embryos as alternatives

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Ecotoxicological risk assessment relies, to a significant part, on the evaluation of chemicals and effluents in fish. Examples of regulatory tests include the fish acute toxicity test (OECD 203) and – as a chronic test – the early life stage toxicity test (OECD 210). In fact, fish are the most widely used vertebrates in environmental risk assessment. Therefore, according to the 3Rs, a refinement or replacement of fish tests is highly desirable. In light of this, we work toward the advancement of fish cell line assays as well as fish embryo tests so that they can be used instead of fish. Two strategies are being followed. The first is to systematically explore an array of fish cell lines for their functional abilities and most sensitive set-up to indicate acute toxicity to fish. One very promising cell line is the rainbow trout gill model, RTgill-W1, which has previously been shown capable of predicting the toxicity of paper mill effluents to fish. The second strategy is to explore the expression of genes in embryos of zebrafish as predictors of prolonged or chronic toxic effects. Focus here is on robust genes that are expressed upon chemical exposure in embryos and later life stages and on the functional characterisation of the role of such genes in the expression or prevention of toxicity. An example is the induction of the genes that code for the metabolic enzyme cytochrome CYP1A and the stress protein HMOX-1. These genes were found to be induced in the embryo in the same range of concentrations that elicit toxic effects in the Early Life Stage Test.

Keywords: fish cell lines, fish embryo, regulatory testing, ecotoxicology, fish tests

Poster: free communications

Cultured meat; advantages of manufacturing meat products through "tissue-engineering" technology

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A new report (2006) from FAO (Food and Agriculture Organization of the United Nations) states that livestock production is one of the major causes of the world's most pressing environmental problems, including global warming, land degradation, air and water pollution, and loss of biodiversity. Regarding climate change, FAO estimates that livestock are responsible for 18% of greenhouse gas emissions, a bigger share than that of transport. The consensus report (2007) from IPCC's (Intergovernmental Panel on Climate Change) confirms that global emissions must start to fall within the next 15 years and then be cut to around half of 1990 levels by 2050 if the world is to have a fair chance of preventing irreversible and possibly catastrophic global changes. In the same time global production of meat is projected to more than double from 229 million tonnes in 1999/2001 to 465 million tonnes in 2050. The environmental impact per unit of livestock production would have to be cut down by three quarters to reach IPCC's goal. FAO proposes promoting research and extension of cutting edge technology. Improved genetics, for example, could greatly reduce emissions of gases (carbon dioxide, methane, etc.) and of nutrients per unit of output. It is foreseeable that these measures will lead to animal welfare problems in research as well as in production itself. Traditional meat production is based on growing whole organisms. The main share of input is lost for maintenance, heat increment, travel, etc. Only 10% to 25% of metabolizeable energy input produces animal biomass. And depending on species only 51% to 78% of animal biomass is useable for human nutrition. Producing meat directly through cell cultures could be much more efficient. Tissue engineered meat could have dramatic environmental advantages. Meat with healthier compositions of fatty acids could be produced. The biggest usage of animals, which affects an annual number of 50 billion individuals worldwide, disregarding water animals, could be reduced and replaced. The market potential is huge: meat has an annual turnover of 250 billions USD. But in spite of existing research and an existing Dutch patent, cultured meat is still a visionary topic. The Future-Food-project (www.futurefood.org was published in July 2007) aims to accelerate research into and development of cultured meat. It is in contact with various researchers and NGOs and can provide their contact addresses as well as potential funding contacts (e.g. in the food-industry).

Keywords: tissue-engineering, livestock, climate change, pollutio

Poster: free communications

Gene expression profiling in dendritic cells to predict skin sensitization

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Up to now, the skin sensitizing capacity of chemicals has been investigated using *in vivo* animal tests. Due to the ethical and economical burden associated with research on animals, the urge for a validated *in vitro* alternative is very high. The aim of the present study was to identify genetic alterations in human dendritic cells in response to (non-)sensitizing chemicals and to extract relevant markers for predicting skin sensitization. In animal studies, Langerhans cells have been identified as potent antigen-presenting cells that play a crucial role in the development of allergic contact dermatitis. We used $CD34^+$ stem cell-derived dendritic cells, isolated from cord blood, as an *in vitro* alternative for Langerhans cells. The cells were exposed to chemicals with known (non-) sensitizing properties (nickel sulphate, dinitrochlorobenzene, oxazolone, eugenol, sodium dodecyl sulphate and benzalkonium chloride) for different time periods. Using microarray analysis, we revealed a set of genes with an expression profile that is strongly associated with the sensitizing character of the exposure compound. For a number of genes, this result was confirmed by real-time RT-PCR. The genes selected here are valuable candidates to predict the sensitizing potential of different classes of chemicals and deserve further validation using a more extended set of (non-)allergic substances.

Keywords: in vitro, skin sensitization, prediction, gene expression

Lecture: free communications

The new German BMBF joint project on the development of predictive *in vitro* tests for developmental neurotoxicity testing

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Given the significant potential of chemicals and drugs to interfere with development of the nervous system, 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 high numbers of laboratory animals. Around 1,000 pups have to be handled in an *in vivo* DNT study and at least 140-mated dams are used to produce enough pups from different litters available to the tests. Moreover, the study design is complex and clear recommendations for optimal methodological approaches in DNT studies are lacking. In addition, under the REACH program of the European Commission it is planned to evaluate approximately 30,000 existing chemicals for their toxicological properties. Prediction of developmental neurotoxic effects is a key feature in the toxicological profile of a compound. This situation will considerably increase the number of laboratory animals. Validated alternative methods for developmental neurotoxicity testing are not available. Thus, standardized, 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 standardized 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. These cell models are (1) embryonic stem cells, (2) human neural progenitor cells, (3) human teratocarcinoma cells, (4) neuro-sensorchips. To assess neural development molecular and mechanistic endpoints like differentiation, migration, proliferation, apoptosis and analysis of electrophysiological data will be established.

Keywords: developmental neurotoxicity, in vitro, embryonic stem cells, human neural progenitor cells, human teratocarcinoma cells, neuro-sensorchips

Lecture: free communications

Mouse precision cut lung slices (PCLS) as alternative for *in vivo* respiratory toxicology testing: focus on immune modulating LPS and allergens

