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ALTEX

ALTERNATIVES TO ANIMAL EXPERIMENTATION

Welcome address
by Horst Spielmann and
Walter Pfaller

ESTIV 2010
16th International Congress on
In Vitro Toxicology

EUSAAT 2010
13th Annual Congress
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LINZ 2010
16th Congress on
Alternatives to
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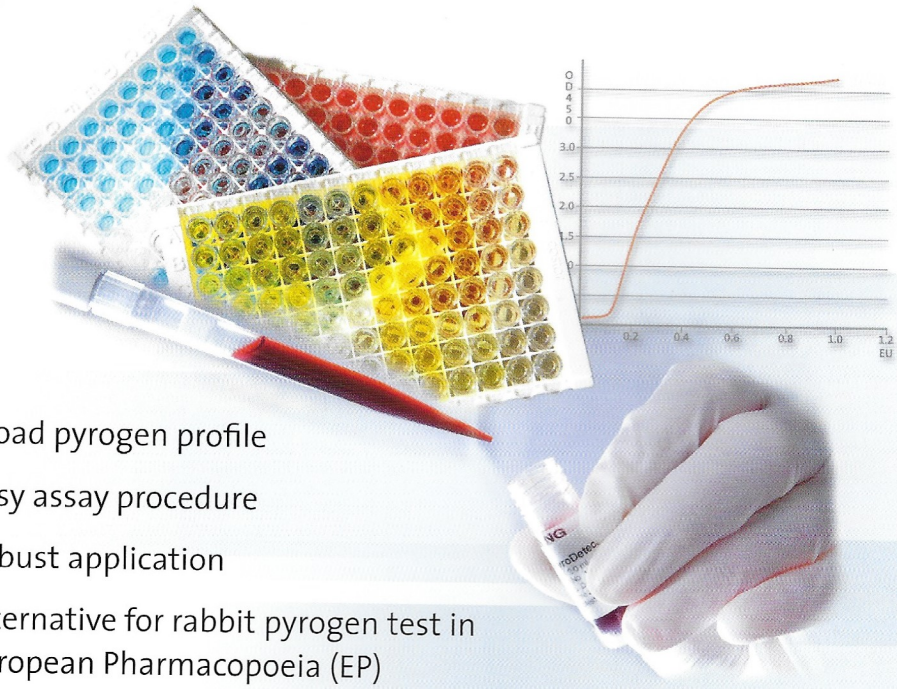

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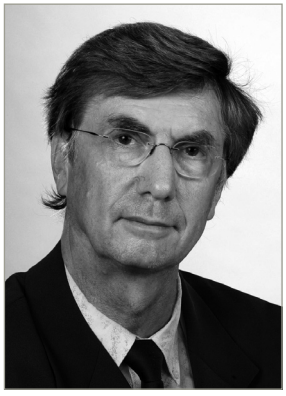
Welcome address to the 16th Congress on "Alternatives to Animal Experimentation" – Linz 2010



Dear colleagues,

on behalf of EUSAAT – the European Society for Alternatives to Animal Testing, ESTIV – the European Society of Toxicology In Vitro and zet - the Austrian Centre for Alternative and Complementary Methods to Animal Testing, I cordially welcome you to the "Linz 2010" congress. This year the "Linz Congress on Alternatives to Animal Testing" is the major international scientific event in the field of the 3Rs. In essence it is this year the European Congress on Alternatives to Animal Testing.

We are very pleased that in 2010 the "*16th Congress on Alternatives in Linz*" will be held as a joint congress of ESTIV and EUSAAT. This means as the "*ESTIV 2010 – the 16th International Congress on In Vitro Toxicology*" and also as the "*13th Annual Congress of EUSAAT*". The cooperation between ESTIV and EUSAAT will ensure a high level of oral presentations and discussions during the poster sessions. Taking into account that we have received significantly more abstracts than in the past, we have reserved considerably more time for the poster sessions than usual. This will allow all of the participants to expose themselves to new ideas, meet old friends and make new ones.

As you can see from the programme, each session will start with a "state of the art" lecture followed by four to five oral presentations selected from the abstracts. Several of the past meetings of ESTIV and EUSAAT have started with satellite meetings. This year we have scheduled several "Pre-Congress Meetings" sponsored by the European Commission as well as by ESTIV and EUSAAT. Since EUSAAT is engaged in legal and ethical aspects of animal experimentation, we are starting the conference with a session on "Legal, ethical and policy aspects of animal experiments". We will then cover "Metabolism and toxicokinetics" and "Skin sensitization and eye irritation". The fourth session is devoted to "Good Cell Culture Practice", which is a joint ESTIV/EUSAAT activity. On the second day we will continue with two sessions on "Acute and long term toxicity" and a session on "Antibody production", which is sponsored by the Dr. Hadwen Trust (UK). The afternoon session, which is devoted to the controversial issue of "Nanotoxicology", will include a round table discussion; this session is sponsored by the "Animalfree Research Trust" (CH). The final day will cover hot topics, e.g. "Reproductive toxicology and stem cells", "New developments and novel methods" and also the US activity "Toxicology in the 21st Century".

As in the past, we are strictly avoiding parallel sessions at the "Linz 2010" congress and we have reserved sufficient time for intensive discussions during the poster sessions. We have always tried to motivate young scientists by giving awards to young authors who present the most innovative and promising posters. We are particularly happy that several funding institutions will present international awards in Linz this year to stimulate the reduction of animal experiments.

Located in the centre of Europe, Linz is a typically charming Austrian city situated on the river Danube. It has a baroque city centre and is famous for its modern museums and the *ARS Electronica* centre. The surrounding Austrian countryside offers unique historic sights as well as cultural and gastronomic highlights.

We will try our best to make your stay enjoyable and are looking forward to seeing you in Linz this autumn.

On behalf of the organisers

Horst Spielmann

President of EUSAAT
Representative of ESTIV

In alphabetical order of the first authors

Poster

The future of human cell culture media

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Human cell culture has become an essential tool for scientific research. The most widely used media for human cell cultures are supplemented with foetal bovine serum (FBS), which contains most of the factors required for *in vitro* cell proliferation and development. Few cell types grow in the absence of FBS and when that happens it is just for a short time. Even when growth occurs, its rate is always better when serum is added to the cell culture medium. This supplement is obtained from blood extracted from foetuses removed, under aseptic conditions, from cows found to be pregnant at slaughter. FBS quality varies between batches and contains many undefined compounds, which carries the risk of undesired protein or pathogens being introduced into the cell culture.

Because of these risks, there is a worldwide drive to develop alternatives using autochthonous proteins or chemically defined compounds. One of the advantages in using FBS is its almost universal growth supplement effect in most types of human and animal cells. Therefore, the use of FBS reduces time and effort in developing a specific media formulation for each cell type. Some of the disadvantages are: high cost; variable composition; presence of harmful toxins; contamination of human cell cultures by bovine contaminants; immune reaction to foreign proteins; difficulty of the downstream purification of proteins products; ethical issue about animal suffering during blood extraction from the heart of the live foetus without anaesthesia.

Although there is no breakthrough discovery of a new alternative cell culture medium, all the new developments and the range of serum free products already on the market suggest that it will only be matter of time before serum is eliminated from use in cell culture.

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Keywords: cell culture, human cell, in vitro

Poster

Transepithelial electrical resistance for *in vitro* toxicology

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Current biochemical or bacterial methods for testing water pollution are expensive and do not test for a wide variety of toxicants (Dayeh et al., 2002). Traditionally, toxicity tests have been done *in vivo* on fish and on other animals as defined in a number of OECD standards (http://www.oecd.org/dataoecd/17/20/1948241.pdf). Recently there has been a huge drive to refine and to develop new *in vitro* methods that could be used to reduce animal testing (Glawdel et al., 2009).

This study aims to develop a cell-based autonomous biosensing microsystem for water quality. Transepithelial electrical resistance measurements (TEER) will be used to monitor the integrity of a monolayer of epithelial cells in an automated fluidics system. The system will be installed at a river or stream and will sample the water. A drop in TEER will be taken as indicative of the presence of toxicants in the water sample and will be automatically relayed to an operator notifying him of a problem.

A simple fluidics system for epithelial cell culture has been developed. It includes a double flow PDMS chamber, with upper and lower compartments separated by a microporous membrane for cell growth. Integrated electrodes allow regular measurements of the TEER of the epithelial cell layer. Comparisons between resistance measurements using standard STX electrodes and transwell polyester inserts and those carried out in the new fluidics system indicate a significant improvement in reproducibility.

Experiments to select the most suitable cell model – preferentially a fish cell model – and optimise cell growth in the fluidics system are now underway. It is preferable to usea fish cell model to utilise characteristics such as low culture temarature and resistance to varying osmoticpressre (Lee et al., 2009). Future work will compare TEER measurements with classical toxicity endpoints.

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Keywords: transepithelial electrical restistance, fish cells, in vitro toxicity, animal testing, biosensing microsystem

Poster

Dissemination of industrial research, screening and testing approaches through validation processes support tools and methodologies contributing to the 3Rs

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In line with the 7th Amendment to the EU Cosmetics Directive, the dissemination of advances on alternative methods represents a step to promote alternatives to animal testing. Over the past decade there have been great efforts in industry and academic laboratories to develop alternative methods, either *in silico* or *in vitro*, that would comply with regulatory constraints. The implementation is still limited, given that it is a great challenge to replace historical approaches by *in vitro* tests with a high predictivity.

L'Oréal has, based on these principles, developed unique test methods to screen and test potential effects of chemicals. We initially focused on approaches for skin irritation, since this is an important endpoint for chemicals used in cosmetics. A peer review on various aspects of alternative techniques was performed at all stages of the research, and development had the focus that *in vitro* methods improve chemical selection (screening) as well as quality of testing. Following the development of the screening test, we implemented some protocols (i.e. EpiSkin and SkinEthic RHE) for pre-validation and validation processes according to ECVAM recommendations. To ensure quality and objectivity, experts from an international committee oversee the content. Details of the approach will be presented. The scope of the present poster will also cover other examples in the areas of eye irritation (SkinEthic HCE) and skin sensitization (MUSST assay).

By participating actively in relevant forums, L'Oréal and other industries will maintain and promote the development of new tools and methodologies as well as improve acceptance of *in vitro* alternatives by authorities.

Keywords: validation

Poster

A new web-portal to support non-animal research: InVitroJobs.com

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The development of non-animal methods in research has advanced rapidly in recent years. The website InVitroJobs.com was created to support this important branch of science. Many researchers have a strong interest in animal-free research, but find information on research groups who use non-animal methods hard to come by. One aim of our portal is to enable scientists and young scholars to access this branch of research more easily.

Alongside the job search portal, we also maintain an up-to-date list of research groups which are active in the development of non-animal research methods or mainly apply these methods. Here, students as well as established scientists will find an overview of the current research landscape. Contact information is provided in order to facilitate the search for a thesis assignment or internship or to establish contact with other research groups. This will help to expand networks and generate new ideas. We regularly update and enlarge our list and groups interested in being listed are welcome to provide their information.

Our website provides the opportunity to post vacancies in the categories: a) Jobs b) Internships c) Thesis assignments. Situations wanted advertisements can also be posted. So you can promote your own competence and you can be sure that the relevant group of people take notice of your proposal. All services provided by InVitroJobs are free of charge.

Moreover the website offers a multiplicity of information: The "News" category supplies comprehensive information on current developments in research and politics. A newsletter provides regular updates on current developments in non-animal research. A literature survey shows publications on specific topics. We also maintain a link list to further information and we give a survey of foundations and organisations that support the development of non-animal methods by grants and funding.

We seek for exchange with competent dialogue partners and we will be glad to inform about current methods in non-animal research. This platform will support an ethically defensible, modern and scientifically reasonable research.

InVitroJobs is a project of People for Animal Rights Germany (Menschen für Tierrechte – Bundesverband der Tierversuchsgegner e.V.). Contact: +49 (0)731 17 67 285 info@invitrojobs.com

Keywords: information sources, research groups, non-animal research methods, jobs, internships, thesis assignments

Poster

In vitro toxicity, mutagenicity and antimutagenicity of South African traditional medicinal plants and their preparations

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There is a continuous search for new medications, e.g. against cancer. The reason is, amongst others, that existing remedies have unwanted side effects and that they may loose their efficacy in the long run. Traditional medicinal plants are seen as an important source of possible new therapeutic drugs. They are still in use all over the world, for example in South Africa, where more than 80% of all medication comes from plants that are provided by traditional healers. Many of these plants or preparations thereof have shown their efficacy over the years. Yet, they are not screened for potential adverse health effects as are our modern medicines.

We have investigated the *in vitro* toxicity, mutagenicity and antimutagenicity of a number of widely used South African traditional medicinal plants or preparations in order to (a) assess their potential adverse health effects (toxicity, mutagenicity) and (b) investigate their possible beneficial (antimutagenic) effects, which would open interesting applications as food additives or in medicines.

We present data showing that some widely used preparations indeed have toxic and mutagenic properties whereas others have pronounced antimutagenic effects against well-known chemical mutagens, indicating that further research is needed to ascertain their safe use or further explore their potential beneficial properties

Published in

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Keywords: toxicity, mutagencity antimutagenic effects, herbal plants0

Poster

In vitro perfusion system for semi-automation of long-term repeat dose toxicity testing

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Repeat dose long-term *in vitro* studies are of particular importance for the hazard and risk assessment of chemicals and pharmaceuticals. However, due to their nature, such studies are time intensive, laborious, tedious and costly. We have adapted a perfusion system (EpiFlow) for semi-automation of repeat dose applications. In this study, we describe the system, the effects of hypoxia and experiments conducted with repeated Cyclosporine A (CsA) dosing of renal proximal tubular cells for 14 days.

The adapted perfusion system holds six 2.5 cm cell carriers in a cell culture chamber. The system is gas equilibrated via a silicone membrane separating the cell culture chamber from the gas system. Medium is delivered to the cell culture chamber using a peristaltic pump. We cultured the human renal proximal tubular cell line RPTEC/TERT1 on aluminium oxide filter inserts and placed them in the perfusion apparatus. CsA was administered to the cells either in a 24 h bolus exchange (18 ml) or in a continuous slow medium perfusion (0.75 ml/h) for 14 days. Additionally, the effect of a 1% oxygen environment was compared to a 21% oxygen environment in the absence of CsA. Medium was collected daily and lactate concentration was quantified. At the end of the 14 days, cells were harvested for transcriptomic and metabolomic analysis.

Under certain types of cellular stress, differentiated RPTEC/TERT1 cells switch from a minimal to an extensive glycolytic metabolism rate that can be monitored by measuring supernatant lactate levels. Increased lactate production was induced by hypoxia in both feeding conditions, whereas only high concentrations of CsA (15 μM) given in 24 h feeding bolus had this effect. The latter observation matches our findings in static cell culture experiments. However, CsA applied in continuous feeding had no effect on lactate production, possibly as a result of the compound binding to the perfusion tubes due to prolonged contact time.

Thus, using CsA as a model nephrotoxin, the perfusion system could reproduce the properties of static 24 h feeding cycles. A deeper analysis of the "omic" profiles will be required for a more complete comparison to the manual static system.

Keywords: semi-automated cell culture system, chronic toxicity, Cyclosporine A, renal proximal tubular cells

Lecture in Session VI: Acute and long term toxicity B

EpiVaginal tissue model for preclinical toxicity screening of chemicals/formulations following single or repeat applications

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A predictive test system for assessing the vaginal irritation potential of chemicals and formulations will have far reaching application in industries involved in women's care products. The vaginal mucosa is commonly exposed to chemicals and therapeutic agents that may result in irritation/inflammation, which can make women susceptible to infections such as HIV-1 and HSV-2. Hence, chemical or therapeutic agent induced vaginal irritation is a public health concern. Testing of such materials has been performed using the rabbit vaginal irritation (RVI) assay. In the current study, we investigated whether the highly differentiated EpiVaginal tissue could be used as a non-animal alternative.

The EpiVaginal tissue was first exposed to single (N=6 chemicals at three concentrations) application of test agents for 24 h. Tissue viability (MTT assay), barrier disruption (measured by trans-epithelial electrical resistance, TEER and sodium fluorescein (FL) leakage) and inflammatory cytokine release (IL-1α, IL-1β, IL-6, and IL-8) were examined. Furthermore, the utility of the tissue model for repeat application of chemicals was tested by exposing tissues with three repeat applications (1 h each time at times 0 h, 24, h, and 48 h) followed by a 24 h recovery time for each exposure. A total of 12 test articles at a single dose (2% of each test article) were used for the repeat application.

When compared to untreated controls, two irritating test articles, benzalkonium chloride and nonoxynol 9, reduced tissue viability to <40% and TEER to <60% and increased FL leakage by 11-24% and IL-1α and IL-1β release by >100% in the single application assay. Four other non-irritating materials had minimal effects on these parameters. Assay reproducibility was confirmed for single applications by testing the chemicals using three different production lots (<10% CV) and by using tissues derived from cells of three different donors (<12% CV). Treatment of the tissue with vaginal fluid stimulant (pH=4.2) did not result in a decrease in tissue viability or an increase in inflammatory cytokines. Release of IL-1α and IL-1β was found to be a strong indicator of toxicity of chemicals. In the repeat (3X) application assay, benzalkonium chloride, nonoxynol 9 and SDS reduced tissue viability to <10% and TEER <20%. For repeat applications either IL-1α or β were found to be indicators of chemical toxicity and the cytokine response was noted in the first 24 h after the initial chemical exposure.

Decreases in MTT and TEER and increases in FL, IL-1α and IL-1β release appear to be useful endpoints for preclinical toxicity screening of chemicals/formulations. The assay method will be cost effective and reduce the use of laboratory animals for experimentation. In the future, the *in vitro* test method could also be a useful tool to assess toxicity of medical devices and drug permeation studies and should be considered as an *in vitro* alternative test for the RVI assay.

Keywords: EpiVaginal, toxicity, IL-1α, IL-1β

Poster

Plasmacytoid dendritic cell-based assay as an *in vitro* alternative assay for chemical allergenicity screening

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Human dendritic cells have been used to evaluate the allergenicity potential of chemicals to serve as an alternative to existing animal models utilised throughout industry to monitor products for contact sensitization. Development of such a non-animal alternative assay system for hazard assessment directly addresses REACH (Registration, Evaluation, and Authorization of Chemicals). In this study, we investigated whether CD86 expression in plasmacytoid dendritic cells (pDC) can be used to identify contact allergens.

Human DC were generated from $CD34^+$ progenitor cells and the pDC fraction ($CD123^+/CD11c^-$) was harvested using FACS sorting. The pDC were exposed to an expanded list of chemical allergens (n=49) or irritants (n=42). Concentrations of each chemical that resulted in >50% viability as determined by FACS analysis of propidium iodide stained cells were used for analysis. Allergens were identified based on stimulation index (SI) calculated by the fold increase in CD86 expression levels. A material that had an SI ≥1.5 in at least 50% of the pDC donors (n=2-5 donors) was considered an allergen.

For 71 of the 91 materials tested, historical mouse local lymph node assay (LLNA) and human clinical data were available. Using the *in vitro* pDC assay, CD86 expression increased ≥1.5 fold for 37 of 39 allergens but not for 26 of 32 non-allergens. Based on these results, a prediction model was developed to classify chemicals as allergens or non-allergens. The *in vitro* assay performance had sensitivity = 95%, specificity = 81%, and accuracy = 89%; these results were slightly better than those obtained using the LLNA assay which showed sensitivity = 85%, specificity = 84%, and accuracy = 85%.

CD86 expression in pDC appears to be a sensitive and specific predictor of allergenicity. The assay is advantageous because high throughput screening of chemicals is possible, donor-to-donor variation can be monitored, the cells are of human origin and the assay is cost effective.

Keywords: dendritic cells, allergenicity, CD86

Poster

Effects of pharmaceuticals and personal care products (PPCPs) on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2)

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Pharmaceuticals and personal care products (PPCPs) are an emerging concern in the aquatic environment. Aquatic organisms exposed to these compounds can be adversely affected, as they are possibly not as efficient as mammals in eliminating lipophilic drugs and oxygen radicals. Risk management necessitates the knowledge of the incurred hazards as well as the quantification of the exposure the organisms, subjected to these hazards, would likely be incurring. At present the toxicological implications of the presence of pharmaceuticals and PPCPs, and the exposure to their mixtures in aquatic organisms remain largely unknown. The most significant entry route for these substances or their metabolites into the aquatic environment is the release from wastewater treatment plants (WWTPs). The purpose of this study was to analyse sub-lethal and cytotoxic effects of pharmaceuticals and PPCPs presents in WWTPs and its mixtures, using the RTG-2 rainbow trout cell line.

Samples were collected from three WWTPs, and the substances detected at the highest concentrations were selected,

including the pharmaceuticals atenolol (β-blocker), bisphenol A (endocrine disruptor), caffeine (CNS stimulant), diclofenac (analgesic), diphenylhydantoin (anti-convulsant), ethinylestradiol (endocrine disruptor), fluoxetine (antideprerssant), hydrochlorothiazide (diuretic), ketoprofen (analgesic), metoprolol (β-blocker), naproxen (analgesic) and propanolol (β-blocker), and the PPCPs musks galaxolide and musk xylene.

RTG-2 cells were plated at a density of 2.5 x 10^4 cells/well in 96-well microtiter plates. For single substances (stock DMSO solutions of 500 mM) different concentrations were prepared by serial EMEM dilutions at a ratio of 1:1 to obtain dose-response curves. Mixtures of pharmaceuticals and PPCPs were prepared according to the concentration in the WWTPs effluents. The induction of cytochrome P450 CYP1A monooxygenase was quantified by their associated ethoxyresorufin-O-deethylase (EROD) activity, using 7-ethoxyresorufin as substrate. The generation of ROS was monitored by employing 2',7'-dichlorodihydrofluorescein diacetate staining. Cellular defence was predicted by a β-galactosidase assay, using MFU-galactoside as substrate. Cell viability was quantified using the NR assay.

Except for atenolol and naproxen, all individually tested compounds showed significant cytotoxic effects in most of the assays, with lowest-observed-effect concentrations and concentrations causing 50% effects (EC_{50}) values within the μg/l range. Clear dose-response curves were found for several of the sub-lethal and cytotoxic endpoints assessed for RTG-2 cells exposed to different mixtures of pharmaceuticals and PPCPs, with lowest-observed-effect concentrations and concentrations causing 50% effect (EC_{50}) values within the ng/l range.

More than 50% of substances tested interacted with CYP1A metabolism pathways in RTG-2 cells through an induction or an inhibition of EROD activity. Such effects might be of environmental concern since alteration of CYP1A activity is known to affect xenobiotic metabolism and toxicity in rainbow trout.

The results indicate that for some compounds, the margin of safety is narrow and that acute and chronic effects, particularly when combined effects of mixtures are taken into account, cannot be ruled out.

This study was funded by the Spanish project CONSOLIDER-INGENIO (TRAGUA) CSD2006-00044 and RTA 2007-00002-00-00.

Keywords: pharmaceuticals, PPCPs, RTG-2

Poster

Criticism of the Limulus Amoebocyte Lysate (LAL) test as a replacement method of the rabbit pyrogen test (RPT) and environmental health implications

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This work intends to debate the acceptance of the Limulus Amebocyte Lysate (LAL) test as a complete alternative method to the rabbit pyrogen test (RPT) in detecting endotoxins. The work is based on literature review and analysis of ethical, methodological and environmental questions.

There is evidence that the extraction of haemolymph from *Limulus polyphemus* (horseshoe crab) causes the death of about 30,000 crabs per year in the United States. That places the biomedical industry as one of the main causes of death of that arthropod. Added to that is our ignorance of the degree to which its phylogenetic scale of consciousness goes. We do not have sufficient knowledge of the sentience of arthropoda and other invertebrates. There is some literature evidence that horse shoe crab eggs serve as intertidal migratory bird food and an increase in *Limulus* death owing to LAL kit production may be a factor of disturbance of theses birds' behaviour. Five tests that are based on the measurement of proinflammatory cytokines (interleukin IL-1β, IL-6) are already validated for the detection of pyrogens, possibly as a replacement of the RPT. Four of these five tests use human blood and the other test uses a human cell line (Mono Mac 6).

In conclusion, there are several ethical advantages in using human blood, since human donors are able to understand the research and give their consent to harmless donation. Since *in vitro* tests with human blood are potential complete replacement methods with no ethical consequences and without environmental disturbances, more studies for catch-up validation of those tests in products different from those used in the validation study should be conducted and encouraged to be later incorporated to pharmacopoeias.

Keywords: Limulus Amebocyte Lysate test (LAL), pyrogen test, sentience of invertebrates

Poster

What do we know about alternative methods to pesticide toxicology? A systematic review

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The use of pesticides to control live organisms that threaten human food supply and health is continuously increasing. Conflicting with that, there is evidence that such use may be detrimental to environmental and human health. In order to achieve proper regulation of pesticide use and exposure, toxicological tests are performed on several animal species, such as hamsters, rabbits, rats, mice, fish and birds. However, the extrapolation of the results obtained from such species to humans and other non-target species is still a matter of intense debate. In the meantime, a large number of animals is used in toxicological tests worldwide. It is therefore an ethical imperative to develop final alternative methods to these tests.

The aim of this study was to systematically review the scientific literature about alternative methods to pesticide assays. It was also investigated which fields of toxicology have been more involved in these efforts to develop alternative toxicological tests to animal use.

Embase, LILACS, IBECS, MedCarib, Wholis, Web of Science and Scielo were searched until April 24, 2010 by means of several databases. The search on MedLine by PubMed used the following Medical Subject Headings (MeSH): "Animal Use Alternatives" with word "pesticid*". Two researchers read all abstracts returned by this search strategy. After abstracts had been analysed, they were categorised into "off-topic", "review", "regulations", "reduction and refinement" and "replacement". Articles dealing with alternative methods to pest control or those aiming to improve animal tests themselves were considered "off-topic". Discordant classifications were analysed by a third researcher.

Search of scientific literature returned 287 abstracts from which 37 repeated articles were excluded. A further 182 abstracts were also excluded as they were considered "off-topic". The reason for this large number of off-topic abstracts was probably the search strategy, which was intentionally wide-ranging in order to include as many relevant articles as possible. In addition, among the 68 remaining abstracts, 20 were reviews and 11 dealt with regulatory issues. Accordingly, after all exclusions, 37 articles were selected for review. Among them, 21 aimed at reducing or refining animal toxicological tests, 15 aimed to replace existing methods to animal tests in pesticide toxicology, and one text was found in the website Nature News. Among all articles classified as replacement methods, six aimed at replacing the acute test (lethal dose), one described methodological validation for reconstructed human epidermis for skin absorption testing, two were about replacement tests for hepatotoxicity, and another two about replacement for ecotoxicity tests.

This systematic review showed that there are only few studies on alternative methods to the use of animals in pesticide toxicological tests. Research on replacement methods needs to be encouraged, as it could lead to a significant reduction of the current massive use of animals in experimental science.

Keywords: systematic review, animal use alternatives, pesticides

Poster

Replacement strategies for animal use in teaching a biotechnology technical course in southern Brazil

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The progress of ethical arguments beyond Cartesian thought – wherewith only humans could have rights and sensation – generates a necessity of rethinking sentient animal use in the life sciences and brings this discussion to science education. Aiming to develop the interest of scientists to replace animal tests, we have worked to demonstrate that the teaching/learning process is possible by using replacement strategies, in vocational education such as in higher education.

Firstly we contacted in the vocation course of biotechnology a teacher of histology technique who did not feel comfortable using *Rattus norvegicus* in her lessons. We suggested, for the practical activities, a replacement using necropsied formaldehyde-fixed dog tissues. We observed those lessons. In the same course, we met a biochemistry teacher who replaced his 16 animal lessons about biochemistry processes with non-animal organisms, like yeasts. He changed his traditional protocols and created alternative protocols, because he did not agree with animal use in education. Later we interviewed the teachers to learn about their ideas for replacement methods for education and to hear whether they felt their lessons successfully taught their topics. We used a focal group method with five students as the class group. We adopted the focal group method because we wanted to study a specific focus and had a defined group with different visions of the subject.

The histology technique teacher mentioned that she felt uncomfortable using animals and considered that the new method "solved the problem" (sic.). She said she would no longer use animals in her classes to avoid creating negative feelings concerning their deaths. She felt that the students obtained comparable knowledge in the alternative lessons. Interviewed students found that the replacement was effective although they had no former knowledge of replacement strategies (or alternative methods). That is possibly because teaching of alternatives to animal use is only starting in Brazil. We discussed other important animal replacement methods for biochemistry lessons. We demonstrated that alternatives used to teach biochemical processes reach the class objectives of understanding metabolic routes and laboratory techniques. The research presented different perceptions from students about animal use in the life sciences, however all students felt that animal use should be replaced where possible. They also felt that new alternative methods should be developed.

Although in Brazil, research on alternative methods is just beginning and teachers often have no prior knowledge of the developments, teachers are able to create alternatives for their lessons out of self motivation. Therefore, we must help and motivate teachers to develop methods to replace animal use in education. We need to disseminate and make available established methods to other teachers and students. In this work, we focused on the principle of replacement, according to the 3Rs "reduce, refine, and replacement" the only one that allows the abolition of the use of animals in scientific practices.

Keywords: alternative methods to animal use, teaching strategy, histological and biochemical techniques, animal ethics

Poster

Use of protein and immune profiles of bacterial veterinary vaccines for batch potency testing as alternative to animal testing

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Veterinary vaccines are based on biological organisms. Inherent molecular complexity and variability necessitate continuous control tests to ensure quality, potency and safety of individual production batches. Currently, product testing often relies on animal experiments including lethal challenge tests. Whereas *in vivo* tests are necessary tools to develop safe and efficacious vaccines, the need for *in vivo* lot release testing is increasingly questioned. Improved quality control systems, new analytical methods and comprehensive post marketing surveillance information justify non-animal approaches in lot release testing of well established products. Molecular analytical methods have been shown to be suitable for thorough qualitative and quantitative characterisation of complex biological products. Electrophoretic protein profiles offer a potential tool to test for batch to batch consistency and equivalence with clinical lots of proven safety and efficacy. Detection of immunogenic fractions of such profiles verifies the immunological functionality of product batches.

Here, we have used SDS-PAGE in conjunction with immunoblotting to evaluate the potential of this scheme for consistency testing of batches of various groups of veterinary vaccines which currently require compromising or lethal in vivo tests in laboratory animals.

Keywords: 3R, consistency approach, veterinary vaccines, protein profile, immuno profile

Lecture in Special Session on antibody production

Exploitation of potential aptamer technology to replace animal antibody against human tumour biomarkers

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Biomarkers are key elements for clinical diagnosis and prognosis of disease, and may also be therapeutic targets. Tumor biomarkers strongly associated with the progression of cancer from benign to malignant disease have already been identified successfully (Barraclough et al., 2010; Liu et al., 2005, 2002, 2000). One promising biomarker is the secreted, steroid hormone-regulated anterior gradient 2 protein, AGR2 (Liu et al., 2005). Although involved in amphibian development and limb regeneration (Kumar et al., 2007), AGR2 is a widely overexpressed protein in human carcinomas (Smirnov et al., 2005; Zhang et al., 2005). Importantly, human AGR2 causes a metastatic phenotype in an *in vivo* model system (Liu et al., 2005) and the presence of AGR2 protein in primary breast tumors is strongly associated with reduced patient survival (Barraclough et al., 2009). Our newly developed AGR2 sandwich ELISA immunoassay has shown for the first time that serum AGR2 protein is elevated in the plasma from patients with early cancer, in this case ovarian cancer (Edgell et al., 2010), and opens up the possibility of a prognostic AGR2 blood test. However, the AGR2 ELISA assay, in common with widely-used ELISA immunoassays generally, has an inherent limit of detection and also uses large amounts of animal-sourced antibodies.

For decades, antibodies, which can only be produced from animals, have played an important role in routine clinical diagnosis and more recently in therapy. However, millions of animals are sacrificed to supply antibodies each year around the world. Thus, it is necessary to find an alternative way to replace animal antibodies.

Antibodies have been used because they can recognise target antigens with high affinity and specificity. Aptamers are small synthetic chemicals that can bind to a target molecule with high affinity and specificity through their highly organised three-dimensional structures(Ellington and Szostak, 1990; Tuerk and Gold, 1990). Most importantly, unlike the production of antibodies, the whole procedure including isolation and production of aptamers does not use animals at all. Therefore, the emerging aptamer technology has the potential to provide an exciting alternative approach to replacing animal antibodies that could save millions of animals being killed each year worldwide in the future.

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Keywords: aptamer technology, alternative way to replace animal antibodies

Lecture in Session III: Skin sensitisation and eye irritation

ECVAM prevalidation study on skin sensitisation alternatives: update on progress

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In the field of *in vitro* alternatives in toxicology, several alternative methods for acute local health effects have already been validated. In contrast, sensitization and other repeated dose endpoints have remained a significant challenge. However, recent progress with *in vitro* assays in skin sensitisation toxicology has resulted in the development of test methods which could make a valuable contribution to the replacement of the existing animal tests. These approaches comprise the Direct Peptide Reactivity Assay (DPRA), the Myeloid U937 Skin Sensitization Test (MUSST) and the human Cell Line Activation Test (h-CLAT). Each of these test methods has been the subject of substantial evaluation, including inter-laboratory assessments, and their status of development has led to their acceptance by ECVAM for inclusion in a Prevalidation Study in which the three test methods will be challenged with a set of coded chemicals in three laboratories each: the lead laboratory which submitted the test to ECVAM and two naïve laboratories. 24 chemicals will be tested once in each laboratory for the assessment of the between-laboratory reproducibility. A subset of 15 chemicals will be assessed a further two times, for evaluation of the within-laboratory reproducibility. It is anticipated that results from the DPRA will be available late in 2010, whereas results from the cell based assays will be delivered for independent review during 2011. Assuming a successful outcome, future activity will require consideration of how to deploy these assays in a structured assessment of skin sensitization potential. An overview of the study organisation and progress will be provided.