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Precision cut lung slices (PCLS) offer the distinctive opportunity to gain insight into lung morphology and physiology under *in vitro* cell culture conditions. Objective of this project is the development of an *in vitro* technique for the assessment of immune modulating (allergic) substances while avoiding animal testing. This study is part of the European Union project SENS-IT-IV. Extraction of lung tissue (mouse) was performed directly post mortem to conserve vitality of the tissue. Lungs were filled and cut with a special microtome. After preparation of PCLS with a thickness of approximately 220 µm slices were cultivated at 37°C under cell culture conditions. PCLS were exposed to respiratory (e.g. ammo-

niumhexachloroplatinate, AHCP), and contact (e.g. cinnamaldehyde) allergens, to positive (LPS) and negative controls (salicylic acid). Vitality of the lung slices was either controlled by measurement of LDH enzyme activity or live/dead staining using confocal laser scanning microscopy (CLSM). Cytokines and chemokines were detected with Luminex technology. Dendritic cell markers CD11c, MHC class II, CD40 and CD86 were investigated in living mouse PCLS *in situ* using CLSM. *Ex vivo* incubation of PCLS with LPS induced both changes in cytokine profile and in expression of cell surface markers. LPS induced and dexamethasone prevented LPS induced release of cytokines and chemokines such as interleukin (IL)-5 (180% for LPS to 0% for LPS/dexamethasone), IL-1 (1550% to 325%), TNF-α (5340% to 470%), IL-12 (723% to 170%) and Rantes (1060% to 120%) in PCLS. Expression of MHC class II, CD40 and CD11c but not CD86 could be observed in naive untreated PCLS. After incubation of PCLS with LPS exclusively a strong enhancement of MHC class II was found. Treatment to AHCP increased IL-10 (220%), TNF-α and IL-1 up to 160% and MIP-1β up to 70%. Eotaxin, G-CSF and IL-12 showed increasing amounts up to 75%. Stimulation with cinnamaldehyde resulted in a significant decrease of MIP-1β up to 50%, but showed no changes in expression of other cytokines. Salicylic acid showed compared to AHCP an increase of TNF-α-expression, but e.g. no increase of IL-1α. Thus, the method of PCLS might provide an *in vitro* technique to predict immune modulating potencies of inhaled substances.

Keywords: PCLS, LPS, allergens

Poster: good cell culture practice

A new approach to study hypoxia *in vitro*

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Alveolar epithelial cells reside in an air liquid interphase and are responsible for maintaining an efficient gas exchange between the blood and alveolar space. Many diseases arise due to the breakdown in this function often involving a severe hypoxic component. As with many reductionist approaches designed to study alveolar dysfunction, technical difficulties arise when attempts are made to model the precise effects of altered oxygen tension on pulmonary cell function when using standard cell culture techniques. Due mainly to the inherent nature of static cell culture cellular oxygen consumption during respiration creates oxygen gradients from atmospheric tensions through the liquid environment of the culture medium to much reduced levels at the surface of the cell. As multiple factors such as cell number and time of culture can influence oxygen tension, such reductionist cell culture techniques are inherently inappropriate for the study of altered oxygen tension on cellular function. At best experiments conducted under static liquid culture are poorly controllable and highly variable, at worst artifactual. Therefore we have developed a system which can mimic the normal environment in the lung and can also be used to alter oxygen tensions. The system is described in detail here. We anticipate this system to be useful in hypoxia regulation studies and in investigations of oxygen tension on alveolar epithelial cell differentiation.

Keywords: lung, air liquid interphase culture, hypoxia

Poster: free communications

Enlargement of the database of the validated embryonic stem cell test (EST): testing of eight substances

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The embryonic stem cell test (EST) is a scientifically validated *in vitro* assay that was established to classify compounds with respect to their embryotoxic potential. This assay assesses the embryotoxic properties of chemicals by the evaluation of inhibitory effects on differentiation of contracting myocardial cells compared to cytotoxic effects on undifferentiated murine embryonic stem cells and differentiated 3T3 fibroblasts. On the basis of test data of 27 compounds a biostatistical prediction model (PM) was developed which enables the EST to predict the embryotoxic potential of test chemicals with an accuracy of 78%. In order to continually improve the predictivity of the EST, we intended to enlarge the data base of the validated EST by testing additional chemicals including pharmaceutical substances. In this study, we have tested eight different substances: benzoic acid, d-penicillamine, methyl azoxymethanol acetate, Y-27632, mometasone furoate, and three pharmaceutical inhouse substances. Different molecular and functional endpoints were assessed: detection of myosin heavy chain expression by flow cytometry analysis, microscopic evaluation of contracting cardiomyocytes and analysis of cytotoxic effects on embryonic stem cells and fibroblasts. On the basis of these *in vitro* endpoints we were able to correctly predict the embryotoxic potential *in vivo.*

Keywords: in vitro, embryotoxicity, embryonic stem cell test, data base enlargement

Poster: free communications

Cryopreserved human whole blood (-80°C): development and optimisation of a stable monocyte source for alternative pyrogen tests (Monocyte activation tests)

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The Paul-Ehrlich-Institute (PEI) is the Federal Agency for Sera, Vaccines and Blood Products in Germany. During the last 10 years we have tested and optimised different Monocyte activation tests (MATs) for the replacement of the rabbit pyrogen test with emphasis on human whole blood as monocyte source. Whereas on a research lab scale the use of freshly drawn human whole blood was convenient, for routine application (industry, agencies) there are severe obstacles like: safety (blood-born infectious diseases), standardisation (donor-to-donor variation), availability and legal issues. Based on our experiences we developed a simple method to cryopreserve human whole blood at -80°C (-80°C freezer). We achieved storage stability at -80°C up to two years. Storage in the vapor of liquid nitrogen (-140°C) is possible, and results in even longer shelf life. The donated blood is tested for infectious markers according to transfusion regulations. Routinely we pool the blood of 10 donors before cryopreservation in order to average the donor-to-donor variation. The susceptibility for non-endotoxin pyrogens is donor-dependent; therefore, we strongly recommend the pooling of monocytes. The frozen blood aliquots can be shipped safe, easy and economic at -80°C on dry ice. The cryopreservation procedure could be applied successfully on human apheresis monocytes, too. The -80°C cryopreservation technique offers an easy and convenient methodology to guarantee a safe, stable and reliable source of monocytes for MATs.

Keywords: alternative pyrogen test, monocyte activation test, cryopreserved blood, cryocopreserved apheresis monocytes

Poster: free communications

Establishment of a three-dimensional cell culture system of the canine endometrium

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Frequently, severe pathological disorders in the adult bitch concern the uterine tract, in particular the uterine glands. Experiments on living animals that help to elucidate their pathogenesis should be substituted by *in vitro* systems due to ethical reasons and practicability. Conventional monolayer cell culture systems are not appropriate to study this purpose as the cells show dedifferentiation and unnatural behaviour. Therefore the aim of our study was to develop a three-dimensional *in vitro* cell culture system that reflects the dynamic processes of the *in vivo* endometrium relating to morphology, epithelial cell polarity, secretion patterns, proliferation, and expression of steroid hormone receptors. Endometrial glands of uteri obtained after routine ovariohysterectomy of anestrous dogs (n=24) were isolated by collagenase-digestion lasting eight hours. Subsequently, they were cultivated on various extracellular matrix components (stromal and basement membrane) to maintain differentiated uterine glands. The cultivation of uterine gland explants on each matrix was extended over four days and repeated two times. Samples were taken every day for immunohistochemical (cytokeratin, vimentin, laminin, Ki-67, progesterone receptor, estrogen receptor) and lectin histochemical stainings and for electron microscopic analysis. The immunohistochemical detection of cytokeratin verified the epithelial nature of the explanted structures, vimentin was used for exclusion. Laminin immunostaining showed a various amount of basement membrane components. Using electron microscopy we could confirm the polarized differentiation of the epithelial cells. Ki-67 immunostaining showed a low mitotic activity of the epithelial cells according to anestrous uterine specimens. For the analysis of the secretion patterns of the extracted glands lectin histochemistry was performed and demonstrated high conformity with the original uterine tissue. A high number of oestrogen receptors was found in endometrial and cultured glands, whereas progesterone receptors were low. With the establishment of this three-dimensional cell culture system we created an analytical tool to mimic the complex endometrial architecture. It should be possible to transfer this system to other tissues and species and therefore represents an important contribution to avoid animal experiments.