Keywords: skin sensitisation, validation

Poster

Quantification of esterase activity in human keratinocytes and fibroblasts, *ex vivo* skin and reconstructed human skin models

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Cutaneous esterases play an important role in the activation of prodrugs and in the biotransformation of xenobiotics. For example, they allow prodrugs to penetrate the skin barrier as lipophilic compounds before being hydrolysed to the active compound to produce the therapeutic effect.

The aim of the study was to develop a quantitative assay measuring esterase activity in human keratinocytes and fibroblasts, *ex vivo* skin and reconstructed human skin models. Fluorescein diacetate (FDA), a non-fluorescent substrate, was chosen as a suitable metabolic probe, which is rapidly hydrolysed by esterases to the fluorescent product fluorescein. Primary cells were obtained from juvenile foreskin and grown on 96-well plates until they reached confluence. Following addition of increasing concentrations of FDA, fluorescein emitted fluorescence was monitored in a microplate reader over time. The specification of reaction time was of crucial importance for the determina-

tion of the enzyme kinetics. Initial reaction rates were used for the calculation of the maximum transformation velocity (V_{max}) and Michaelis Menten constant (K_m). Data were normalised for total protein content. As expected, a higher esterase activity was observed in keratinocytes, which are superior in the cleavage of e.g. glucocorticoid esters.

Latest investigations addressed the esterase activity in human skin *ex vivo* and commercially available 3D skin models. In conclusion, the assay for FDA hydrolysis provides an accurate, reliable, and reproducible *in vitro* test to determine esterase activity in human monolayer cell cultures and reconstructed human skin models. Homogenised skin tissues show an increased esterase activity compared to monolayer cell cultures.

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Keywords: esterase, fluorescein diacetate, human skin, skin models, enzyme activity

Poster

Establishment of an *in vitro* test battery for the prediction of skin sensitizing potentials of chemicals

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Allergic contact dermatitis is induced by repeated skin contact with an allergen (hapten, pro-hapten or complete allergen). Assessment of the skin sensitizing potential of chemicals, agrochemicals and cosmetic ingredients is crucial to define their safe handling and use. Up to now, guinea pigs have been used to identify a skin sensitizing potential. And, more recently, the LLNA was established as an alternative method in mice. Animal welfare (which is also reflected in current legislation, such as the 7th Amendment to the Cosmetics Directive as well as REACH) demands animal free alternatives. The mechanisms of induction and elicitation of skin sensitization are complex. Briefly, the contact allergen needs to penetrate the skin and to be either reactive towards endogenous proteins itself (hapten) or must be metabolically activated in the skin (pro-hapten). The following events involve the activation of dendritic cells (DC), the upregulation of co-stimulatory cell surface markers (e.g. CD86), the secretion of cytokines and migration of DC into lymph nodes. To account for the multitude of events in the induction of skin sensitization, an *in vitro* test system will consist of a battery of different tests.

Within our laboratories we have established methods to assess dermal penetration *in vitro* (OECD test guideline 428) and metabolic capacities of rat, human and porcine skin (reviewed by Oesch et al. 2007). Currently, we are performing in house validations of four *in vitro* assays addressing three different events during induction of skin sensitization. 1) The peptide reactivity assay according to Gerberick et al. (2004) using synthetic peptides and HPLC analysis. 2) Two dendritic cell activation assays (DCAA) based on the dendritic cell like cell lines U-937 and THP-1 and flow cytometric detection of the maturation markers CD54 and/or CD86 (Ashikaga et al. 2006; Python et al. 2007; Sakaguchi et al. 2006). 3) ARE-dependent gene activity in a HaCaT reporter gene cell line. We present the results of our validation of these assays with more than 40 substances of known sensitizing potential (including the performance standards defined for the LLNA). The sensitivity, specificity and accuracy of the individual tests were obtained by comparison to human epidemiological data as well as to data from animal tests, such as the local lymph node assay.

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Keywords: sensitization, in vitro, in house validation, dendritic cell activation, peptide reactivity, ARE reporter cell line

Poster

Introduction of a new reconstructed human epidermis with integrated functional primary melanocytes

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The development of novel cosmetics for skin tanning or bleaching is an important issue for cosmetic and chemical industry. The active ingredients used in these products have to be tested for efficacy and product safety.

Here we present a new reconstructed epidermis with integrated melanocytes, which is based on the already established technology of Epidermal Skin Test 1000 (EST1000, CellSystems, Germany). We tested different ratios of melanocytes and keratinocytes for the production of skin models and used melanocytes from different ethnic groups. To demonstrate the physiological behaviour of the newly developed epidermal model we successfully stimulated the melanin synthesis with chemical inducers or UV-radiation. Blocking the melanin synthesis by chemical inhibition of the tyrosinase activity demonstrated that tanning of the models depends on the specific activity of melanocytes. Finally, the melanin content could be correlated with different tanning intensities.

This epidermis model can be cultivated for at least weeks and allows short term as well as long term studies. We conclude from these results that CellSystems' newly reconstructed epidermis with integrated melanocytes is a good tool to study tanning and bleaching of the skin.

Keywords: EST1000, human reconstituted epidermis, melanocyte, tanning

Poster

The potential of the oocyte *in vitro* maturation system (IVM) for reproductive toxicology

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There is rising concern about the effects of environmental contaminants (EC), many of them oestrogen-like substances, on the reproductive function of animals and humans. Although standard screening assays for EC are available, they might not reveal the full physiological relevance of the potential harm of those compounds with respect to oocyte maturation and fertilisation. Moreover, to screen for reproductive and developmental toxicity, a substantial amount of experimental animals is being used. The immature oocyte (in germinal vesicle stage) is a very susceptible cell considering that it still needs to complete the final stages of nuclear and cytoplasmic maturation before it can properly support fertilisation and the early stages of embryo development. Previously, we demonstrated by using the *in vitro* oocyte maturation system (IVM) that even physiological concentrations of 17β-estradiol can be deleterious for the final maturation of oocytes (malformation of the meiotic spindle) if those oocytes are exposed in an abnormal window of exposure. Therefore, it can be expected that a chronic exposure of woman to environmental oestrogens may affect spindle formation and chromosome segregation *in vivo* as well.

Our hypothesis is that the IVM could contribute to an integrated testing strategy (ITS) for screening chemicals that affect female fertility. Furthermore, by using ovaries from slaughterhouse waste, it

could also contribute to reducing the number of experimental animals being used for such studies.

We have selected compounds with a known mechanism of action (i.e. cycloheximide, estradiol, DES, cadmium chloride) to be tested in this system. As the first step to investigate this proof of principle, we cultured bovine oocytes, harvested from slaughterhouse ovaries, in M199 medium at 39°C for 22 h under 5% CO and in the presence of the following concentrations of cycloheximide (CHX), a known protein synthesis inhibitor: 0.75; 0.60; 0.45; 0.30; 0.15 and 0 μM. The toxicological endpoint was the ability of the oocyte to reach the metaphase II stage (i.e. the final stage of maturation). In addition, we investigated the general cytotoxicity of CHX using the MTT test.

Our results show no general cytotoxicity of CHX at any of the concentrations used. Meiosis was inhibited in a dose-dependent manner and the EC_{50} for CHX was 0.55 μM, much lower than the concentration used to block protein synthesis in somatic cells, indicating the high sensitivity of the oocyte to express toxicity.

The next step is to extend this investigation to other selected compounds and to combine functional parameters with the use of proteomics and transcriptomics to obtain mechanistic insights into oocyte maturation and the effect of chemicals hereon. It is expected that markers derived, along with functional parameters, can form a powerful assay for the *in vitro* prediction of reproductive toxicity. In conclusion, we believe that the use of the IVM assay can grant a powerful and physiological model in an ITS of effects of chemicals on female fertility.

Research supported by TNO Research Program on Hazardous Substances and Occupational Safety; and The Netherlands Toxicogenomics Center.

Keywords: reproductive toxicity, oocyte, in vitro maturation, environmental contaminants

Poster

Plasma Medicine – a new field of research on the biomedical applications of plasma technologies requires screening methods: the modified CAM assay and the Reconstructed Epidermis as useful models to assess the effects on angiogenesis, inflammation and cytotoxicity caused by nonthermal atmospheric pressure plasma

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Plasma Medicine is a fairly new, highly interdisciplinary field of research on the numerous biomedical applications of plasma technologies. Reports from many countries prove that low temperature plasma can assume a highly beneficial position in different aspects of medicine, e.g. sterilisation/decontamination, wound healing (Kramer et al., 2009; Matthes et al., 2009), oncology (induction of apoptosis), dermatology (Lademann et al., 2009), dental applications and some veterinary fields. Even though knowledge on plasma-based biomedical mechanisms, the right dosages and treatment times is sparse, animal experiments have been carried out and many more have been applied for. To follow the 3Rs, it is unconditionally necessary to define methods for the screening of plasma parameters.

To determine a reliable test and validate the use of nonthermal atmospheric pressure plasma on chronic wounds, a new approach for testing plasma interactions was chosen. We tested *in vivo* effects using the CAM (chorioallantoic membrane) of the developing chicken embryo as an alternative to traditional mammalian models and we have adopted the Reconstructed human Epidermis (RhE) to assess cytotoxicity *in vitro*, so that the different qualities of the conclusions complement one another. The modified CAM-assay served as a model for the plasma's effects on angiogenesis and as an alternative inflammation model.

Effects of varying intensities, provoked by using an HF Plasma Jet, corresponded to exposure times. In the CAM-assay, depending on the intensity of the induced inflammation, the effects were completely or partially reversible or led to scarring (Bender et al., 2010). The RhE was used to assess the time dependent cytotoxicity effects of plasma on NHEK cells (normal human epidermal keratinocytes), also regarding reversibility.

To conclude, the modified CAM-assay and the RhE are suitable for screening plasma sources and parameters for medical applications of plasma: the additional findings allow the determination of plasma parameters for application of plasma to chronic wounds.

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Keywords: Plasma Medicine, HET-CAM, CAM-assay, Reconstructed human Epidermis, inflammation, angiogenesis, cytotoxicity

Poster

Reporting animal research in journals: contribution to the 3Rs

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Some see the compulsory inclusion of an animal welfare section in papers describing animal experiments as another burden for scientists (Jimenez, 2007). Fortunately, others disagree with this. Not only would such a section allow scientists to detail their measures to minimise suffering and numbers of animals used (Würbel, 2007), since many factors influence the animal's biological functioning (Sherwin, 2007), but it would also improve the reproducibility of the experiments by others or even prevent the repetition of animal experiments. Furthermore, the detailed and proper description of any measure to minimise pain and distress would not only benefit the experimental animals, but also other researchers as an educational tool (Olssson et al., 2007).

Several studies reported on journal policies with regard to animal experiments and the welfare of the animals used. The current report follows up on three of these: Boisvert, 1977; Festing and Van Zutphen, 1999, and Van Boxel and Hendriksen, 2007.

This study involved 139 journals, covering the whole field of biomedical research. Journal instructions to authors were assessed regarding the presence of several criteria including: statement that a relevant animal ethics committee has approved the animal study, specific guidelines have been followed, description of anaesthesia and euthanasia and consideration of 3Rs alternatives. Depending on the quality and specificity of the journal's instructions, weighing factors were awarded for each criterion. The maximum value that could be obtained was 26.

None of the studied journals reached this value. To summarise, 28% of the studied journals have no specific requirements for describing animal experiments, 47% of the journals request brief information and 22% request comprehensive information on the animal studies performed. In comparison to the previous studies an increased number of journals required information: from 49% (Boisvert, 1997) and 62% (Van Boxel and Hendriksen, 2007) to 81% (current study). Also with regard to comprehensive information an improvement was observed: 16% (Festing and Van Zutphen, 1999), 13% (Van Boxel and Hendriksen) and 22% (current study). In addition, an improvement is seen with regard to brief requirements: 25% (Festing and Van Zutphen, 1999), 46% (Van Boxel and Hendriksen, 2007) and 47% (current study).

In conclusion, progress is seen with regard to requirements of journals to detail animal studies in published studies with experimental animals. Still, most journals lack sufficient criteria to establish whether all 3Rs have been considered and how these were applied.

The next step would be that both referees and editors seriously assess whether the requirements have been fulfilled. It has been shown that some journals with strict requirements do not seem to adhere to these (Olsson, Hansen and Sandoe, 2007).

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Keywords: 3Rs, animal experiments, methods, journals, animal welfare, reporting

Poster

Phototoxic effect of TPPS4 and MgTPP4 on DNA fragmentation of HeLa cells

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Photodynamic therapy presents a promising alternative method of cancer treatment. This therapy is based on selective uptake or retention of a sensitizer in the tumour tissue and cytotoxic effects of the sensitizer in combination with light. Porphyrins present a group of photodynamic agents which sensitize cells so that they are damaged when exposed to light. Light leads to a photochemical reaction which converts O_2 to cytotoxic $^1O_2$ and other forms of reactive oxygen species (ROS).

The comet assay (also called single-cell gel electrophoresis, SCGE) is a sensitive, simple and quantitative technique for detection of DNA damage. The comet assay can be used for detection of DNA damage caused by single and double strand breaks, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-drug cross-linking or DNA repair. The DNA damage is presented by the amount of unwound DNA fragments which resembles a "comet" with a distinct head and tail. The head consists of intact DNA, while the tail is created of broken fragments of DNA or relaxed chromatin. The amount of the DNA damage is directly proportional to the amount of DNA liberated from the head. The degree of DNA damage can be assessed manually or automatically using appropriate scoring software.

In our study we investigated the phototoxicity of the two porphyrin photosensitizers TPPS4 and MgTPPS4 on HeLa cells. Three different irradiation doses of 1, 15 and 30 Jcm^{-2} emitting from LEDs with an emission wavelength maximum of 414 nm were used. The concentrations of the photosensitizers were 0.1, 0.5, 1, 5, 10, 50 and 100 μmol. Our results show that the DNA of the cells treated with the TPPS4 and MgTPPS4 at concentrations higher than 5 μmol was highly fragmented, indicating a strong phototoxic effect resulting in cell apoptosis. On the basis of our results we can hypothesize that even the irradiation dose of 1 Jcm^{-2} is sufficient to induce DNA fragmentation.

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Keywords: PDT, ZnTPPS4, MGTPPS4, comet assay, HeLa cells

Poster

Persistent organic pollutants have CAG repeat and dose dependent effects on androgen receptor activity *in vitro*

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High exposure to persistent organic pollutants (POPs) have in subjects with a short androgen receptor (AR) CAG repeat (<20) been associated with low sperm counts (Giwercman et al., 2007). Since the CAG repeat regulates the AR's transcriptional activity (Nenonen et al., 2009), which these pollutants have the ability to inhibit (Kelce et al., 1995), we investigated their direct effects on the hormone induced AR activity and content.

The ability of ARs containing CAG repeats within the normal range (16, 22 and 28) to activate a reporter gene in the presence of 10 nM 5α-dihydrotestosterone and the POP markers 2,2´,4,4´,5,5´-hexachlorobiphenyl (CB-153) and 1,1-bis-(4-chlorophenyl)-2,2-dichloroethene (p,p'-DDE) separately or in combination was measured. The concentrations used had previously been measured in serum from the highly POP exposed Greenland Inuit population, but also 10 and 100 times higher concentrations were tested. AR protein content was analysed by enzyme-linked immunosorbent assay (ELISA) and total protein was analysed by Bradford protein assay. The experiments, which were carried out 6-8 times in duplicates, were adjusted for total protein amount. Friedman's and sign tests were used for statistical analyses.

A short CAG together with high p,p'-DDE exposure affected AR activity with 23% repression (p=0.02). No statistically significant changes in activity were observed after single exposure to CB-153. A combination of p,p'-DDE and CB-153 hampered the activity of the long CAG (-24%, p=0.02). After addition of 10x the concentration of the POP combination, a decrease in activity for all variants was observed, although not statistically significant. An even higher concentration of the POP combination (100x) resulted in markedly reduced activity for all CAG repeat lengths (16CAG,-76%; 22CAG,-74%; 28CAG,-62%; p=0.03 for all). AR protein was decreased at both 10x and 100x the POP concentration.

These results indicate that POPs, in concentrations humans are exposed to, can inhibit AR action depending on CAG repeat length, whereas higher doses inhibit both the AR function and restrain AR protein formation.