Keywords: endometrium, in vitro, dog

Poster: free communications

Comparison of percutaneous permeation with epidermal lipid composition and histological parameters in four different species

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In vitro permeation experiments are important alternative methods to reduce *in vivo* animal tests for toxicological risk assessment and pharmacological research experiments. Although experiments on animal skin are accepted models, many unknown influences on permeation rates complicate a prediction of permeation through human skin based on *in vitro* experiments in other species. Therefore, we investigated the epidermal lipid composition and histological parameters (e.g. morphology and density of hair follicles, stratum corneum thickness) in dogs, cattle, pigs, and rats. To elucidate the influence of these parameters on transdermal drug transport we studied percutaneous permeation rates in the four species using test substances chosen based on different lipophilicity and molecular weight (flufenamic acid, ibuprofen, indomethacine, and salicylic acid). Permeation parameters were obtained in Franz diffusion cells with split skin (thickness 500 µm) over a period of 30 hours. Epidermal lipids were quantified after extraction with chloroform-methanol using high-performance thin-layer chromatography. Furthermore, histological parameters were obtained from 8 µm thick vertical and horizontal slices (haematoxylin-eosin staining). The permeation experiments showed an equal ranking of permeabilities for all substances (rat > cattle > dog > pig). Significant differences were found in cholesterol, cholesterol ester, cholesterol

sulfate, ceramide 3, galactosylcerebrosides, and phospholipids. Ceramide 4 could only be found in pig skin. Moreover, the histology showed a complex variety of results. From the large number of factors possibly influencing percutaneous permeability a correlation was only found to stratum corneum thickness (positively correlated with the permeation rate) and total epidermal lipid content (inversely correlated with the permeation rate). As the latter result is contradicting to other investigations, further studies will be required to clarify species related influences on skin permeability.

Keywords: percutaneous permeation, epidermal lipids, histology, epidermis, stratum corneum, hair follicles, comparative study

Poster: good cell culture practice

A cell culture model for the detection of chemical or physical agents with potential pro- or antioxidative properties using 8-hydroxy-2′-deoxyguanosine in cellular DNA as toxicological endpoint

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Oxidative DNA damage is widely accepted to be linked to mutation and malignant transformation in eukaryotic cells. Although *in vitro* as well as *in vivo* test systems are in use for the identification of oxidants there is still a vital need for feasible *in vitro* screening systems. In order to test their suitability to detect oxidative cytotoxicity we exposed lymphoblasts to a chemical and a physical agent both with known pro-oxidative activity mediated by the generation of reactive oxygen species (ROS) and by the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in cellular DNA. Commercially available murine lymphoma cells (YAC-1) were exposed to hydrogen peroxide (H_2O_2, 1 mM) and $FeCl_2$ (50 µM) or UV-C-irradiation (peak wavelength 253.7 nm; dose: 6 mJ/min x cm^2) for distinct periods of time. In separate experiments, the free radical scavenging compound TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) was added (1 mM) in order to demonstrate its protective activity. A HPLC system equipped with UV and electrochemical detection was employed to measure the levels of 8-OH-dG in isolated cellular DNA. DNA of untreated YAC-1 cells contained 5.95 ± 3.06 µmol 8-OH-dG/mol guanine. Following exposure to H_2O_2/FeCl2 for 10, 25, 60 or 180 min levels of 8-OH-dG increased time-dependently to more than threefold. Exposure to UV-C light also caused a time-dependent rise in 8-OH-dG by a factor of 40 (60 min) to 100 (360 min). In both cases, the addition of TEMPO almost completely inhibited the increase in concentrations of 8-OH-dG. Based on these results, YAC-1 cells represent a promising and feasible *in vitro* system for the detection of DNA oxidants in terms of an acute generation of 8-OH-dG. Ongoing investigations aim at the possible oxidative effects of nitroheterocyclic compounds of the nitroimidazole and nitrofuran type and the identification of further compounds with anti-oxidative activity. Also the capability of YAC-1 cells to repair oxidative DNA-lesions is presently under investigation.

Keywords: 8-hydroxy-2'-deoxyguanosine, pro-oxidative, DNA damage, antioxidative, in vitro

Lecture: free communications

Discrimination of contact allergens and irritants employing the ARC immune toxicity chip

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The aim of our present research is the establishment of an in vitro test system to reveal the sensitizing potential of chemicals. Therefore, we developed the ARC immune toxicity chip, a DNA microarray containing 65 immune genes in addition to a series of housekeeping genes, negative controls and normalization controls. Contact sensitizers (nickel, Bandrowski's base, sodium-2,4-dinitrobenzenesulfonate, 1-chloro-2,4-dinitrobenzene, alpha-hexyl-cinnamaldehyde, hydroquinone, eugenol) and irritants (sodium dodecyl sulphate, methyl salicylate, TritonX-100) were applied to immature dendritic cells (iDC) cultured from human peripheral blood monocytes. Gene expression in chemical-treated cells was compared to gene expression in solvent-treated samples. Taking into account the problem of donor variability, cells from 3 or 4 donors were exposed to each chemical to identify immune genes differentially expressed in all biological replicates. In addition, transcriptional changes in dendritic cells exposed to selected chemicals were analyzed on whole genome microarrays to investigate effects on genes not represented on our chip. Real-time PCRs were performed to confirm treatment-related differences in gene expression detected on microarrays. Employing the ARC immune toxicity chip, we could identify several immune-relevant genes that were up- or down-regulated after exposure of iDC to sensitizers, while no significant effect was detected after application of irritants. Allergens well-known as strong sensitizers induced differential expression of a higher number of genes than treatment with moderate and weak allergens. Our results indicate that our system could be used as an *in vitro* alternative to animal tests, provided marker genes not affected by donor variability are employed.

Keywords: DNA microarrays, dendritic cells, immunotoxicological testing, contact sensitizer

Poster: ethical and legal aspects in animal experimentation

Dramatic increase in numbers of transgenic mice – we must take action now!