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Keywords: androgen receptor activity, CAG polymorphism, persistent organic pollutants, p,p'-DDE, CB-153

Lecture in Session II: Metabolism and Toxicokinetics

Effect of trichostatin A on miR-122 expression in primary hepatocyte cultures

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Primary hepatocytes in culture undergo a progressive deterioration of their specific phenotype, including xenobiotic biotransformation capacity, which largely restricts their application to short-term studies (Vanhaecke, 2005). Our group has shown that interfering with epigenetic determinants of hepatocellular gene expression, e.g. reversible histone acetylation and DNA methylation, is a promising strategy to maintain the *in vivo*-like differentiated phenotype of cultured hepatocytes over the long term (patent CT/EP2004/0012134). Since microRNA species have recently joined in as master regulators of the epigenetic machinery (Costa, 2008), it was thought conceivable that their modulation could also be a potential strategy to achieve this purpose.

In the present study, we therefore investigated the effect of the histone deacetylation inhibitor Trichostatin A (TSA) on the expression of this family of small non-coding RNAs, with special focus on miR-122, being the most important liver-specific microRNA (Chang et al., 2004).

As shown by microarray analysis and qRT-PCR, the expression of miR-122 decreases as a function of time in primary rat hepatocyte cultures. However, upon exposure to TSA, miR-122 expression was retained at a higher level compared to control conditions. This effect was accompanied by a better maintenance of the *in vivo*-like morphological features of hepatocytes, as well as their albumin secretion capacity.

In conclusion, these preliminary results indicate that miR-122 expression is affected by epigenetic regulators such as TSA, resulting in prolonged phenotypic stability of the hepatocytes in culture. A direct causal relationship between this differential expression of miR-122 and possible liver-specific

target genes and their biological functions remains to be established.

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Keywords: microRNA, miR-122, HDAC inhibitor, TSA, epigenetics

Lecture in Session VII: Nanotoxicology

Air-liquid exposure of gold nanoparticles to study effects, uptake and intracellular distribution in a human 3D epithelial airway model

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Nanoparticles (NPs < 100nm) show promising features for pharmaceutical applications. However, biomedical applications of NPs require a detailed understanding of potential interactions with biological systems. One important route of entry into the body is pulmonary inhalation, which could potentially be used for biomedical applications. To analyse particle-cell interactions within *in vitro* cell studies, a dose-controlled system for delivery of NPs to lung cell cultures is required. A new air-liquid interface cell exposure (ALICE) system has been developed, allowing a dose-controlled deposition of NPs on top of the cells (Lenz et al., 2009). This system has been shown to mimic the aerosol inhalation very realistically.

The ALICE system was combined with either a human triple cell co-culture system, composed of epithelial cells, macrophages and dendritic cells simulating the most important barrier functions of the lung epithelial airway (Rothen-Rutishauser, 2008), or with epithelial mono-cultures only. The cells were exposed to 15 nm gold particles, which show promising features for biomedical applications. Oxidative stress and pro-inflammatory effects were analysed at concentrations of 61 ng Au/cm^2 and 561 ng Au/cm^2 deposition, but no adverse effects were found after 4 h and 24 h exposure time (Brandenberger et al., 2010).

The intracellular particle distribution over time was quantitatively evaluated by stereology on electron microscopic images and compared to particle uptake under submerged conditions. The analysis revealed a significant, non-random intracellular NP distribution. NPs were localised in intracellular vesicles, but not in the nucleus, mitochondria, endoplasmic reticulum or Golgi apparatus. Only a minority of gold NPs was found free in the cytosol. However, when NPs were exposed under submerged conditions, an increased NP agglomeration was observed. This can lead to differences in cellular NP uptake mechanisms, hence influencing particle-cell interactions. In addition, it was found that particle-cell interaction was reduced under submerged exposure conditions, since not all NPs suspended in the cell culture medium were able to deposit on the cells.

With the ALICE system, it is therefore possible to reflect the physiological conditions of aerosol inhalation and deposition more precisely. No adverse effects from gold NPs were observed. By using a stereological analysis the intracellular particle number, localisation and intracellular trafficking can be quantified.

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Keywords: nanoparticles, particle-cell interaction, exposure systems, air-liquid interface, respiratory epithelial barrier model

Poster

Transport of chlorpromazine across Caco-2 cells and measuring the free concentration by nd-SPME

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During drug development, the pharmacokinetic parameters of a compound are determined in animal experiments. To reduce the number of animal experiments, *in vitro* and *in silico* approaches are needed that can help predict the kinetics *in vivo*. To make full use of these *in vitro* kinetic data, it is also necessary to fully understand the kinetic behaviour of the compound in the *in vitro* system. Therefore, the aim of this project, part of the European project Predict-IV, is to improve our understanding of the kinetics of compounds in *in vitro* test systems.

The absorptive and secretory transport of chlorpromazine hydrochloride (CPZ) across Caco-2 cell monolayers was measured and the influence of bovine serum albumin (BSA) applied basolaterally was determined. A negligible depletion-solid phase micro extraction (nd-SPME) method was developed to measure the free concentration of CPZ in the medium and to correct the Caco-2 permeability results for protein binding. All samples were measured by HPLC-UV.

The nd-SPME measurements showed that 90% of chlorpromazine is bound to proteins (KBSA=276 l/kg) and that the solubility limit in HBSS is 450 mg/l. Transport of CPZ was concentration-dependent; the average efflux ratio was 3.5, indicating active efflux transport. The basolateral addition of BSA decreased the secretory transport values, lowering the efflux ratio to below 1. However, correcting the secretory transport values for protein binding increased transport values, giving an efflux ratio above 1 again. Because of low recoveries, the amount of CPZ inside the Caco-2 cells and bound to the plastic well were taken into account. This increased CPZ recovery from 50-89% to 77-97%.

In conclusion, BSA needs to be added to the basolateral compartment of the Caco-2 system to better mimic the *in vivo* situation. Then, when transport of lipophilic compounds is measured, the transport rate should be corrected for protein binding. Additionally, when a poor mass balance is found, the amount of compound inside the cells and bound to the plastic well should be taken into account.

Keywords: Caco-2 cells, chlorpromazine, nd-SPME, free concentration

Poster

Specificity of the *in vitro* cell-based assay for marine toxins detection in natural samples

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The development of cell-based assays (CBAs) for toxin detection in natural samples (fish, shellfish, microalgae) has important methodological implications and represents a clear advancement in hazard identification. In natural samples, both the toxic compounds and the accompanying matrix itself may cause cell death, and thus the availability of a specific CBA for a given toxin family is crucial for diagnosis purposes. Specificity can be understood as the modulation of determined signalling pathways involved in the action of toxic compounds in order to enhance or hinder their cytotoxicity, an effect that will not be observed in the absence of the given toxin. The neuroblastoma (Neuro-2a) CBA for neurotoxins (ciguatoxin, saxitoxin, brevetoxin) as originally described in Manger et al. (1993) is one of the best established and most widely used CBA for neurotoxin detection in natural samples. The assay is based on the use of ouabain and veratridine that enhance or block Na^+ influx induced by neurotoxins. Another CBA with Neuro-2a cells is used for the detection of maitotoxin based on the antagonistic effect of SK&F 96365 (blockage of the intracellular Ca^{2+} influx) on the MTX-induced toxic effects. Examples of application of both CBA in fish and microalgal samples are presented. Those assays are likely to identify the presence of ciguatoxins (P-CTX-1) and maitotoxin in natural samples with high specificity and high sensitivity (limit of quantification for P-CTX-1 in fish sample = 0.0096 ppb).

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Keywords: cell-based assay toxin detection

Poster

The BALB/c 3T3 cell transformation assay to assess the carcinogenic activity of chemicals

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The new EU regulation for chemicals, REACH, specifically requires the development of alternatives in order to reduce and eventually replace studies on vertebrates. At the moment the cell transformation assay performed on rodent cell lines (BALB/c 3T3 or C3H10T1/2) or primary cells from Syrian Hamster is regarded as the only possible *in vitro* alternative to animal testing for carcinogenesis studies. Cell transformation assays have been proposed as screening tests for the carcinogenic potential of compounds for which there is no evidence of genotoxicity (OECD, 2007) and are listed among the REACH methods, as reported in EU regulation EC 440/2008.

For the last 20 years we have tested many chemicals and complex mixtures by using BALB/c 3T3 A 31 cells in different experimental protocols (Colacci et al., 1990; Mascolo et al., 2010). Recently, several scientists proposed to switch to the BALB/c 3T3 A 31-1-1 cell clone to develop a validated protocol to fulfil REACH requirements. The present study was performed with the aim of comparing the results obtained with the two different clones. Cells were treated with PAHs (3-MCA 2.5 μg/ml, B(a)P 2.5 μg/ml) and aloethanes (1,2-DBE 50 μg/ml). The induction of cytotoxicity and the onset of chemically transformed foci were evaluated by two different experimental protocols: i) the originally recommended protocol (Kakunaga, 1973; IARC/NCI/EPA Working Group, 1985), where cells were seeded at 10,000 cells/60 mm dish and exposed to chemicals in the culture medium for 72 h; ii) an improved protocol aiming at reducing the toxicity of the chemical treatment, where the number of seeded cells was increased from 10,000 to 30,000 per dish and the cell treatment started two days later and lasted 48 h instead of 72 h (Matthews et al., 1993). In both treatment schedules, at the end of the exposure, the treatment medium was replaced with complete medium and the cultures were maintained for 4-6 weeks to allow the expression of transformed foci.

The two clones differed regarding their response to chemicals, probably because of the different metabolising capacity. The A31-1-1 cells showed a higher inherent transformation rate after PAH treatment, but they were insensitive to 1,2-DBE. As DBE is bioactivated to reactive forms able to bind DNA mainly through the conjugation with intracellular glutathione (Guengerich, 2003), these results suggested a reduced activity of phase-2 enzymes involved in gluthatione conjugation in A31-1-1 cells.

Our results seem to suggest that *in vitro* cell transformation protocols performed under REACH regulation should take into account the different sensitivity of BALB/c 3T3 clones to different classes of chemicals.

Keywords: transformation, alternative methods, carcinogens

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Lecture in Session V: Acute and long term toxicity A

Assessment of acute, long-term and chronic respiratory toxicity using a long shelf-life 3D model of the human airway epithelium

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Most *in vitro* cell models for long term testing of chemicals suffer from at least two shortcomings: 1) The failure of reproducing the *in vivo* physiological characteristics of the corresponding tissues, such as in the case of immortalised cell lines. 2) A limited shelf-life, for example, freshly established primary cell cultures.

Epithelix has developed and is commercialising on a world-wide scale a standardised air-liquid interface *in vitro* cell model of the human airway epithelium (MucilAir™) that is free from these limitations. MucilAir™ is morphologically and functionally differentiated and can be maintained at a homeostatic state for more than one year. The typical ultra-structures of the human airway epithelium, such as the tight junctions, the cilia, the basal cells, the mucous cells, can be observed. The epithelium is electrically tight (TEER ≈ 450 ohm.cm2). Classical airway transporters and CypP450s are expressed and functional. The ion channels are fully functional and respond normally to their specific inhibitors and activators. Moreover, the epithelial cells react to pro-inflammatory mediators in a physiological manner (Huang and Caulfuty, 2009). The epithelia can be stimulated regularly with inflammatory substances to simulate chronic inflammatory reactions for up to several months. A large panel of cytokines, chemokines and metalloproteinases has been detected in MucilAir™ (e.g. IL-8, IL-6, GM-CSF, MMP-9, GRO-α, etc.). Remarkably, the epithelium has a strong capacity for regeneration after mechanical or chemical injuries. Epithelia from several different pathologies can be reconstructed (e.g. asthma, COPD, CF, smoker, etc.) (Huang et al., 2009).

Due to its unique long shelf-life of one year, this model is used for studying human respiratory diseases and to test long-term/chronic effects of drugs/chemicals on the respiratory tract (Maier, 2010). Late effects of chemicals (several weeks after exposure) can be observed. Several applications of MucilAir™ relevant to inhalation toxicity assessment will be presented:

– Acute, long-term and chronic toxicity testing
– Inflammatory effect assessment – examples of 2 months exposure study
– Assessment of reversible vs. irreversible effects of chemicals
– Recent advances in the detection of airway sensitization and irritation

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Keywords: airway toxicity, airway sensitization, long term toxicity, chronic toxicity, 3D human airway epithelium

Poster

Evolution of *Pseudomonas aeruginosa* virulence in infected patients revealed in a *Dictyostelium* host model

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Pseudomonas aeruginosa may cause acute infections in intubated patients or chronically infected patients with cystic fibrosis (CF). *P. aeruginosa* has been shown to adapt to these particular lung environments by progressive phenotypic modulation, in particular genetic inactivation of LasR. Whether these adaptive changes affect virulence is classically tested by infecting model

animals (typically mice or rats). This creates a serious ethical problem and at the same time a strong limitation to research, since only a limited number of strains can be analysed in this way. We show here that the use of an alternative model organism, the amoeba *Dictyostelium*, circumvents the need for animal experiments while providing high-quality data that could not be obtained using animals.

We analysed a collection of clinical isolates from 16 CF patients and from 10 intubated patients for their virulence in a *Dictyostelium discoideum* amoeba model. Virulence was assessed by following growth of *D. discoideum* on a lawn of P. aeruginosa bacteria for 10 days, and scored on a scale from 0 (avirulent) to 8 (virulent).

With one exception (a CF patient), all initial isolates, whether from CF or from intubated patients, were virulent (score = 8). For eight CF patients, no decrease in virulence was observed in the two late samples, while for the remaining seven CF patients at least one of the two late isolates was significantly less virulent. Isolates from eight out of the ten intubated patients were virulent at all times analysed. In two intubated patients fewer virulent isolates were seen within a few days (score 4 to 0). Mutations in the quorum sensing regulator LasR were identified both in CF and non-CF isolates; however their presence did not correlate with loss of virulence.

Loss of virulence is not the main driving force for the adaptation of *P. aeruginosa* to the human host and does not occur predominantly through mutations in LasR. This study illustrates how use of an alternative host model can replace the use of animals to investigate key questions concerning bacterial infection of patients.

Keywords: Pseudomonas infection, cystic fibrosis, Dictyostelium, virulence

Poster

Acceptance of *in vitro* EpiSkin and SkinEthic RHE skin irritation test methods for hazard identification of chemicals

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In line with the 7th amendment of the Cosmetics Directive deadlines, the European Union is banning the *in vivo* skin irritation assessment on ingredients for cosmetic purposes. European legislation has heightened the need for accepted *in vitro* skin irritation tests to replace the regulatory Draize skin irritation test. The reconstructed human epidermis EpiSkin and SkinEthic RHE "42 bis" test methods were validated by ECVAM as stand alone replacement tests for the prediction of acute skin irritation, discriminating irritant (category 2) and not classified substances according to both former and recently implemented Global Harmonization System (GHS) classifications applied in Europe. Therefore, the test methods were endorsed in the EU test method B.46 and in the draft *in vitro* skin irritation OECD Test Guideline. Evaluation using these methods directly addresses the initial step of the inflammatory cascade/mechanism of action, i.e. cell and tissue damage resulting in localised trauma. The global predictive capacities (specificity, sensitivity and accuracy) of the EpiSkin (designated the Validated Reference Method in the OECD guideline) and the SkinEthic RHE methodologies demonstrate that they are applicable to a wide range of physico-chemical substances and mixtures.

Keywords: skin irritation, EpiSkin, SkinEthic RHE, validation

Lecture in Session IX: New developments and novel methods

Toxicity profiling of nephrotoxins in the human proximal tubular RPTEC-TERT1 cell line: influence of hypoxia

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The process of drug development involves specific testing protocols assessing the toxicity of lead compounds and their derivatives. Current preclinical toxicity testing which has predictive value is mainly carried out in animal models. It has however some major disadvantages, being highly time consuming and requires a large amount of animals and test compound and is therefore associated with high costs. The development of an alternative/complement to *in vivo* animal toxicity testing, namely *in vitro* human cell line alternatives is an important effort to optimise costs and deliver more predictive outcomes.

To this end we have developed such an *in vitro* human cell line system for renal toxicity testing. Importantly the kidney, and particularly the proximal tubule epithelium, is sensitive to alterations in oxygen tension. Many chronic renal diseases have elements of hypoxia due either to vasoconstriction or fibrosis. Signalling mechanisms and cellular process altered by hypoxia are known targets for nephrotoxin action. Therefore, in addition to profiling the toxicity of a panel of nephrotoxins, we examined the influence of hypoxia as a model of compromised renal tissue in the human proximal tubule cell line RPTEC-TERT1.

RPTEC-TERT1 cells were grown on 96 well plates cultured for more than 10 days after confluence to achieve a differentiated proximal tubule phenotype. Cells were treated with Adefovir Dipivoxil, Adefovir, Cidofovir, Tenofovir, Ibandronate, Zoledronate, Clodronate, Acyclovir, Vancomycin, Cadmium, Cisplatin, Cycosporine A and Diquat across a dose response range (5 concentrations) every day for 14 days (repeat bolus). Cell culture media was removed at 1, 3, 7 and 14 days and analysed for lactate levels. At 14 days, cells were processed for toxicity profiling through the analysis of Resazurin, ATP, Glutathione, LDH release and protein content.

The profile of lactate release revealed a dose and time dependent effect with nephrotoxin exposure in RPTEC-TERT1 cells for a number of compounds and was most evident with Diquat. Analysis of the other toxicity endpoints revealed a complex profile of dose dependent effects with compounds such as Adefovir Dipivoxil, Cidofovir, Zoledronate and Diquat displaying greater toxicity compared to others such as Vancomycin, which did not significantly alter any measured endpoint. For a large number of compounds concomitant exposure to hypoxia increased toxicity. This was most apparent with Cisplatin, Zoledronate and Adefovir Dipivoxil. Compounds such as Diquat displayed no alteration in toxicity in the presence of hypoxia, indicating compound specific toxic susceptibility. The mechanism through which increased toxicity in hypoxia occurs is likely through a common target such as the mitochondria, injury to which is additive in conditions of reduced oxygen and nephrotoxin treatment. The contribution of altered glycolytic and ROS / glutathione activities may also be an important factor.

Therefore it is clear that environmental factors such as oxygen can alter toxicity profiles in renal proximal tubular epithelial cells and suggests an important consideration when developing *in vitro* toxicity models. This is crucial in extrapolating to human pharmaceutical treatment as many patients have compromised renal function to begin with.

Keywords: nephrotoxin, hypoxia, oxygen, proximal tubule

Lecture in Session IX: New developments and novel methods

An *in vitro* ocular testing strategy for US EPA registration of anti-microbial cleaning products

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In the United States, household and industrial cleaning products which make an "anti-microbial" claim must be registered with the EPA. This registration process generally requires the performance of animal tests, including ones for eye irritation, in order to provide the appropriate hazard labelling for the product. However there was strong interest from both the EPA and several companies producing cleaning products to investigate whether an *in vitro* testing strategy for ocular irritation of anti-microbial cleaning products (AMCP) could be developed which would provide sufficient information to determine an EPA hazard category. As a result seven companies (Clorox, Colgate-Palmolive Company, The Dial Corporation, EcoLabs, JohnsonDiversey, Inc., The Procter & Gamble Company, and S. C. Johnson & Son, Inc.) plus The Accord Group and the Institute for In Vitro Sciences (IIVS) began a collaboration to jointly develop a data set demonstrating the predictive capacity of an *in vitro* ocular testing strategy for the restricted applicability domain of AMCPs. Historical animal data for AMCPs and similarly formulated products were compared to data from three *in vitro* ocular test methods – the Bovine Cornea Opacity and Permeability (BCOP) assay, the Cytosensor™ microphysiometer (CM; Molecular Devices, Menlo Park, CA, USA) and the EpiOcular™ 3-dimensional tissue model (MatTek Corp., Ashland, MA, USA). Some of the *in vitro* data were from previously conducted studies, but new *in vitro* data were also generated using coded compounds to prevent bias within the testing laboratory. As part of a weight-of-evidence approach, additional data from common AMCP ingredients and similarly formulated personal care products were analysed to help support the proposed prediction models (cut-off values) for the three proposed *in vitro* methods. Although exact names for the products of the participating companies were not given, a descriptive formulation was provided for almost all of these products (or combinations of ingredients). As a result of the data analysis, a testing strategy was proposed which utilised either a top/down, or a bottom/up approach. No test was shown to adequately differentiate between all four of the EPA hazard categories, but a combination approach using the BCOP assay and either the CM or EpiOcular assay could place a product into any one of the four hazard categories. After an EPA internal review, the EPA has proposed an 18-month pilot program under which new AMCPs can undergo the registration process using the *in vitro* ocular testing strategy described above.

Keywords: eye irritation, regulatory use, testing strategies

Poster

Current status of the COLIPA prevalidation of the Reconstructed Human Skin Micronucleus Assay (RSMN)

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The current battery of *in vitro* genotoxicity assays are thought to produce a high level of false positive results. Subsequent investigation of these results is generally done with *in vivo* genotoxicity studies which are costly and time consuming. As of March 2009, the 7th Amendment to the EU Cosmetics Directive prohibits the use of *in vivo* genotoxicity tests in safety assessments for cosmetics, meaning that many safe ingredients may be discarded because further investigation of *in vitro* positive results with animal studies is now impossible. To address this, the European Cosmetic Toiletry and Perfumery Association (COLIPA) has initiated an international project to establish and evaluate more predictive *in vitro* genotoxicity assays using 3D human tissues. One focus has been on the 3D human skin micronucleus assay (RSMN) in EpiDerm™. Since skin is the first site of contact with maximum exposure to many different products including cosmetics, the RSMN assay offers the po-

tential for a more realistic application/metabolism of test compounds for evaluating genotoxicity (Curren et al., 2006; Mun et al., 2009; Hu et al., 2009). The COLIPA RSMN project is a multi-lab initiative which has involved Procter & Gamble (US), L'Oréal (France), Henkel (Germany) and the Institute for In vitro Sciences (IIVS, US). Intra-laboratory and inter-laboratory reproducibility have been investigated with model genotoxins mitomycin C and vinblastine sulfate, as well as a variety of chemicals that require metabolic activation. The first phase of testing with coded compounds has been successfully completed and a second round has begun. Results so far support this model as a promising new *in vitro* method for detecting micronuclei induction in human skin.

This work is funded by the European Cosmetic Industry Association COLIPA.

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Mun et al. (2009). *Mut, Res. 673*, 92-99.

Keywords: skin models, genetic toxicology, 7th amendment to the cosmetics directive

Poster

Implications of postponement of the 2013 deadline for implementation of the 7th amendment to the Cosmetics Directive

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The 7th amendment to the Cosmetics Directive (76/768/EEC), adopted in 2003, was a political and social landmark in codifying widespread concern among the European public about the use of animals in cosmetics testing.

The amendment was the result of a lengthy political process and has faced a number of challenges since. In addition to prohibiting animal testing within the European Union, the amendment introduced a two-stage ban on the marketing of cosmetics containing ingredients tested on animals for human health effects, irrespective of the availability or regulatory acceptance of alternatives, with a final deadline of March 2013.

The existence of these deadlines has provided a positive impetus for the development of alternatives and their use outside the cosmetics sector, including meeting the requirements of other legislation such as REACH. There is, however, widespread consensus that validated alternatives to the tests still permitted will not be available by 2013 and the European Commission is therefore expected to propose legislation in 2011 that may postpone this deadline.

In addition to the scientific challenges posed by developing alternative methods, the continued use of animals for the testing of cosmetics raises strong ethical and legal questions and can be seen as a test case for the principle of "harm-benefit" analysis enshrined in the new EU Directive on animal experimentation.

This presentation examines the background to the current situation, the approaches taken by national governments and international bodies to the existing provisions and the implications of postponement of the deadline, especially in the context of the additional animal testing expected in order to meet REACH requirements in the period until 2018. It concludes that the ethical case against postponement remains compelling and that other advantages accrue from maintaining the current date. These factors must be addressed by scientists, companies and policymakers in formulating a response to the technical, commercial and political challenge posed by the deadline.

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Keywords: animal testing, alternatives, cosmetics directive, REACH

Poster

Center for Alternatives to Animal Testing – Europe

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Two universities renowned for their experience in the area of alternative methods in Europe and the US have joined forces to complement the 29-year-old Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University with a corresponding Center for Europe (CAAT-EU) at the University of Konstanz.

The "Excellence University" of Konstanz has twenty years of experience in alternatives to animal experiments, employing five professors in pharmacology and toxicology along with numerous coworkers researching human-relevant alternative methods. CAAT-EU brings together industry and academics to address the needs for human-relevant methods, to use strategic funds to fill gaps in the development and implementation of alternative methods, to coordinate information days in Europe on relevant developments in the area of alternatives, to develop strategic projects with sponsors for the promotion of humane science and "new toxicology" and to set up a joint education programme between Johns Hopkins and the University of Konstanz.

Keywords: CAAT, transatlantic cooperation, CAAT-EU, Johns Hopkins University, University of Konstanz

Poster

Study of photodynamic effect on NIH 3T3 cells and bacteria

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Photodynamic therapy is a relatively new, minimally invasive therapeutic treatment of malign and nonmalign diseases. This method has historically been used to treat cancerous tumours but has recently also been used to kill bacterial cells. The therapy is based on the combination of a light-absorbing photosensitizer and irradiation with visible light of an appropriate wavelength. Upon irradiation with the light, the photosensitizer undergoes a transition from a low energy (ground state) to a higher energy (triplet state) and generates free radicals or singlet oxygen, which are toxic to target microorganisms. Today therapy with antibiotics has become unpopular because the resistance of bacteria to antibiotics is increased. In this study ZnTPPS4C, TPPS4C and TM porphyrin were chosen for testing as potential sensitizers for a photodynamic effect study on NIH 3T3 cell lines and 4 bacterial strains.

We report the production of reactive oxygen species and phototoxicity on NIH 3T3 cells, Gram(+) and Gram(-) bacteria. The production of ROS was investigated using the molecular probe CM-H2DCFDA. Phototoxicity was evaluated by MTT test. The light emitting diodes (LEDs 414 nm) were used as a source for evocation of the photodynamic effect at total doses from 0.5 to 25 Jcm^{-2}. The cells or bacteria were grown in the presence of sensitizers at concentrations from 0.5 to 100 μM. Fluorescence of CM-DCF was calibrated according to the fluorescence response of the probe to the addition of external H_2O_2.

Our results show that ROS production in NIH 3T3 cells is dependent on the sensitizer concentration and dose of irradiation.

In summary, we found that all of three tested sensitizers were efficient. TM porphyrin is the best for evocation of the photodynamic effect and it is efficient in NIH 3T3 cells and Gram(+) bacteria.

This work was supported by the grant projects NS9648-4/2008 from the Ministry of Health, MSM 6198959216 from the Ministry of Education, Youth and Sports of the Czech Republic, 303/09/H048 from the Czech Science Foundation and CZ.1.05/2.1.00/01.0030.

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Keywords: photodynamic effect, NIH 3T3 cell line, bacteria

Poster

Evaluation of the multipotent character of human skin-derived precursors

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Evidence is accumulating of the existence of multipotent mesenchymal stem cells/progenitor cells in various niches within the adult human body. Their favourable immunologic properties and plastic behaviour in response to specific stimuli provide new perspectives with respect to tissue engineering and create expectations for their usefulness in future protocols of autologous transplantation, gene therapy and tissue reconstitution in a number of pathological processes. A promising mesenchymal stem cell source consists of human skin-derived precursor cells (hSKP), a novel population of neural-crest related precursor cells isolated from human embryonic, infant or adult skin with minimal discomfort to the donor. hSKP represent a multipotent pool of stem cells capable of generating neuronal, glial, mesodermal and endodermal progeny. Indeed, the fact that hSKP derivatives such as Schwann cells and neuronal cells display *in vitro* and *in vivo* function raises the possibility of hSKP being both an experimental and a therapeutic resource for disease modelling and regenerative medicine (Biernaskie et al., 2007; Higashida et al., 2010; Lavoie et al., 2009; De Kock et al., 2009).

In the present study, the trilineage multipotent differentiation capacity of hSKP is screened by means of immunocytochemistry upon exposure to various (non-)commercial differentiation formulations. More specifically, hSKP are exposed to 2 commercial neural media (Lonza, Stem Cell technologies), 5 commercial keratinocyte media (CellnTec, Epilife), 3 modified commercial adipocyte media and 4 "in house" prepared hepatic differentiation media. Special attention is paid to their suitability in terms of differentiation efficacy.

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Keywords: hSKP, skin-derived precursor, plasticity, endoderm, ectoderm, mesoderm

Lecture in Session II: Metabolism and Toxicokinetics

An *in vivo* like expression pattern of CYP450 enzymes found in a 3D *in vitro* model of the human airway epithelium – MucilAir™

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Most of the *in vitro* primary cell and tissue models rapidly lose the morphological and functional characteristics of the original native tissues. This is also the case for the expression of the cytochrome P450 (CYPs) enzymes, essential for metabolising and eliminating xenobiotics. Epithelix has developed and offers a novel 3D *in vitro* model of the human airway epithelium, which maintains the fully differentiated, morphologically and functionally, characteristics of the native tissues.

We present here some data showing that MucilAir exhibits an *in vivo* like expression pattern of CYP450 enzymes. Indeed, high levels of 2A13, 2B6 and 2F1, moderate levels of 2A6 and 2S1, and low levels of 2C8-1 were found in the 3D model-Mu-

cilAir (3 different donors), as reported for their expression in the native tissues (Coskela et al., 1999; Su et al.,2000; Weems et al., 2010). This *in vivo* like expression profile of CYP450s reflects the fully differentiated nature of MucilAir, since this expression pattern was not found in the monolayer culture conditions of the same epithelial cells. In contrast, these cells show a totally different profile: high levels of 2C8-2 and 2E1, low levels of 2B6, whereas 2A6, 2A13, 2C8-1, 2F1, 3A4 and 3A5 are almost undetectable (Seo et al., 2008; Ha et al., 2007). Furthermore, this *in vivo*-like expression pattern can be maintained *in vitro* for at least 6 months.

Taken together, MucilAir is a good candidate as an *in vitro* model for studying the metabolism and toxicology of chemical compounds.

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Keywords: CYP450, human airway, primary cells, bronchial epithelium, in vitro, 3D

Lecture in Session IV: Good cell culture practice

STR DNA typing: intra- and interspecies cross-contamination detection of human cell lines

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Inter- and intra-species cross-contaminations (CC) of human and animal cells represent a chronic problem in cell cultures leading to false data. Microsatellite loci in the human genome harbouring short tandem repeat (STR) DNA markers allow individualisation of cell lines at the DNA level. Thus, fluorescence polymerase chain reaction amplification of STR loci D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX, CSF1PO and Amelogenin (AMEL) for gender determination is meanwhile the gold standard for authentication of human cell lines and represents an international reference technique (Masters et al., 2001). Furthermore, an additional tetraplex PCR has been established in order to detect mitochondrial DNA sequences (mtDNA) of animal rodent cells within a human cell culture population.

The major cell banks of USA, Germany and Japan (ATCC, DSMZ, JCRB and RIKEN, respectively) have built compatible STR databases to ensure the availability of STR reference profiles (Dirks et al., 2010). Upon determination of an STR profile of a human cell line the suspected identity can be proven by an online verification of customer-made STR data sets on the homepage of the DSMZ institute. Since authentic cell lines are the main pre-requisite for rational research and biotechnology, the presentation describes a rapid and reliable method available to students, technicians and scientists for certifying identity and purity of human cell lines of interest.

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Keywords: authentication, cross-contamination, DNA STR typing, human cell lines, mtDNA typing, misidentification, quality control

Lecture in Session IV: Good cell culture practice

Use of autologous serum improves yield and quality of neohepatocytes for *in vitro* toxicity testing

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Altering the molecular structure of chemicals to increase polarity is essential for their elimination from the human body. Most biotransformation reactions are defined by phase I and II drug metabolising enzymes. Thus, expression and regulation of these enzymes are essential for the determination of pharmacologic and toxic effects, which are conventionally measured in primary human hepatocytes. However, their availability is limited by donor organ scarcity. As a possible alternative, NeoHepatocytes, generated by trans-differentiation of peripheral blood monocytes (PBMCs), have been suggested, although the basal phase I and II enzyme activities are significantly lower compared to primary human hepatocytes. In the last years it has be critically discussed whether gaining hepatocyte features is associated with trans-differentiation of monocytes or their activation towards a macrophage phenotype.

NeoHepatocytes were generated using six different human AB-sera, FCS or autologous serum. For de-differentiation cells were treated with IL-3 and M-CSF for six days, following differentiation for 14 days with FGF-4. Yield of resulting cells was determined by sulphorhodamine B (SRB) staining. For characterisation of NeoHepatocytes we measured phase I and II enzyme expression and activities. In addition we measured toxicity of five different pro-teratogenic substances and their metabolites, e.g. cyclophosphamide and acroleine or valpromide and valproic acid. Primary human hepatocytes were used as gold-standard. Macrophage activation was investigated by measuring the levels of TNF-α, TGF-β and RANKL secretion, matrix metalloproteinase (MMP) activity as well as mRNA levels of interleukin-1α, -1β, -6, -8 and -10 in "programmable cells of monocytic origin" (PCMOs).

The yield and quality of NeoHepatocytes varied considerably depending on the different sera. The highest number of cells was consistently obtained with the autologous approach. These cells also showed the highest activity of phase I (CYP1A1/2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4) and phase II enzymes (e.g. glutathione-S-transferase). The lowest yield and quality of NeoHepatocytes was obtained using FCS, while in the corresponding PCMOs macrophage activation was highest (TNF-α↑, TGF-β↑, RANKL↑, MMP activity↑, IL-1α↑, -1β↑, -6↑, -8↑ and -10↑). Treatment of NeoHepatocytes generated using autologous serum with five different pro-teratogenic substances and corresponding metabolites showed toxicity comparable to that determined in primary human hepatocytes. Also, biotransformation rates of the substances, assessed by measuring metabolite formation and release into the culture supernatant, were comparable in both cell types.