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Transgenic animals, mostly transgenic mice, are important models to study (human) gene function and disease in biomedical basic research. With the rapid advances in sequencing the whole human genome, the great challenge today is to ascribe the function of the thousands of genes in normal physiology, development, and disease. One powerful approach to decipher the functions of genes is the manipulation of genes in intact animals. The production of transgenic animals that enable to transmit the genetic modification (gene knock-out, knock-in, or mutation) to their offspring requires germline transformation. The most commonly used techniques are: (1) microinjection of the transgene DNA into the pronucleus of fertilized eggs, (2) embryonic stem (ES) cell manipulation, and (3) the cre-lox technology for targeted homologous recombination. All methodologies have success rates below 10%. Thus, the process of transgenesis is wasteful and involves the infliction of pain, distress and suffering of the genetically modified (GM) animals. Besides that, the subsequent establishment of homozygous transgenic strains from founder animals is also a wasteful process, generating a large number of surplus animals, for which the strange term "waste animals" is used in the literature. A careful mangement of breeding colonies is mandatory in order to reduce these numbers (Robinson et al., 2003). The number of laboratories and companies generating knock-out mice has steadily increased since invention of this technique in the 1980s. At present, about 3,000 knock-out strains are available, and the numbers are growing exponentially (Knight and Abbot., 2002). In the table below, the numbers of transgenic animals used in basic research in Germany, Switzerland and the UK are listed. While the use of genetically modified animals increased dramatically in the last decade,

	2000	2001	2002	2003	2004	2005	%
Germany: transgenic mice	149,859	200,776	218,072	244,588	302,143	348,399	**233 %**
Switzerland: transgenic animals (>99 % mice)	59,000	61,000	68,500	63,500	81,000	94,000	**159 %**
UK: transgenic animals (>95 % mice)	581,740	630,759	709,979	764,095	914,023	957,451	**165 %**

there was a general decline in the overall number of experimental animals, which is mainly due to following the 3R principles and the invention of alternative methods. Thus, there is an urgent need to reduce at least the dramatic increase in the numbers of transgenic mice used in the EU and overseas. In 1997, an ECVAM Workshop on The Use of Transgenic Animals in the EU was held, and a Workshop Report together with a series of recommendations was published in 1998 (Mepham et al., 1998). Further recommendations on welfare of GM mice and on reduction of animal numbers have been published elsewhere (Robinson et al., 2003). Among these it was clearly stated, "that adequate and clear statistics on the numbers, species, types, production, breeding and uses of transgenic animals should be collected and published at least bi-annually by each EU Member State, and by the European Commission to promote transparency and accountability" (Mepham et al., 1998). It is the primary aim of the present contribution, to provide the latest statistics on the numbers of transgenic animals and to create awareness of this steadily increasing problem in the scientific community and in the public.

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Keywords: transgenic animals, animal numbers, animal statistics, animal protection law

Poster: free communications

Use of an alternative test battery to predict ocular irritancy of agrochemicals

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The Draize rabbit eye test, or some modification of this test, is essentially the only method for determining ocular irritation that is acceptable to the various regulatory agencies. Several *in vitro* methods have been used to investigate the toxicity of potential eye irritant with a view to replacing *in vitro* eye irritation testing. This study reports the results of an alternative approach for predicting irritation potential of agrochemicals. The approach was a two-stage test battery *in vitro*. The first stage was a cytotoxicity test, the MTT assay. The second stage was the HET-CAM test. The chick chorioallantoic membrane (CAM), being a connective tissue sheet with a visible blood supply, has been proposed as a substrate to identify the eye irritation potential of chemicals. During the HET-CAM test the chemicals are placed directly onto the chorioallantoic membrane. The changes of the vascular injury (haemorrhage, lysis or coagulation) are indications of the potential of the chemical to damage mucous membranes *in vivo.* MTT assay is a simple method to determine the viability / number of cells in culture, through the formation of a coloured product to which the cell membrane is impermeable. Determination of the ability of cells to reduce MTT to the formazan product after exposure to test compounds enables the relative toxicity of test chemicals to be assessed. In our studies comparative screening was performed with 6 agrochemicals to establish parallel data on alternative test battery (HET-CAM, MTT) and *in vivo* (Draize) results. In most cases, this study showed a good correlation between *in vitro* and *in vivo* data. By these results the present form of alternative test battery (HET-CAM and MTT) can be proposed as a pre-screen method for predicting ocular irritancy.

Keywords: alternative, HET-CAM, MTT, agrochemicals, eye irritancy

Poster: endocrine disruptors

In vitro and *in vivo* screening of azole fungicides for antiandrogenic effects

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In general, azole fungicides have a low acute toxicity, but we have only little knowledge about their potential health risks at low chronic exposures. Previously, we have shown that prochloraz has multiple potential mechanisms of action in cell-based assays, and prochloraz possessed antiestrogenic (Andersen et al., 2002) and antiandrogenic effects both *in vitro* and *in vivo* (Vinggaard et al., 2002). Two other azole fungicides, tebuconazole and epoxiconazole, have now been investigated for antiandrogenic effects *in vitro* and *in vivo* as well. The fungicides were screened in two well-established cell assays, including testing for agonistic and antagonistic effects on AR in transfected CHO cells, using an AR reporter gene assay. The compounds were also analyzed for effects on steroidogenesis in H295R cells, a human adrenocorticocarcinoma cell line, used to detect effects on steroid production. *In vitro* tebuconazole and epoxiconazole proved to be antagonists of the AR, and in the H295R cell assay, they were able to inhibit testosterone and estradiol levels, and increase progesterone levels. In an *in vivo* study, designed to test for developmental effects on rat offspring after prenatal exposure, the effects on hormone levels in male fetuses and morphological signs of feminization of the male offspring were investigated. Tebuconazole caused an increase in testicular 17alfa-hydroxyprogesterone and progesterone levels, and a decrease in testosterone levels in male fetuses. Epoxiconazole had no effect on any of the mesured hormonelevels. Furthermore, tebuconazole increased the AGD in female pups and resulted in an increased number of nipples in male pups, a tendency that was also seen for epoxiconazole, though it was not statistically significant (Taxvig et al., subm.). In conclusion the results obtained *in vitro* are in good agreement with the effects observed *in vivo*. Tebuconazole showed antiandrogenic effects both *in vitro* and *in vivo.* Antiandrogenic effects were also seen for epoxiconazole *in vitro*, however the dominating effect observed *in vivo* was a high frequency of stillbirths at the highest dose.

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Keywords: tebuconazole, epoxiconazole, reproductive toxicity, azole fungicides, endocrine disrupters

Lecture: nanotoxicology

In vitro techniques and nanotoxicity – the way forward

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Nanoparticles are already in our environment; we use them in our cosmetics products and medical researchers use them in novel drug delivery systems. Because of their small size they have the potential to cross biological barriers and produce exponentially greater effects than their non-nano counterparts. There is therefore increasing concern about the toxicity of nanomaterials and increasing pressure on governments and regulators to ensure that appropriate testing methods are in place. The European Scientific Committee on Emerging and Newly Identified Health Risks have recently assessed the appropriateness of the current chemical testing guidelines for nanomaterials and, in the UK, DEFRA have proposed a voluntary reporting scheme for the results of nanotoxicity tests. Animal protection organisations have an important role to play in highlighting the unreliability of existing animal models for the safety testing of non-nanomaterials (and by inference the inadequacy of these for nanomaterials) and the ethical objection to increased animal use. The relatively new field of nanotoxicity is ideally placed to change the current testing paradigm away from animal models. This already appears to be

happening, with a greater proportion of *in vitro* studies being published. For example, techniques have been developed to investigate the translocation of nanoparticles through the blood-brain barrier, the respiratory epithelia and the skin, completely *in vitro*. Such methods have the advantage in that they enable not only the indication of likely cytotoxic effects but the mechanism by which they might occur (e.g. oxidative stress). In addition, the use of diseased human tissues can identify any likely enhanced toxicity due to increased sensitivity and deterioration in cellular and tissue functions, without the additional problems of extrapolation from animal models. An intelligent or tiered testing strategy has been adopted under REACH and can form the basis for a similar strategic approach to testing nanomaterials. An example for how these methods may fit into a testing strategy for nanotoxicity is offered. In addition, priorities for research in the future are discussed.