Our data reveal that the use of autologous serum reduced macrophage activation in PCMOs which, as a consequence, led to improved yield and function of NeoHepatocytes. As biotransformation and activity and toxicity studies were comparable between NeoHepatocytes and primary human hepatocytes, their easy accessibility makes them ideal candidates for *in vitro* toxicity studies. With respect to the autologous approach one can even speculate about their use for personalised medicine.

This project was partially supported by the BMBF (0315208A and 01GN0984)

Keywords: primary human hepatocytes, NeoHepatocytes, toxicity tests, macrophage activation

Poster

Alternatives outreach and a new student movement for humane veterinary education in Egypt

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A new student movement for alternatives in veterinary education and training has been founded in Egypt. Cairo University Vets for Alternatives (CUVA) was established in March 2010 following the 1st North Africa and Middle East Seminar on Alternatives in Education and Training and during InterNICHE outreach activity across the country. Over 40 presentations and meetings were held between InterNICHE and university deans, teachers, students and others. Negotiations between InterNICHE and Cairo University Faculty of Veterinary Medicine addressed the potential for replacement of harmful animal use in practical classes by innovative alternative methods such as training mannekins and advanced software. Parallel to these discussions, students established CUVA to help achieve full replacement and to enhance the quality of their education and training. CUVA membership includes over 400 students and junior teachers, facilitated by the social networking site Facebook. An Organising Committee of 10 students manages the organisation, and three workgroups progress its objectives. In partnership with InterNICHE, the workgroup on clinical rotations and body donation programmes is developing collaborative projects with shelters and veterinary outreach organisations. Under supervision, students will participate in sterilisation and other procedures on patients at shelters and join veterinary convoys providing animal care in villages across the country. These initiatives address the concerns by animal campaigners, shelter owners and the students themselves at the significant lack of practical experience and animal welfare awareness often shown by graduates. Although extra-curricular at first, it is hoped that such involvement in clinical work will help replace animal experiments within clinical skills and surgery training. The body donation programmes will help provide cadavers that are ethically sourced according to the InterNICHE and CUVA policies, and which may help replace the killing of animals in several disciplines. The workgroup on alternatives is collating information on animal use and potential replacements, organising self-training in alternatives and investigating the opportunities for local production of alternatives. The publicity workgroup promotes the meetings and activities. Aware of the empowerment and leadership potential within the movement, CUVA intends to help initiate student self-organisation at other faculties of veterinary medicine in Egypt and across the region once the projects are established and once experience in overcoming the obstacles has been gained.

Keywords: animals, education, veterinary, replacement, alternatives, Egypt, CUVA, InterNICHE

Lecture in Session II: : Metabolism and Toxicokinetics

Human pluripotent stem cells and their derivatives in assays for chemical risk assessment

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Traditionally, safety assessments in drug discovery and evaluations of environmental stressors on human health are based on non-human material or limited sources of human tissue samples. The use of large numbers of experimental animals and big inter-experimental variations negatively impacts the process, and there is a great need to evaluate alternative options. During the last decade, human pluripotent stem cells (hPSCs) have received increasing appreciation for their use in a wide range of experimental settings. Cellartis is a biotech company specialised in hPSCs technologies and has developed several hPSC-based products for the *in vitro* and tools market space.

Since many compound effects are species-specific, reliable toxicity assays based on human cells are of high relevance. Cellartis has developed cytotoxicity models based on human undifferentiated stem cells, partially differentiated cells and terminally differentiated cells. Notably, *in vitro* differentiation

of hPSCs resembles the early stages of human embryonic development and thus offers unique possibilities for alternative *in vitro* screening of compounds for their potential risks of adverse effects on the growing embryo. At Cellartis, the unique features of hPSCs are taken advantage of in a cellular model for developmental toxicity testing. Furthermore, hepatocytes and cardiomyocytes derived from hPSCs are provided by Cellartis in multi-well plate formats in an unlimited supply suitable for a wide range of applications. The cells exhibit specific markers and functional properties similar to their adult counterparts and can be cultured for extended times *in vitro* without loss of basic functionality.

Hence, it is anticipated that new improved *in vitro* models based on physiologically relevant human cells will result in more cost-effective assays, ultimately leading to safer new drugs and better evaluations of the effects of chemicals on human health. In this presentation, the differentiation of hPSCs to specialised cells and results from safety assessment analyses utilising hPSCs will be discussed.

Keywords: human pluripotent stem cells, hepatocyte, cardiomyocyte, toxicity, safety assessment, embryo

Poster

A hemi-cornea model for eye irritation testing: quality control of production and prevalidation of prediction models

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A hemi-cornea model exclusively based on immortalised human corneal cell lines (Araki-Sasaki et al., 1995; Zorn-Kruppa et al., 2004, 2005; Manzer et al., 2009; Seeber et al., 2008) has been developed to provide an animal-free tool for eye irritation testing. The hemi-cornea model comprises a multi-layered epithelium and a stroma of collagen-embedded keratocytes. During the current prevalidation study we have successfully demonstrated the protocol transfer for the hemi-cornea construction. This means that it could be reproducibly produced according to standard operation procedures and defined acceptance criteria in the three participating laboratories. The low intra- and inter-laboratory variation of our results show that the hemi-cornea maintains reproducible quality markers over many production lots and can be constructed in every well-equipped laboratory experienced in cell culture.

For the assessment of the predictive capacity and reliability of the test system for eye irritation we developed preliminary prediction models based on the results achieved with 13 reference chemicals (RC; 4 x R36/Cat.2 and 9 x NL/NC). Tissue viability, determined with an MTT assay, was defined as the sole toxicological endpoint. According to the test protocol, the hemi-corneas were exposed to the RCs for 3 different time intervals (10, 20 and 60 min). Alterations of tissue viability over time after topical substance application can be described best using an asymptotic exponential decay approximation. Our prediction models are either based on the variables of the exponential decay function or on the relative tissue viability after a 10 min exposition interval. With either of the three prediction models, eight out of nine chemicals classified as non-irritant (NL/NC) and three out of four chemicals classified as irritant (R36/Cat.2) were predicted correctly.

The test system is now under prevalidation in three participating laboratories using 20 reference chemicals under double-blind coded conditions.

The project was financially supported by the Federal Ministry of Research and Education (BMBF, FKZ 0313913; FKZ 0315504)

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Keywords: hemi-cornea, eye irritation, quality control, prevalidation, prediction model

Poster

Attenuating effect of genistein on polychlorinated biphenyl induced oxidative toxicity in TM3 Leydig cells

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Polychlorinated biphenyls, which are produced for industrial purposes, are organic chlorinated compounds. The use of polychlorinated biphenyls was banned in several countries when they were determined to be persistent pollutants in the environment. Many polychlorinated biphenyl containing industrial products and equipment (industrial fluids, flame-retardants, diluents and fluids for capacitors, transformers, hydraulic pumps, pesticide and dielectric fluids) are still in use and thus pose a threat to the environment and human health. They bio-accumulate both in the environment and in living organisms owing to their lipophilic features and chemical stability. It is determined that polychlorinated biphenyls have various negative effects, such as toxic, carcinogenic, immunosuppressive, teratogenic and endocrine disruptive activities.

Genistein is a natural isoflavone phytoestrogen present in soya and soybean products. Isoflavones may prevent oxidative DNA damage via affecting free radicals and antioxidant enzymes. Genistein is the compound with the highest antioxidant activity within the isoflavones. Therefore, it was investigated whether genistein has protective effects on polychlorinated biphenyl-induced oxidative damage.

In this study antioxidant effects of genistein on polychlorinated biphenyl-induced oxidative toxicity on TM3 Leydig cells were investigated. Enzymatic antioxidants, lipid peroxidations and reactive oxygen species were measured.

Keywords: polychlorinated biphenyls, genistein, oxidative toxicity

Poster

A cell-free protein synthesis (translation) system based on reactor grown cells

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Cell-free systems for the study of protein synthesis (translation) have already been obtained from many sources, including bacteria, wheat germ, rabbit reticulocytes, murine ascites fluid, frog oocytes and insect cells. We have established an innovative expression system originating from mammalian cells that are grown in large-scale cultures in bioreactors and in the absence of serum. The production cost is low. These translation extracts allow either the direct translation or the coupled transcription and translation of an exogenously added mRNA or cDNA, respectively. The expression system can be either stored hydrated at low temperatures (-80ºC) or it can be lyophilised and kept dehydrated in a freezer (up to -20ºC) for prolonged periods without loss of translational activity after replenishment with water. The system can be produced as a commercial kit and could replace the existing expression systems based on animal extracts, such as the Krebs ascites fluid or the rabbit reticulocyte lysate. It could improve the optimisation, automation and miniaturisation of fed-batch and semi-continuous reactions and would allow *in vitro* synthesis of proteins in a large variety of low- and high-throughput applications suitable for functional and structural proteomics.

Keywords: protein synthesis, translation, in vitro, bioreactors, mammalian cells, suspension, cell-free

Poster

Cultivation of different mammalian cell lines in a serum-free chemically defined medium

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Many reports deal with the cultivation of mammalian cells in serum-free media of defined composition. Healy (Healy et al., 1954) was probably the first to achieve continuous growth of mouse L cells in such a defined medium. Later on Higuchi (Higuchi, 1963; Higuchi and Robinson, 1973) developed the serum-free chemically defined medium. This enabled the cultivation of eight lines of human origin, three of primate origin and five lines of rodent origin. Hormones like insulin, cortisone and thyroxin were added to the defined medium. Vitamin B12 and biotin were needed for growth. Additionally, lipids, such as oleic acid, lecithin and cholesterol, also promote cell growth.

We aimed to improve the Higuchi defined medium (Higuchi, 1963; Higuchi and Robinson, 1973) to enable the cultivation of a greater number of cell lines, especially primary cells like human muscle and chicken embryonal fibroblasts. The following substances were added to the final form of Higuchi medium: 1) Vitamin C (ascorbic acid), 2) dexamethasone, 3) platelet-derived growth factor and 4) fibroblast growth factor.

Cultivation of different cell lines showed that the best growth was obtained with highly transformed cell lines, like CaCo-2. Cells of a lower level of transformation, i.e. VERO cells, could also grow in this medium. The growth of primary cells/cell lines was promoted partially with the addition of fibroblast growth factor, platelet-derived growth factor and ascorbic acid.

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Keywords: cultivation in vitro, cell lines, serum-free chemically defined medium

Poster

Study of keratinocyte response after exposure to sensitizers in a reconstructed human epidermis

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The two most frequent manifestations of some toxicity induced to the skin are irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Reconstructed human Epidermis (RhE) was used to assess the irritation and sensitization potentials of chemicals through the investigation of cellular release of IL-1α and IL-8 from keratinocytes into culture medium. New attempts are presented to understand certain molecular and cellular mechanisms apparently involved in human epidermal keratinocytes exposed to skin irritant and sensitizers. More precisely, we have tried to underline the potential mechanisms responsible for the differential release of IL-1α and IL-8 from RhE treated with chemicals of typical sensitizing potencies, i.e. dinitrofluorobenzene (DNFB), oxazolone (Oxa) or cinnamaldehyde (Cin), in comparison with RhE treated with a well known irritant, i.e. benzalkonium chloride (BC).

In the present study, we used RhE, which was produced in-house following our published protocol (Poumay et al., 2004), in which culture parameters and origins of cells can be controlled independently of commercial suppliers.

We confirm that irritant (benzalkonium chloride) and sensitizers (dinitrofluorobenzene (DNFB), oxazolone, cinnamaldehyde) induce different profiles of interleukin-1α and interleukin-8 expression and release from the RhE (Coquette et al., 2003). Indeed, the ratio of released IL-8/IL-1α, measured after 22 h of treatment

at a concentration of the chemicals that preserves 50% of cell viability, is proposed to easily differentiate irritant from sensitizers. Intracellular signalling pathways were analysed by Western blot analysis in order to investigate potential involvement of signalling in the differential release of interleukins. Our current data illustrate important differences in EGFR, p38 and ERK1/2 MAPKs activation sustained with sensitizing treatments. Exposure of RhE to specific inhibitors of EGFR, p38 and ERK1/2 kinase activities suggest that the activation of EGF receptor only is significantly involved in the induction of IL-8 release after treatment with sensitizers. The potential involvement of H_2O_2 as an intermediate determining the responses of RhE towards sensitizers was also investigated. Using the anti-oxidant agent N-acetyl cysteine as well as performing intracellular measurement of ROS, results were obtained that reveal strong evidence of oxidative stress in events triggering IL-8 release after the contact of the epidermis with the DNFB sensitizer. The toxicity Keap1-Nrf2-ARE pathway was also investigated in response to a panel of sensitizers and irritant. Indeed, the transcription factor Nrf2 is responsive to oxidative stress and induces expression of antioxidant genes that attenuate tissue injury. Furthermore the expression of IL-8 is under the control of Nrf2 in multiple cell types (Zhang et al., 2005). Therefore, expression of Nrf2-dependent ARE-responsive genes, such as HMXO, NQO1 and GCS, was measured using q-PCR and the activity of Nrf2 transcription factor was evaluated with TransAM tests.

Although some clues already allow partial discrimination between irritant and sensitizing chemicals, more investigations are still required in order to refine the identification of involved cellular mechanisms that will permit rules for classification of chemicals. Behind the replacement of laboratory animals in the cutaneous toxicological evaluation of chemicals, the development of alternative methods is triggering refinement in the approach of toxicology at cell and tissue levels.

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Keywords: keratinocytes, reconstructed human epidermis (RHE), sensitization, cell responses

Poster

Challenges in cytotoxicity testing of nanoparticles

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The use of nano-sized materials (NMs) offers exciting new options in technical and medical applications. These materials, however, have been reported to cause adverse effects in cells and organs. Conventional testing is complicated by the fact that NMs may interfere with conventional assays, especially with the MTT assay. As many NMs are not degraded, they may, in addition to acute cytotoxicity, cause cell dysfunction by biopersistance. These effects cannot be evaluated in the set-up used for conventional cytotoxicity testing. Potential effects of long-term exposure may include basic cytotoxicity and dysfunction of specific organelles or of specific cell functions. As most NMs are taken up by the endosomal-lysosomal system, changes in lysosomal function may occur in cells upon chronic exposure to NMs.

By testing various types of nanoparticles produced in two national and international consortia we evaluated a panel of cytotoxicity assays (WST-1, MTT, MTS, Neutral Red, TOX-6, leucine incorporation and ATP content) for potential assay interference. Additional controls, for interference with the cells and for interference with the assay compounds, were added to the conventional set-up to identify false positive or false negative results. For the assessment of chronic effects fluorescently labelled polystyrene particles (FluoSpheres®) in sizes of 20 nm and 200 nm were used. The influence of NMs on the endosomal-lysosomal system was studied by pH-dependent dyes, by detection of cathepsin B activity and of lysosomal sulphatase activity and by exposure of LAMP-1-RFP transfected cells. These experiments were performed in serum-reduced medium to prevent dilution of the NMs by cell proliferation.

Formation of aggregates occurred quite frequently. The correlation of cytotoxic effects to the size of the primary particles may possibly not be correct. The inclusion of additional controls helped to identify interference of NMs with the assay. Most NMs, however, did not interact with the assays. For these NMs inter-assay differences of the IC_{50} concentrations were low. Although the assays used different detection principles and read-outs only small variations in the IC_{50} concentrations were noted. MTT and TOX-6 assay were the only assays suited to detect cytotoxicity of high concentrations of floating, coloured NMs.

Comparison between the different endosomal-lysosomal stains showed that more acidic and LAMP-1 positive than cathepsin B- and lysosomal sulphatase-positive organelles were seen. 20 nm and 200 nm FluoSpheres® were taken up with different velocity and stored at different locations in the cell. They co-localised mainly, but not exclusively, with markers of the endosomal-lysosomal compartment. FluoSpheres® were detected inside the cells without obvious loss of intensity for the entire observation period of one week. Exposure of cells to 20 nm CPS for seven days resulted in a decrease of cathepsin B staining.

In conclusion, the inclusion of additional controls for cytotoxicity testing of NMs is recommended. Detection of lysosomal activity may be a model to address chronic cellular effects of non-biodegradable NMs.

Keywords: nano-sized materials, cytotoxicity testing

Poster

Is gene expression profiling a useful tool for assessing the influence of ultrafine particles in *in vitro* tests?

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The New Energy and Industrial Technology Development Organization (NEDO), Japan started a 5-year project in the fiscal year 2006 entitled "Research and development of nanoparticle characterization methods". The goals are 1) to establish preparation methods of test samples, to develop methods to measure the shape and size of tested nanomaterials (NMs), to test their toxicity, analyse exposure and to publish the results obtained in the form of manuals; 2) to carry out the risk assessment of typical NMs, propose a risk management policy on the basis of the risk assessments and formulate the risk assessment documents.

In order to estimate the biological effects of ultrafine particles, an optimal *in vitro* assay system using well-characterised dispersions was developed (Kato et al., 2009; Kato et al., 2010). We have examined cellular responses of about 30 kinds of ultrafine metal oxide particles, such as TiO_2, ZnO, NiO, CeO_2, SiO_2, and Fe_2O_3, etc. (Horie et al., 2009a, 2009b). Moreover, cellular responses induced by C60 fullerenes, carbon nanotubes (CNTs) and ultrafine metals are being examined.

In this study, we utilised the DNA microarray analysis technique to determine the gene expression profiles of human keratinocyte HaCaT cells exposed to anatase titanium dioxide (TiO_2) particles of different (7 nm, 20 nm and 200 nm) average diameters without illumination (Fujita et al., 2009). Cells were incubated for 24 h with TiO_2 particles, which were dispersed in the culture medium and size-fractionated such that the concentration of titanium in all the fractionated samples was nearly equivalent. According to the cluster analysis, only genes involved in the "inflammatory response" and "cell adhesion", but not the genes involved in "oxidative stress" and "apoptosis", were over-represented among the genes that were up-regulated in HaCaT cells. After 24 h exposure to ultrafine 7 nm TiO_2 particles, we observed altered expression levels of genes involved in matrix metalloproteinase activity (MMP-9 and MMP-10) and cell adhesion (fibronectin FN-1, integrin ITGB-6, and mucin MUC-4). These results suggest that the ultrafine TiO_2 particles without illumination have no significant impact on ROS-associated oxidative damage, but affect cell-matrix adhesion in keratinocytes for extracellular matrix remodelling.

We suggest that assessing *in vitro* cellular responses to molecular events is an effective means to elucidate the toxicological behaviour of ultrafine particles.

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Keywords: ultrafine particles, gene expression, DNA microarray, titanium dioxide, keratinocytes, matrix metalloproteinases

Lecture in Session VII: Nanotoxicology

The role of alternative test methods in nanotoxicology

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The OECD WPMN has established a steering group on Alternative Methods in Nanotoxicology with the objectives of developing testing strategies and identifying suitable methods for the hazard assessment of pristine manufactured nanomaterials (MN). So far two main areas of concern related to the use of MN have been discussed, i.e. possible translocation across cellular barriers and proinflammatory effects. Currently, effects on potential target cell types are investigated with an emphasis on cytotoxicity, genotoxicity, immunotoxicity and cell proliferation. However, test methods are far from being validated and harmonised. With respect to the adaptation of existing alternative methods to the field of MN, the physico-chemical characterisation of the test item and test medium, harmonisation of relevant cell models and control against well characterised reference materials are indispensable criteria for the generation of dose response relationships that are relevant for risk characterisation. In addition to toxicological test method development, there is a need for the establishment of analytical test procedures to enable characterisation of MN in products *in situ*.

There is a need for concerted actions, especially in the field of cosmetics safety testing with respect to identification and quantification of MN in cosmetics by 2014 according to the new cosmetics regulation; a list of MN in cosmetics shall be made publicly available by the European Commission. Special concern might be raised in case of a foreseeable or intended use of sprays containing MN. Translocation through the lung is more likely than through the healthy skin. Thus, lung model development might become an area of increased relevance for the investigation of local cellular effects and translocation likelihood of MN upon inhalative exposure.

Keywords: testing strategies, alternative methods, translocation of nanomaterials, toxic concerns

Poster

Androgenic properties of Halowax 1051 in ovarian follicles

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Polychlorinated naphthalenes (PCNs) are a group of substances containing a naphthalene ring system. PCNs have been extensively produced and used as mixtures with various commercial names like Halowax, Nibren Waxes, Seekay Wakes. They were used mainly for cable insulation, wood preservation but also as engine oil additives and feedstock for dye production and capacitors and refracting index testing oils. Today, PCNs are regarded as ubiquitous environmental contaminants and are classified as priority pollutants for consideration in several countries. To our knowledge there are no data concerning the action of Halowax as an endocrine disruptors in the ovary. The aim of the presented data was to show dose and time action of Halowax on follicular cell steroid secretion.

Ovarian follicles (5-7 mm in diameter) collected from cycling pigs were pressed down and separated from connective tissue. Whole follicles were cut with small scissors and placed individually in 24-well plates (Nunc) with 2 ml Parker medium supplemented with 5% FBS/CD. To examine the influence of dose dependent action of Halowax 1051 on steroid secretion, diced-up follicular wall including theca and granulosa, without follicular fluid, was incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 with 1, 10, 100, 1000 and 10,000 ng/ml Halowax 1051 for 24, 48 and 72 h. After incubation, the conditioned media were collected for determination of steroids levels. Progesterone (P4), testosterone (T) and estradiol (E2) levels were determined by EIA using a commercially available ELISA kit (DRG Diagnostic, GmbH, Germany).

An inhibitory action on P4 and E2 secretion parallel with stimulatory action on T secretion was observed from 24 until 72 h of incubation.

Data on the toxicity of PCNs in experimental animals are very rare. Åkerblom et al. (2000) showed that PCNs had negative

effects on ovaries of Baltic salmon, including delayed development. Moreover they indicated that PCNs are not capable of causing effects similar to E2. This indirectly confirms our observations of an inhibitory effect on estradiol secretion. Additionally, taking into consideration that the toxic responses associated with PCNs are mediated through an aryl hydrocarbon receptor (AhR)-dependent mechanism of action (Poland and Knutson, 1982; Villeneuve et al., 2000), an antiestrogenic effect of PCN could be expected. We observed previously, that TCDD added to testosterone supplemented cultures additionally increased testosterone secretion with a concomitant decrease in estradiol secretion (Gregoraszczuk, 2002). Omura et al. (2000), examining the semen quality of males exposed during their embryonic life to 1,2,3,4,6,7-hexachloronaftalen, showed accelerated spermatogenesis in postnatal development. This confirms the androgenic effects PCNs.

Taken together, our findings for the first time show PCNs acting as endocrine disruptors in the ovary. A decrease of the P4: T and E2: T ratio parallel with a statistically significant stimulatory action on testosterone secretion suggests androgenic properties of PCNs. As a consequence, PCNs exposure should be taken into consideration as an additional factor responsible for polycystic ovarian syndrome (PCOS).

Keywords: Halowax 1051, ovarian follicles, steroid secretion

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Lecture in Session Go3R

Go3R – intelligent internet searches for alternatives to animal testing

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EU Directive 86/609/EEC for the protection of laboratory animals obliges scientists to consider whether a planned animal experiment can be replaced, reduced or refined. To meet this regulatory obligation, scientists must consult the relevant scientific literature prior to any experimental study using laboratory animals. Several million potentially 3Rs relevant documents are spread over the World Wide Web, biomedical literature and patent databases. Accessing this information simultaneously in a fast and exhaustive manner is still impossible with traditional search technologies. This is where semantic web technologies may offer solutions.

Go3R, the first knowledge-based search engine for alternative methods to animal experiments, was developed to enable scientists and regulatory authorities involved in the planning, authorisation and performance of animal experiments to determine the availability of 3Rs relevant information from PubMed in a fast and comprehensive manner.

Knowledge-based search engines integrate human expert domain knowledge into retrieval procedures. The technical basis of Go3R is specific 3Rs expert knowledge captured within a 3Rs specific ontology. This ontology is a hierarchically structured network of terms, such as toxicological endpoints, 3Rs methods and cell lines.

When a user performs a search query with Go3R, the search engine expands this request using the ontology's structure and terminology, which has been previously used to index the documents. It highlights relevant terms or their synonyms that occur in both and uses them to arrange the documents within an "intelligent" directory of contents. The directory is presented alongside the search results and serves as a convenient means of navigation.

The Go3R beta-version is available online free of charge (www.Go3R.org). This beta-version right now contains no less than 17,000 3R-relevant terms structured in 26 branches with biomedical headings, e.g. "Cell Culture Technology", "3Rs Methods in the Life Sciences" and others. Scientific documents that mention one of these terms or a synonym are automatically assigned to the respective branch.

Go3R was developed to allow retrieval of 3Rs relevant documents, even if they do not explicitly mention a 3Rs-relevant term. This will be achieved by a technique called "indirect mapping". Here, related terms pointing to a certain topic without mentioning it are exploited for the assignment, e.g. the combination of "3T3-cells", "neutral red", "irradiation", for assignment to the term "3Rs in phototoxicity testing".

Thanks to funding by the German Federal Ministry of Education and Research (BMBF), the search engine will be re-engineered and extended to meet the requirements of scientists and competent authorities even better. An essential extension will be the additional inclusion of scientific resources other than PubMed in the near future. The ultimate aim, however, is to open up resources that right now are non-accessible to an indispensability search due to their non-indexed and/or unstructured nature.

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Keywords: 3Rs principle, replacement, reduction, refinement, information retrieval, literature search, animal testing alternatives, semantic search technology, ontology

Poster

Concentration or dose: What is the proper measure for quantitative *in vitro-in vivo* extrapolation of toxic potency?

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Toxicological hazard assessment is largely based on exposure-toxicity-data obtained in animal experiments. Depending on the route of administration, the measure of exposure is either the dose administered or the concentration of the chemical in the ambient air or water. If *in vitro* data shall be used for toxicological hazard assessment, *in vitro* toxic exposures have to be converted into external exposures of animals or men. To this end, the biokinetics of the substance must be taken into account. This may be done by using biokinetic modelling relating internal exposure of cells and tissues to external exposure of the body. An important question is: What measure of exposure of cells *in vitro* is the proper one to be used for *in vitro-in vivo* extrapolation?

The exposure of cells *in vitro*, in general, is characterised by the nominal concentration of the test substance in the incubation medium. Based on experimental evidence on cellular and extracellular binding of chemicals, we have recently argued that the exposure measure suitable for use for *in vitro-in vivo* extrapolation is the free concentration in the medium (Gülden and Seibert, 2006). We have developed an equilibrium distribution model which, based on substance and system specific data relevant for distribution, can be used to estimate the free from the nominal effective concentration of lipophilic and albumin bound compounds, respectively (Gülden and Seibert, 2003; Gülden and Seibert, 2005). These considerations and models were explicitly confined to chemical compounds not markedly eliminated from the *in vitro* systems during the exposure time.

Using C6 astroglioma cells and the example of hydrogen peroxide (H_2O_2) we have investigated how exposure *in vitro* can be characterised if the substances are metabolised by the exposed cells. C6 cells rapidly eliminate H_2O_2 from the culture medium, i.e. the nominal concentrations of administered H_2O_2 are initial concentrations that decline continuously. The elimination rate depends on both the H_2O_2 and the cell concentration. The nominal cytotoxic concentration (EC_{50}) of H_2O_2 is likewise cell concentration dependent. Exposure to changing concentrations of H_2O_2 can be quantified by the area under the concentration versus time curve (AUC). While the EC_{50} varies with the cell concentration, the corresponding AUC_{50} is almost constant. From the relation between EC_{50} values and cell concentration a cytotoxic cell dose (ED_{50}) can be derived. This dose is in-

dependent of the cell concentration. These results indicate that the cell dose or the AUC but not the nominal concentration are suited to quantify the exposure of cells *in vitro* towards chemicals eliminated from the *in vitro* system by the cells themselves. When the cytotoxic cell dose of H_2O_2 is used to estimate an equivalent body dose, the result is very close to the LD_{50} values determined with rodents.

This and the previous investigations let us conclude that the proper measure of exposure of cells *in vitro* to be used for *in vitro-in vivo* extrapolation depends on the fate of the substance in the *in vitro* test system and can be either the free concentration, the AUC or the cell dose.

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Keywords: in vitro-in vivo extrapolation, cell dose, in vitro dosimetry, free concentration, AUC in vitro

Poster

Cytotoxicity of beauvericin and fusaproliferin mycotoxins in Caco-2, HT-29 and Hep-G2 cells

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Mycotoxins are naturally occurring secondary metabolites produced by the mycelial structures of either toxigenic or non-toxigenic filamentous fungi. Beauvericin (BEA) is a depsipeptide that was first identified in a soil-borne entomopathogenic (insect-pathogenic) fungus, *Beauveria bassiana*, which induces significant cell death in insect, murine and human tumour cell lines. Furthermore, BEA is a potent and specific cholesterol acyltransferase inhibitor in rat liver microsomes. Fusaproliferin (FUS) is a recently described mycotoxin that is produced by two closely related species, *Fusarium subglutinans* and *F. proliferatum*. Both species are important pathogens of maize and other economically important crop plants and may be isolated together from the same plant. FUS is toxic to *Artemia salina*, IARC/LCL 171 human B lymphocytes and SF-9 insect cells and has teratogenic and pathogenic effects on chicken embryos. Recently, the occurrence of FUS in several naturally contaminated samples of corn was reported.

Considering the few toxicological studies executed on these new classes of contaminants, the aim of this study was to investigate to what extent the toxicity of BEA and FUS are involved in their effects on cell proliferation and viability. These parameters were determined using human epithelial colorectal adenocarcinoma (Caco-2) cells, human colon adenocarcinoma grade II (HT-29) cells and human hepatocellular liver carcinoma (Hep-G2) cells. Human colorectal and hepatocellular cells were chosen because the intestine should be considered as a target of toxins as it is in contact with them when oral exposure occurs. The viability and proliferation of cells after 24 and 48 h of incubation were measured using the MTT assay. BEA and FUS concentrations from 0.6 to 30 μM were tested.

Results obtained show that only HT-29 and Caco-2 cells, but not Hep-G2 cells, reduced cell viability after BEA exposure. The DL_{50} of the BEA was 15.0 μM on the HT-29 cells and 20.6 μM on the Caco-2 cells at 24 h exposure. However, the 48 h exposure showed more sensitivity of these cell lines. The DL_{50} values were 9.7 and 12.7 μM for HT-29 and Caco-2 cells, respectively. The FUS mycotoxin was not cytotoxic in the three cell lines utilised in this study, neither over the range of concentrations tested nor at the different exposure times.

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Keywords: cytotoxicity, beauvericin, fusaproliferin, mycotoxins, in vitro assays

Poster

Effects of valproic acid (VPA) and leviteracetam (LEV) on human choriocarcinoma cell line BeWo proliferation, apoptosis and hormone secretion

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Maternal antiepileptic drug use is associated with an increased frequency of adverse pregnancy outcome including congenital malformations. Sodium valproate (VPA) is the most widely prescribed antiepileptic drug in the world (Perucca, 2002). Levetiracetam (LEV) is a relatively new antiepileptic drug that has seen extensive use during recent years. The current study, using a human choriocarcinoma cell line (BeWo), was performed to assess the effects of long time exposure to VPA and LEV upon cell proliferation, apoptosis and hormone production (progesterone, estradiol and of human chorionic gonadotropin).

BeWo human choriocarcinoma cells (ATCC, Manassas, VA, USA) were treated with different concentrations of VPA (0.45, 0.6, 1.5 or 2 mM) or LEV (0.07, 0.12, 0.3 or 0.5 mM) for 24, 48, 72 or 96 h. DNA synthesis in cells was determined by measuring BrdU incorporation with the Cell Proliferation ELISA System (Roche Molecular Biochemicals, Mannheim, Germany). Caspase-3 activity was measured as described previously by Nicholson et al. (1995) using the fluorescent substrate Ac-DEVD-AMC. The concentrations of progesterone (P4), 17β-estradiol (E2) and human chorionic gonadotrophin (hCG) in the media were determined using commercial ELISA kits (DRG Diagnostic, Germany).

In VPA treated cells, no effect of small doses but inhibitory action on cell proliferation of high doses parallel with stimulatory action on cell apoptosis was observed. It was accompanied by a reduction in the secretion of HCG, E2 and P4. In LEV exposed cells no effect on cell proliferation but an inhibitory action of small doses on cell apoptosis only after 96 h of exposition was noted. P4 production of LEV exposed cells was not affected, but a decrease in β-hCG and E2 secretion was also observed.

In recent years it has become clear that apoptosis is a normal physiological process throughout gestation (Huppertz and Kingdom, 2004) and disturbances in programmed cell death in placenta seem to be associated with an abnormal pregnancy outcome (Jerzak and Bischof, 2000). Decreased proliferation and abnormal apoptosis noted in VPA exposed cells could lead to faulty placentation, involved in several gestational disorders including preeclampsia (Allaire et al., 2000; Crocker et al., 2003). Inhibition of P4 and E2 secretion may help explain a high rate of spontaneous abortions and premature births following VPA exposure in pregnancy (Mawer et al., 2002; Pittschieler et al., 2008). Progesterone plays a physiologic role in trophoblast differentiation (Malassiné and Cronier, 2002), while estrogens have important physiological roles in regulating functional differentiation of the placental villous trophoblast (Bukovsky et al., 2003; Cronier et al., 1999; Rama et al., 2004). The low hCG levels probably reflect a derangement of trophoblast function that will culminate in placental insufficiency and fetal growth restriction (FGR). Taking into consideration direct inhibition of cell proliferation and hormone secretion parallel with apoptotic action suggest that exposure to therapeutic doses of VPA during early pregnancy should be approached with caution. Trophoblast cells seem to be less sensitive to LEV, however a further study with placental tissue is necessary to decide whether the use of LEV during pregnancy is safe.