Keywords: nanotechnology, nanotoxicology, in vitro, non-animal methods, testing strategy

Poster: ethical and legal aspects in animal experimentation

How do we assess the value of fundamental research using animals?

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A large proportion of laboratory animal use is in fundamental or basic research (35% in the EU). This is not carried out in direct relation to the testing of medical products but to understand more about the body and how, and why, it may malfunction. Under the proposed revision of European legislation for laboratory animals (EEC86/609), each research project may have to undergo a "cost: benefit" assessment prior to authorisation. In some countries (such as the UK) this already occurs but is done in a rather rudimentary manner. Although under-appreciated, more confidence is placed on our ability to assess the "cost" to animal. In contrast, the assessment of the "benefit" of animal research has been little studied, with the result that unsubstantiated claims to possible improvements to human health may be used in licence applications. This paper critically evaluates how it is possible to assess the value of fundamental research that uses animals retrospectively, particularly in relation to actual human health advances. A range of techniques are described including assessment of publication rate, citation analysis and systematic review. Additional problems that arise with the assessment of genuinely fundamental projects are also discussed. In particular, the possibility that applied benefits may not be realised for a number of years (if at all) or may be difficult to trace, given the non-linear nature of research and citation. This raises the genuine question of whether procedures, in which the cost to the animal can be predicted quantitatively but the benefit to humans cannot, should logically, be permissible.

Keywords: ethical review, basic research, citation analysis, systematic review, publication

Poster: revision of Directive 86/609/EEC

ECEAE recommendations for the revision of Directive 86/609/EEC relating to animals in science

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The European Coalition to End Animal Experiments (ECEAE) is an alliance of animal protection organisations from 17 European countries. The 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: alternatives, EU legislation, animal experiments

Lecture: free communications

Novel cell culture model for prevention of estrogen receptor negative, chemotherapy-resistant breast cancer: an alternative approach for animal experimentation

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In progressive pathogenesis of clinical breast cancer, comedo ductal carcinoma *in situ* (DCIS) represents a high risk lesion for chemo-endocrine therapy-resistant breast cancer. This preinvasive lesion is characterized by the presence of HER-2+, EGFR+, p53+, and ER- / PR- cells. Human tissue-derived cell culture models expressing clinically relevant genetic defects and exhibiting quantifiable carcinogenic risk should provide a facile paradigm to reduce, refine and/or replace the use of existing preclinical animal models involving xenotransplants of human breast cancer cells in athymic mice.

The EGFR+, p53+, and ER-/PR- human breast epithelial cells with stable expression of HER-2 oncogene (184-B5/HER cell line) represented the model for comedo DCIS. Status of cell proliferation, cell cycle progression and cellular apoptosis, represented the modifiable mechanistic end point biomarkers.

Aberrantly proliferative preneoplastic 184-B5/HER cells exhibited defective homeostatic growth control as evidenced by enhanced cell proliferation and down-regulated cellular apoptosis. Acute treatment of 184-B5/HER cells with synthetic chemopreventive agent, select dietary phytochemicals, or their active components/structural analogues, either inhibited cell cycle progression, or induced cellular apoptosis, thus re-establishing homeostatic growth control, predominantly *via* cytostatic growth arrest. Long-term treatment with these chemopreventive test compounds inhibited anchorage-dependent and anchorage-independent colony formation, thereby decreasing the risk for carcinogenesis.

These data validate an alternative and complementary approach to animal experiments for rapid prioritization of dietary phytochemicals for primary prevention or adjuvant therapy of ER- /HER-2+ breast cancer [Support: NCI CN-5029-63, DOD-BCRP DAMD 17-94-J-4208 and Irving Weinstein Foundation].

Keywords: human breast epithelial cell culture, HER-2 oncogene, growth arrest, apoptosis, cancer prevention

Poster: free communications

Towards the validation of Caco-2 cell line as model of intestinal absorption

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Caco-2 bi-dimensional system allows the evaluation of chemicals ability to cross the intestinal barrier, as well as to study their transport mechanism. Several studies, comparing Caco-2 permeability values of a large number of compounds (mainly drugs) with different physico-chemical characteristics with the corresponding *in vivo* human oral fraction

absorbed (Fa) values, have shown a good correlation between *in vitro* and *in vivo* data. Based on these results, the Caco-2 cell model, already extensively used in different laboratories to predict the oral absorption and permeability of different compounds in humans, is a good candidate for its validation for regulatory purposes. Recently, two different inter-laboratory studies have been founded by ECVAM: the first one was aimed at evaluating the best Caco-2 line and/or clone in terms of reproducibility and sensitivity. The latter (still in progress) is running in two different laboratories, with the purpose to evaluate the predictive capacity of this *in vitro* model (e.g., sensitivity, specificity, concordance), that is, its ability to predict unknown oral Fa. An appropriate number of compounds, for which good human and/or animal oral absorption data are available, were chosen as representatives of different *in vivo* response and/or use category (industrial chemicals and drugs), and have been tested either on the parental Caco-2 cell line (ATCC-derived) or on the TC7 clone (derived from late passage of parental Caco-2), in strictly defined experimental conditions. Absorption of the chemicals through Caco-2 monolayer, cultured on insert, is determined in a two-steps procedure: a) the highest non toxic concentration on cellular barrier is calculated for each compound using 14C-mannitol permeability, in presence of the chemical, as marker of integrity; b) the absorption kinetic (influx and efflux direction) is evaluated at the concentration selected in the previous step and expressed as permeability coefficient (Papp = dQ/dt*1/(A*C0). Results obtained in the present study with selected reference compounds for high, medium and low absorption (propanolol, cimetidine and atenolol respectively) are in agreement with the ranking reported for human oral Fa values, and look promising for the validation of Caco-2 system. This study is supported by ISS- ECVAM study contract CCR.IHCP.C431224.X0

Keywords: Caco-2, intestinal absorption, validation

Lecture: nanotoxicology

Nanotoxicology: *In vitro* model development for nanomaterial toxicity assessment on human health

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The global market of engineered nanoparticles is consequent and in perpetual progress, this exponential proliferation of nanomaterials may generate a new class of risk on health and environment not only for workers chronically exposed to nanoparticles, but also for population in general indirectly exposed. In order to better determine the potential harmful effects of nanoparticles on health, a new field of toxicology defined as nanotoxicology has emerged worldwide to investigate the risks of exposition to nanomaterials. The questioning and the awareness of authorities, industries and scientific community encourage investigations of the potential hazards of nanoparticles to avoid a similar disaster as with the use of asbestos which happened in the past. If the literature dealing with nanoparticles has been exploding during the last decade, there is no sufficient analysis of the toxicity of such particles till now due to inadequate understanding of a very complex reality and the absence of relevant standards. Therefore, additional toxicological studies are necessary to understand interactions between nanoparticles and living cells. The purpose of the Nanotoxico project managed by an excellence centre consists in the toxicological study of three types of nanoparticles having a real economic benefit in Belgium (carbon nanotubes, exfoliated nanoclays, silicon and titanium carbides). The multidisciplinary research team including chemists, physicists, biologists, physicians and pharmacists has five years to develop representative *in vitro* models using cell cultures mimicking tissues exposed to nanomaterials. These *in vitro* tissues models include skin, lung and intestine, which constitute the main potential routes of contact with nanoparticles. With this project, a 3D reconstituted human epidermis (fully differentiated) and new relevant cell models of reconstituted respiratory and gastrointestinal epithelia (with differentiated intestinal epithelia absorption cells and Follicle Associated Epithelium) has been developed to characterise the origin of the toxicity related to nanoparticles using histology, cytotoxicity and genotoxicity tests.

Keywords: nanoparticles, 3D cell cultures, skin, intestine, lung, histology, cytotoxicity, genotoxicity

Poster: nanotoxicology

Follicular penetration – *in vitro* testing of rigid and flexible liposomes

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The follicular penetration of two different liposomal formulations (rigid and flexible) was compared. The critical parameters occlusion, body area and humidity were examined. The Franz Diffusion Cell (FD-C) is an *in vitro* method often used for skin absorption tests. In this study, a new *in vitro* method was developed to mimic the dermal application of formulations with this test system. After donor application, human full thickness skin fixed in the FD-C was massaged for 3 minutes (female subjects, plastic surgery). The liposome membranes were labelled with Lissamine Rhodamine B and the core contained carboxyfluoresceine, allowing the analysis of the penetration pathway using Confocal Laser Scanning Microscopy. The recovery rate was determined for each skin layer (donor, heat separated epidermis, dermis and receptor). If the humidity was 75% (Organisation for Economic Co-operation and Development Test guideline (OECD TG) 428, skin absorption), it was not possible to detect differences between occlusion and no occlusion. If the skin is observed as an entity, no body area differences were detectable, while significant differences were found between the skin layers. If the humidity was varied, a significant influence for the liposomal formulation was detectable. The influence of the liposomal formulations depends on humidity (summer *vs*. winter situations in living quarters) as well as the different body areas in the deeper skin layers (as the follicle act as reservoirs for liposomal dermal applications). The follicular pathway was influenced indirectly by the humidity and the properties of the examined liposomes.

Remark: Trauer Sindy: Center of Experimental and Applied Cutaneous Physiology, Department of Dermatology, Universitätsmedizin Charité and ZEBET at the Federal Institute of Risk Assessment (BfR)

Keywords: follicular penetration, in vitro skin absorption test, rigid and flexible liposomes, in vitro massage technique

Poster: free communications

Development and pre-validation of an *in vitro* method for the prediction of drug-drug interactions and intestinal absorptions of P-glycoprotein substrates using cell monolayers

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Drug efflux transporters like the multidrug resistance protein MDR1 can significantly affect oral absorption and bioavailability, tissue distribution excretion, pharmacodynamics of drugs and take part in the drug-drug interactions. Bidirectional transport experiments using cell culture models that mimics the characteristics of physiological barriers such as intestines have shown good performance for passively transported drugs, but for drug molecules with carrier mediated transport there is still a need for an accurate and repeatable method for the prediction of *in vivo* absorption and drug-drug interactions. In this study our aim is to establish and pre-validate an *in vitro* bi-directional cell culture model using MDCKII, MDCKII-Pgp and Caco-2 cells for the prediction of *in vivo* absorption, drug-drug interactions and pharmacokinetics of efflux system substrates. In bi-directional transport experiments designed to evaluate the suitability of the Caco-2 model used in this study low, middle and high molecular markers: propranolol, atenolol and mannitol, respec-

tively were used and apparent permeability coefficient (Papp) clearly decreased with the increasing molecular weight of the markers. The low permeability coefficients of mannitol observed throughout all experiments demonstrated the tightness and tight junction integrity of the Caco-2 cell monolayers used during this study. Functional expression of the P-gp in this cell line was shown with the bidirectional transport experiments done with Rhodamine 123 which resulted in the asymmetric transport of the substance with an efflux ratio of 16.5. In the experiments with Digoxin (1 µM), the efflux ratio was found to be 21.7 which is decreased to 1.24 and 1.07 in the presence of Cyclosporine A (12 µM) and Verapamil (200 µM), respectively. Substrates of P-gp, Fexofenadine, Saquinavir, Paclitaxel, Loperamide, Quinidine, Talinolol and Celiprolol along with the specific inhibitor LY-335979 will be used as reference substances in the further experiments in compliance with recently relieved draft FDA Guideline on *in vitro* testing of drug-drug interactions. Nowadays many animal experiments are conducted in order to be able to determine the leading drugs from a large number of drug candidates thus this study will allow reduction in the number of animal experiments in testing the drug-drug interactions. Financial support from the EU project MEMTRANS is gratefully acknowledged.

Keywords: efflux proteins, permeability, drug-drug interaction, cell monolayers

Poster: free communications

Establishment and pre-validation of a pulmonary *in vitro* model for transport and toxicity studies

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Within the light of revised EU directive (86/609/EEC) it is of utmost importance to develop new *in vitro* methods as a possible replacement for laboratory animals. Calu-3 cells have been described to have numerous features to be used as suitable in vitro model for upper epithelial airway (Foster et al., 2000) but there are no validated methods available. We report here the influence of DMSO, pH on Calu-3 cells and selection of a suitable buffer for transport experiment. TEER (Transepithelial resistance) and epithelial transport were the parameters we focused on. DMSO, a popular solvent for lipophilic compounds, was found to have considerable influence over TEER and transport of compounds across the monolayer in a concentration and time dependent manner. When applied apically DMSO had modest effect on TEER, decrease in TEER was more prominent when DMSO was present only in the basolateral side. When DMSO was present in both compartments the TEER values were comparable to negative controls. DMSO increases transport of rhodamine (RHO) from basolateral to apical direction but had no influence over fluorescein (FLU). It is known that pH influences tight junctions, which in turn govern the TEER. We observed TEER values over a range of pH (6.6-8.0) in two buffers Hanks' Balanced Salt Solution (HBSS) and Krebs-Ringer Bicarbonate Buffer (KRB). TEER values were more stable in HBSS over the entire range. Whereas KRB showed more anomalous behavior as compared to HBSS, and TEER values showed inconsistent increase and decrease over different pH values moreover at low pH values (6.8-7.0), TEER values were relatively higher. TEER values were stable at pH 7.4 in both buffers. Based on these results, HBSS was chosen as a buffer for future experiments. Our findings clearly demonstrate that even apparently simple steps could have profound influence over cell behavior and can govern the fate of future experiments. Buffer selection, effect of pH and DMSO, are the landmarks, which will help better validation and establishment of Calu-3 as *in vitro* models for possible replacement of animal trials in studying pathogenesis of the lung and assessing potential toxins as well as future drugs. Financial support by EU project PULMONET is gratefully acknowledged.

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Keywords: lung, Calu-3, epithelial barrier, permeability, DMSO toxicity, TEER

Poster: free communications

New bioethical forms of educational process in universities of Armenia

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Education in the field of biology, medicine and veterinary science was always accompanied by experiments on the animals which were far from humane and ethical grasps. Both students and lecturers criticized such educational methods.

During lessons there are protests among students who don't want to take part in severe experiments on animals because of moral, ethical and religious reasons. However, students don't have a choice and are obliged to attend such lessons, because otherwise they won't be admitted to the examinations. Such situation is contrary to the principles of freedom of student's conscience and breaks his human rights.

This problem has been unsolvable for many years. But today, owing to activity of the numerous nongovernmental organizations, there are libraries of alternatives to experiences on animals. Alternative methods imply thematic computer programs, modeling, plaster casts which substitute use of animals.

Now National Center on Bioethics successfully realizes the project on application of progressive educational methods, humane alternative programs, which are mentioned above, in education system of universities of Armenia.

Keywords: education, Armenia

Poster: free communications

Towards an *in vitro* test system for developmental neurotoxicity: a new test method using mouse embryonic stem cells

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Today, on a large number of chemicals we lack information on their adverse effects on the developing nervous system. Existing *in vivo* test methods are time consuming, laborious, expensive and require high numbers of laboratory animals. Thus, standardized, predictive *in vitro* screens for the evaluation of developmental neurotoxicity need to be available. The long-term goal is to increase efficiency in terms of reduced animal use and higher throughput compared to whole-animal testing using the existing regulatory guidelines. In this study we established a method for differentiation of mouse embryonic stem cells into neural cells, designed with special regard to the testing of chemicals. It is based on a modified protocol for adherent monolayer cultures in a defined medium and offers the advantage of a reproducible development of neural cells in a comparatively short time. The differentiation of D3 cells into neural cells was determined by analysis of neuron-specific marker protein expression using flow cytometry. In addition, the developing neurons were characterized by immunofluorescence staining using neuron- and glia-specific antibodies. In this way, we also identified neurotransmitter subtypes. As a result, we were able to define neural-specific molecular endpoints for the detection of chemical effects on neural development. To investigate the assay performance, we assessed concentration-dependent effects on neurons and glial cells using a limited number of model substances. This *in vitro* method might prove to be a useful component of a more complex modular *in vitro* test system assessing the impact of neurotoxicants on the developing nervous system.

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

Lecture: EU-chemicals policy (REACH)

The need for improvement of the integrated testing strategy for reproductive toxicity under REACH

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Under the new EU chemicals legislation REACH 30,000 existing chemicals should be evaluated within a period of 15 years. About 3,000 high production volume chemicals have to be assessed during the first 3 years. For most of these chemicals large data gaps must be closed by conducting various studies, repeated dose studies in particular. According to estimates of the European Commission, around 70% of all experimental animals are used to test chemicals for toxicity on reproduction. This is also where about 70% of the costs are generated – particularly for testing substances on impairment of fertility and reproductive ability, because REACH prescribes a two-generation test in rats (OECD Test Guideline 416) as the standard procedure for chemicals with production volumes of more than 1,000 tonnes. Around 3,000 animals are needed to test one substance using OECD TG 416. ZEBET proposes a procedure with a considerable lower number of animals: The standard test could be the one-generation test. There is growing evidence that the examination of only one generation hardly leads to any loss of information for decision making. However, before a one-generation test could be used for regulatory purposes, the corresponding OECD TG 415 would have to be updated. Preliminary work for the use of a test of this kind in a graduated programme of pesticide testing was done by the International Life Science Institute/Health and Environmental Sciences, USA and by ZEBET. If the standard testing requirement could be limited to a one-generation study, around 1,400 experimental animals for each substance tested would be saved. With an estimated 2,000 substances that have to be tested over the next three years under REACH, this would mean a 2.8 million reduction in the number of laboratory animals used.

Keywords: REACH, reproductive toxicology, testing strategy, generation study

Lecture: free communications

LCSA, loose-fit coculture-based sensitization assay

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Protection against contact allergy begins with the collection of reliable data about the sensitizing potential of chemicals. Today, the LLNA (local lymph node assay) in mice is widely used to identify sensitizing substances. For several reasons, an *in vitro* assay could be preferable to animal experiments. We propose an *in vitro* test for the detection of a sensitizing potential of a chemical composed of a single layer of human non-differentiating keratinocytes and of allogenic floating monocytes which are cocultured in serum-free medium in the presence of a cytokine cocktail. Within days, the coculture develops to an allergen-sensitive system consisting of activated keratinocytes and of mobile dendritic cell-related cells. The sensitizing potential can be determined by analyzing the expression of the dendritic cell maturation marker CD86. For the model contact allergens tested so far (trinitrobenzenesulfonic acid (TNBS), phenylendiamine (PPD), 4-aminoacetanilide), the strength of the reaction was in concordance with results from the LLNA. Sensitivity of the assay allowed testing at concentrations without general cytotoxicity. Thus, a differentiation between allergens and irritants was possible. Regarding cytokine secretion, the assay distinguished between the allergen TNBS and the Toll-like receptor ligand LPS (lipopolysaccharide). The coculture can be set up from cryopreserved cells. The assay is easy to perform and reproducible. Donor-variance is negligible. This *in vitro* assay based on a loose-fit coculture is a reasonable approach to screen for the sensitizing potential of xenobiotics and might partially replace the LLNA and other animal tests.

Keywords: in vitro assay, sensitization, contact allergen, cell culture

Poster: free communications

Construction and functional testing of a three-dimensional cornea equivalent using primary canine corneal cells

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A wide range of ophthalmic drugs are being used in veterinary ophthalmology. Their dosage regime is often directly transferred from humans to companion animals without formal testing. To provide a model to be used for *in vitro* studies on drug effects in dogs, this study was conducted to establish a protocol for the primary culture of canine corneal cells (i.e. endothelium, fibroblasts, epithelium) and the construction of a three-dimensional cornea equivalent. Pharmacological effects of dexamethasone were used to test the functionality of this equivalent. Corneal cells were isolated using a combined enzymatic and mechanical technique. In culture, the different cell types were verified with phase contrast microscopy, immunofluorescense and western blotting. The cornea equivalent was constructed step by step in membrane inserts of a six-well plate. Stromal fibroblast in a collagen matrix were seeded onto a confluent endothelial cell layer and cultured for 6-8 days. Then epithelial cells were added and grown to confluence in a submerged culture. Finally, the equivalent was lifted to the air-liquid-interface for two more weeks to allow a differentiation of the epithelial cells. To study the effects of dexamethasone, the glucocorticoid receptor was investigated in the cells and the equivalents using RT-PCR and immunohistochemistry. Furthermore, both systems were stimulated with lipopolysaccharide (LPS) and treated with dexamethasone; the effects of the treatment were measured as a change in prostaglandin E2 (PGE2) concentration in the culture medium. A protocol for the isolation and culture of canine corneal cells as well as their reassembly in a vital cornea equivalent was successfully established. The cornea equivalents could be cultured for a total of five weeks. The glucocorticoid receptor was detected in both the cultured cells and the cornea equivalents. Dexamethasone led to a dose-dependent reduction of PGE2 in both systems. The primary culture of the canine corneal cells and the cornea equivalent are interesting systems to test drug effects on corneal cells. Interestingly the cornea equivalents were more sensitive to the dexamethasone treatment than the single cell cultures. Studies using the equivalent may reveal further insights on pathophysiological and therapeutic mechanisms in ocular disease.

Keywords: cornea equivalent, dexamethasone, glucocorticoid receptor, LPS, canine

Lecture: free communications

EC funding of alternative testing strategies

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The European Union supports research and development activities covering almost all scientific disciplines. Framework programmes are the main financial tool for RTD support. The current Seventh Framework Programme (FP7) is organised in four programmes corresponding to four basic components of European research: Coorperation, Ideas, People and Capacities. Within the Cooperation Programme life science related research is supported by the Health Theme, the Environment Theme and the Food, Agriculture and Fisheries and Biotechnology Theme. The European Union is Europe's major sponsor of research in alternative testing strategies. Joint European research in this area is expected to mobilise and integrate research excellence necessary for the promotion of new strategies for alternative testing. The research should progress towards alternative methods in order to reduce, refine or replace substantially the number of tests and laboratory animals involved in pharmaceutical discovery and development. Obtained results should establish a basis for international validation.

Keywords: European Commission, FP7, collaborative research

Poster: endocrine disruptors

Prevalidation of the MELN assay, a reporter cell line to screen for estrogenic activity

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The screening for estrogenicity and androgenicity of existing and new chemicals has been assigned priority in the determination of endocrine activity of compounds. Insight into the mechanism of action of steroid hormones has provided tools to develop alternatives to classical *in vivo* assays. Several *in vitro* assays are available and prevalidation for two estrogen receptor (ER-) and two androgen receptor (AR-) gene reporter assays is going on as part of the EU 6th FP project REPROTECT, coordinated by ECVAM. VITO has been appointed as the leading lab for the MELN assay and results of the 1st & 2nd module of the ECVAM validation scheme are presented. For this assay, which makes use of estrogen-sensitive human breast cancer cells (MCF-7) stably transfected with an estrogen-responsive Luc-gene (Balaguer et al., 1999), test procedures were optimised and intralaboratory variability was evaluated. As cytotoxicity should be evaluated adjacent to estrogen receptor transactivation, the Cyto-TOX-OneTM homogenous membrane integrity assay, a sensitive fluorescent LDH-leakage test was included for cost-efficient dual endpoint measurements using the same cells (Berckmans et al., 2007). Standard operating procedures considering cell culture conditions and evaluation of estrogenic activity are available. For chemicals with unknown properties, first a pre-screen procedure should be performed. The latter, based on a broad range finding from 10-13 M up to 10-5 M, does include a test for mode of action (agonist or antagonist), a test for evaluation of cytotoxicity and a test for unspecific toxicity. Next, the non-toxic working range for estrogenicity is defined, and the test procedure for agonistic mode using -estradiol as a positive control or for antagonistic mode using OH-Tamoxifenβ17 as a positive control can be applied. The test results for a panel of 16 chemicals evaluated according to these procedures of the MELN assay will be presented. EC_{50} calculations will be made and used (1) to evaluate intralaboratory performance and (2) to classify the chemicals according to their potency and compare these with available literature data. The other steps in prevalidation, the transferability of the procedures and evaluation of interlaboratory variability have started, and data will be available by the end of this year.

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Keywords: estrogens, MELN cell line, prevalidation

Poster: nanotoxicology

Ecotoxicological hazard of ZnO, TiO_2 and CuO (nano)powders: effects on bacteria, crustaceans and protozoa

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Metal oxides are generally considered non-harmful primarily because of their low solubility. It is known however, that if reduced to nanoscale (<100 nm), chemicals start to feature characteristics that differ from those of the bulk material. The greater specific surface area renders nanoparticles (NPs) biologically more active. Currently the research concerning environmental hazards of engineered NPs is limited: There are no standardized methods and the question remains whether the techniques used for the toxicity testing of chemicals are applicable for NPs. Insolubility and aggregation of particles in aqueous media make the testing technically difficult and give rise to the dilemma whether to simulate environmentally relevant conditions or use solvents/dispergants to stabilize the suspensions.

The aim of this study was to evaluate the influence of size and hydrolysis of metal oxides on ecotoxicity with bacterial (*Vibrio fischeri*) luminescence and growth inhibition, crustacean (*Daphnia magna, Thamnocephalus platyurus*) mortality and protozoan (*Tetrahymena thermophila*) growth inhibition. Tested chemicals were nanosized ZnO (50-70 nm), TiO_2 (25-70 nm), CuO (30 nm). Bulk forms of the metal oxides and ionic forms of the metals ($ZnSO_4*7H_2O$ and $CuSO_4$) were assessed in parallel. Bioavailable ions from hydrolysis of oxides as potential contributors to the toxic effects were quantified by luminescent recombinant metal-specific *E.coli* sensor bacteria. The metal oxide suspensions were studied for particle size distribution by using Nanosight LM10 nanoparticle characterisation system.

Our study is the first comparison of toxicity of nanosized and bulk ZnO and CuO for *V. fischeri, D. magna, T. platyurus* and *T. thermophila* – representatives of different trophic levels. Our data showed that in case of all organisms nano and bulk ZnO exhibited practically the same toxicity, whereas nano CuO was up to100-fold more toxic than its bulk form. Nearly all the toxicity of ZnO and a great part of toxicity of CuO was due to dissolved toxic concentrations of metal ions. TiO_2 had no effect (up to 20.000 mg/l) for all the test organisms. Visual appearance of test solutions and Nanosight LM10 analysis revealed that NPs had aggregated to the size of their bulk forms, which may also occur in natural conditions.

Keywords: ecotoxicology, nanoparticles, CuO, TiO_2, ZnO, bacteria, protozoa

Calendar of events

» 4th International SkinEthic Workshop,
September 6-7, 2007,
Nice, France.
http://www.skinethic.com

» 18th Meeting of the International Society for Livestock Husbandry (Internationale Gesellschaft für Nutztierhaltung, IGN),
September 20-21, 2007,
Giessen/Germany.

» 14th Congress on Alternatives to Animal Testing – Linz 2007,
September 28-30, 2007,
University of Linz, Austria.
http://www.zet.or.at/kongress/Linz2007/index.html

» EUROTOX 2007,
October 7-10,
Amsterdam, The Netherlands.
http://www.eurotox2007.org

» First International Forum Towards an Evidence-Based Toxicology (BT),
October 15-18, 2007,
Spazio Villa Erba, near Cernobbio, Lake of Como, Italy.
http://www.ebtox.org, ebt.forum@jrc.it

» AALAS National Meeting 2007,
October 14-18, 2007,
Charlotte, NC.
http://www.aalas.org/index.aspx

» 2. REACH-Symposium,
November 22, 2007,
Berlin, Germany.
http://www.gd-online.de