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Keywords: antiepileptic drugs, BeWo placenta cell line, hormone secretion, proliferation and apoptosis

Poster

The human bronchial epithelial cell line Calu-3 as *in vitro* model of the human airway epithelial barrier to study the transepithelial transport of insulin

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Over the past decades, increasing research efforts have been made to address the administration of therapeutic peptides and proteins via the pulmonary route. Validated *in vitro* models of the respiratory tract allowing for the assessment of drug transport and local toxicity are thus urgently needed. The Calu-3 cell line is a well established cell culture model for drug transport and absorption studies (Grainger et al., 2006). Calu-3 cells produce features of differentiated human airway epithelial cells, such as formation of tight junctions and Cl- secretion (Wan et al., 2000; Ilek et al., 2008). Insulin was chosen as a water soluble model peptide. The aim of the present study was to compare the effect of liquid covered culture (LCC) and air interfaced culture (AIC) as well as different sampling methods on the transport of insulin across Calu-3 monolayers. Furthermore, the effects of pulmonary surfactant on the absorption of insulin were studied.

Calu-3 cells were grown on Transwell inserts with 0.4 μm pore size. Tight junction integrity was checked by measuring the transepithelial electrical resistance (TEER) using an EVOM® epithelial voltohmmeter. Secretion of mucus-like substances was analysed by a lectin binding assay. Transport experiments were done on day 11-13 in culture. In the case of AIC cultures, application of insulin aerosols onto the air liquid interface of Calu-3 monolayers was done using a MicroSprayer™. The transport was analysed in the apical to basolateral direction under sink-conditions, i.e. the receiver compartment was exchanged at each time point, and with serial samplings. To investigate the effect of surfactant on insulin transport, Curosurf®, a natural porcine surfactant preparation, was added to the apical surface. Insulin was quantified in the receiver compartment by radioimmunoassay (RIA). Secretion of proinflammatory IL-8 was measured by ELISA.

Calu-3 cells cultured using LCC exhibited higher TEER values when compared to AIC conditions, the levels being 928 ±292 vs. 339 ±120 Ohm/cm² on day 11. Mucus secretion was approximately two-fold higher in AIC cultures (day 11). Cumulative insulin transport was considerably higher under AIC conditions. The transport kinetics proved to be highly dependent on culture conditions and the sampling regime. Linear transport was only observed in LCC cultures using serial sampling. Addition of 15 mg/ml Curosurf® increased the cumulative insulin transport approximately nine-fold. This effect was obviously not caused by increased tight junction permeability, as judged by TEER measurements. IL-8 secretion was not influenced by insulin.

The Calu-3 cell line is a dynamic model and the culture conditions employed are critical to differentiation and transport properties. Sampling regime and culture conditions have thus to be tailored to the experimental question in order to produce meaningful results.

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Keywords: Calu-3, insulin, surfactant

Lecture in Session IX: New developments and novel methods

Lessons learned from alternative methods and their validation for a new toxicology in the 21st century

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Europe and the U.S. further the development of new toxicological tools in very different ways: While the replacement of animal tests has been promoted strongly in Europe over the last decades (following the 3Rs principles – reduce, replace, refine), in the U.S. the vision for a toxicology in the 21st century (Tox-21c), which was prompted by the National Research Council document only three years ago, dominates the discussion. In both cases, there is significant political support. However, while in Europe the horizontal animal welfare legislation from 1986 (which urges the use of 3Rs methods wherever possible) currently under revision and cosmetics and chemical legislation are the primary drivers, in the U.S. mainly federal agencies, most prominently the Environmental Protection Agency (EPA), made the implementation of the NRC report their toxicity testing strategy only in 2009. This pre-empts such likely legislative measures as the reauthorisation of the Toxic Substances Control Act (TSCA) in the U.S. The European implementation is characterised by substantial broad funding programs to develop 3Rs methods and can be termed a "bottom-up" approach; the Tox-21 programme, in contrast, represents a "top-down" approach, where programmed research is carried out and commissioned. The author argues here that the two approaches are two sides of the same coin. They can instruct and complement each other. More importantly, however, if brought together they can result in a Human Toxicology Project and a real revolution in regulatory toxicology.

Keywords: toxicology, regulation, chemicals

Poster: Cross-cutting issues

A tiered approach combining the short time exposure (STE) test and the bovine corneal opacity and permeability (BCOP) assay for predicting eye irritation potential of chemicals

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For the assessment of ocular irritation, one *in vitro* alternative test may not completely replace the Draize test. Therefore, a tiered approach combining several *in vitro* assays, including cytotoxicity assays, is proposed in order to estimate the irritation potential for a wide range of chemical classes. The Short Time Exposure (STE) test, an alternative eye irritation test, involves exposing SIRC (rabbit corneal cell line) cells for 5 min to two concentrations (5% and 0.05%) of test material. In the present study, we examined the predictive potential of a tiered approach analysing the results from the STE test and then the results of the BCOP assay for assessing GHS eye irritation rankings.

Over 50 chemicals listed in the BCOP background review document (BCOP BRD, ICCVAM-NICEATM, 2006) were selected for the present study. The GHS rankings non-irritant (NI) category 2 (Cat. 2) or category 1 (Cat. 1) for the chemicals were evaluated in the STE test as described by Takahashi et al., 2008. In addition, the overall classification of the GHS rankings was also evaluated using the combined results of the STE test and the BCOP assay. A BCOP assay was not repeated for the 50 chemicals in this study. Instead, the data for the BCOP classification and GHS eye irritation rankings of NI, Cat. 2 or Cat. 1 were adopted from the BCOP BRD. To determine the GHS classification using the tiered approach, the results of the STE test were reviewed first. If the chemical was classified as a "non-irritant" in the STE test, the chemical was considered to have a GHS ranking of NI. If the chemical was classified as an "irritant", the classification for this chemical was subsequently confirmed by reviewing the BCOP BRD data as to whether the

chemical was a non-severe or severe irritant. If the BCOP classification was "severe", the chemical was considered GHS Cat. 1. For those chemicals whose classifications were "non-severe", these were considered to be GHS Cat. 2.

The STE rankings of 1, 2 and 3 correlated well with the GHS rankings of NI, Cat.2, and Cat.1, respectively, for the chemicals evaluated with the STE test. Furthermore, the accuracy of predicting the GHS rankings was slightly improved when the tiered approach combination of STE test and BCOP assay was used. The improved accuracy was attributed both to the higher specificity of the STE test and the higher sensitivity of the BCOP assay after screening out the chemicals classified as NI by STE. Moreover, the underprediction rate was markedly-improved when this tiered approach was used. Therefore, the bottom-up approach combination of the STE test followed by the BCOP assay might improve the accuracy of predicting the eye irritation potential of chemicals *in vitro*. From these results, the tiered approach of combining the data analysis of the STE test and BCOP assay might be a promising alternative eye irritation test strategy.

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Keywords: STE, BCOP, GHS, tiered approach, eye irritation, alternative method

Poster

The EpiDerm-FT™ full-thickness *in vitro* human skin model: an animal alternative model for cutaneous wound healing research

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

Normal human epidermal keratinocytes (KC) and dermal fibroblasts (FB) were cultured to produce the highly differentiated full-thickness skin model. Small wounds of several mm diameter were induced in the epithelial model by means of a battery operated cauteriser or a dermal biopsy punch. Biopsy punches were used to produce wounds that removed only the epidermis. The wounded EpiDerm-FT™ cultures were fixed at various time points and H&E stained paraffin sections were prepared to evaluate the wound and the wound healing process. QPCR arrays were utilised to evaluate gene expression changes occurring during the wound healing process.

Immediately after burn wounding, necrotic epithelium and denatured collagen dermal matrix were evident. Within one day, the denatured collagen matrix began to degrade and epithelial KC were observed migrating inward from the wound edges. Over a time course of seven days, migrating KC repopulated the wounded area to form a fully covered epithelium. Dermal fibroblasts were also observed to be proliferating within the wound area and generating new dermal matrix material. These wounds also healed within a timeframe of 3-7 days. Increased FB proliferation in dermal areas directly adjacent to migrating KC was observed. Gene expression profiling of the wounded area showed temporally regulated increases in mRNA expression of basement membrane components, collagens and genes involved in extracellular matrix remodelling. FB proliferation and epidermal healing were severely impaired in the presence of an EGFR tyrosine kinase inhibitor or a TGFα neutralising antibody.

These results demonstrate that EpiDerm-FT™ is a useful animal alternative *in vitro* human skin model for investigating dermal-epidermal interactions during wound healing and evaluation of the role of specific growth factors or new therapeutics in the dermal wound healing process

Keywords: skin equivalent, wound healing

Poster

Development of a vapour cup dosing method for evaluation of chemical toxicity in the EpiAirway™ organotypic *in vitro* human airway model: an animal alternative model for inhalation toxicity evaluation

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Determination of airway irritation potential is an international regulatory requirement to ensure safe handling, packaging, labelling, use and transport of chemicals, cosmetics and household products. Recent REACH legislation has heightened the need for validated *in vitro* airway models for toxicity testing. *In vitro* determination of airway irritation and toxicity potential is problematic since traditional submerged monolayer cell culture systems are not amenable to *in vivo*-like vapour phase dosing. Organotypic air-liquid interface airway models more adequately reproduce *in vivo* morphology and are more amenable to vapour phase dosing since the apical surface is exposed to the atmosphere. However, simple and reliable dosing methods that are applicable to a broad range of chemicals and correlation of *in vitro* results to *in vivo* irritation/toxicity potential are still lacking. Here we describe a simple and reliable vapour cup method for *in vitro* dosing of the EpiAirway™ organotypic *in vitro* human culture model.

Liquid test chemical solutions are applied to a fibre pad contained within the vapour cup, and the cup is then inverted onto the tissue culture insert to form a sealed vapour phase exposure system. Dose response experiments are conducted to determine EC_{50} concentrations for toxicity responses, including loss of culture viability, loss of transepithelial electrical resistance or release of inflammatory mediators such as IL-8. EC_{50} concentrations were correlated with OSHA established Permissible Exposure Limits (PEL) and Immediately Dangerous to Life and Health (IDLH) concentrations.

Toxicity responses of the EpiAirway™ model to eight volatile chemicals including formaldehyde, acetic acid, allyl alcohol, ethyl formate, isoamyl alcohol, tetrachloroethylene, choroacetaldehyde and acetone were evaluated with the vapour cup method. A correlation coefficient of $r^2 = 0.8029$ was obtained.

Further testing and refinement of the EpiAirway™ vapour cup method may provide a useful method for *in vitro* airway toxicity/irritation determination.

Keywords: airway toxicity, airway irritation

Lecture in Session VIII: Reproductive toxicology and stem cells

Mouse embryonic stem cells in developmental neurotoxicity testing: aiming at higher throughput and specificity

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Testing for developmental neurotoxicity (DNT) is a key element in the toxicological portfolio of a compound and triggered DNT studies are recommended under REACH. This situation will considerably increase the number of laboratory animals used in chemical safety testing. Validated alternative methods for DNT testing are currently not available. Thus, standardised, predictive screens for the evaluation of DNT need to be established with the ultimate goal of increased efficiency in terms of reduced animal use and higher throughput compared to whole-animal testing using existing guidelines. Mouse embryonic stem cells (mESCs) are pluripotent cells with an unlimited capacity for differentiation and thus provide an attractive cellular system for

in vitro studies in developmental biology as well as toxicology. In a previous study we demonstrated that these cells differentiate efficiently into neural cells, including neural progenitors, neurons, astrocytes, oligodendrocytes and radial glial cells, and might have the potential to develop into functionally active networks. Therefore, mESCs offer the opportunity to study adverse effects on neural cell development after exposure to toxic compounds in an *in vitro* setting.

Murine ESCs (line D3) were differentiated into neural cell types. Proliferation in differentiating and undifferentiated stem cell cultures was determined using a BrdU-ELISA system with a chemiluminescence read-out. Cell viability was analysed as the ability of the cells to reduce resazurin into the fluorescent product resorufin. For quantification of neuron differentiation the expression of the neuron-specific marker protein beta-III tubulin was assessed in a new cell-based ELISA method, which we developed specifically for this purpose.

Recently, we developed an *in vitro* test method suitable to assess adverse effects of chemicals and other compounds on neural development. Molecular and mechanistic endpoints for differentiation and proliferation were successfully established. Furthermore, our model seemed applicable to detect a diverse group of positive and negative developmental neurotoxicants. However, it also turned out that the detection of specific neurodevelopmental effects depends on the exposure regimes used. Therefore different exposure regimes, along with the selected set of positive and negative developmental neurotoxicants, were evaluated. In this way we were able to establish the most specific and predictive endpoints for DNT testing in our model, clearly separating specific DNT effects from general cytotoxicity.

The mESC model represents a useful component in an *in vitro* testing strategy for developmental neurotoxicity. The tools described here provide the possibility for a higher throughput and for the detection of developmental neurotoxic effects with increased specificity, thus facilitating application in extensive chemical testing.

Keywords: in vitro test method, developmental neurotoxicity, embryonic stem cells, higher throughput, chemical testing

Lecture in Special Session on antibody production

Generation of antibodies *in vitro*: no living cells required

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Antibodies can be selected *in vitro* from a large DNA library using display technologies without animals. This is achieved by linking the genotype (DNA or mRNA) to phenotype (protein) in the display methods, which allows isolation of the genetic material via functional selection of the protein it encodes. The genetic material can then be used for further manipulation, such as DNA sequencing and expression. Repeating the process favours the enrichment of high affinity molecules, leading to isolation of improved molecules when DNA mutagenesis is used (Hoogenboom, 2005).

Ribosome display is an *in vitro* display technology which is widely used for antibody generation and optimisation. In ribosome display, individual antibodies (phenotype) are linked to their encoding mRNAs (genotype) through the stalling of translating ribosome in a cell-free system. The formation of antibody-ribosome-mRNA complexes permits simultaneous selection of the displayed antibody together with its corresponding mRNA, which is recovered as DNA by RT-PCR (He and Khan, 2005). As a cell-free system without cell transformation, ribosome display overcomes the size limitation encountered by *in vivo* display methods. Consequently, it screens larger libraries (>1012 members) and enables continuous search for newly-expanded diversity between display cycles. The latter feature provides an efficient route for *in vitro* protein evolution, generating novel antibody combining sites which are not possible by *in vivo* methods.

However, the current ribosome display procedure still requires *E. coli* expression after the selection for screening of desirable molecules. This laborious process often causes loss of selected antibody candidates. In order to bypass the bacterial cloning step and generate antibodies in a fully *in vitro* manner, we have developed an ultra-sensitive RT-PCR procedure, making it possible to amplify individual molecules from ribosome complexes as "PCR clones". Using a cell-free expression system, these "PCR clones" can be directly expressed for functional screening. By integrating these procedures, we are able to generate specific antibodies starting from a PCR library without use of any living cells.

Using progesterone as the antigen, we have shown that a panel of high affinity anti-progesterone antibodies were rapidly isolated from a large PCR antibody native library in two weeks through a single round of ribosome selection, followed by "*in vitro* PCR cloning" and cell-free expression. DNA sequencing has confirmed the identity of the *in vitro* cloned antibodies and biochemical analysis showed the successful isolation of antibodies highly specific to progesterone.

In summary, we have demonstrated a rapid procedure for generation of recombinant antibodies in a fully cell-free manner. The method offers advantages over existing techniques in terms of speed and efficiency. In addition, it can potentially be adapted for automation or a high throughput procedure for large scale production of antibodies. Although only antibodies were tested, this approach should be generally applicable for other proteins.

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Keywords: ribosome display, antibody generation in vitro, cell-free expression

Lecture in Session IX: New developments and novel methods

Evaluation of *in vitro* models for investigation of endocrine disruptive effects on insulin metabolism using omics

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Recently it has been suggested that besides reproduction and thyroid function, also other pathways of the endocrine system could be affected by environmental pollutants. Compounds that affect the endocrine regulating systems of the energy metabolism could cause fundamental problems (e.g. diabetes, obesity, wasting syndrome, etc.) and thus could have profound consequences at higher levels of biological organisation and species survival.

In this study we evaluate effects of environmental pollutants on insulin metabolism, which is central in energy homeostasis regulation. Epidemiological studies have shown that compounds such as phthalates (Stahlhut et al., 2007), perfluoroalkyl chemicals (Lin et al., 2009), dioxin-like compounds (Remillard and Bunce, 2002), brominated flame retardants (Lim et al., 2008), etc. could be correlated to the prevalence of diabetes and other metabolic diseases. Since these toxicants are ubiquitously present and tend to accumulate in humans and because toxicity data are often missing or incomplete, these compounds are studied with regard to their effect on insulin secretion (of pancreas β-cells) and insulin signalling/insulin sensitivity (in hepatocytes).

In practice, these environmental pollutants are first screened for their ability to disrupt insulin secretion using the INS-1 832/13 pancreas β-cell model. Secondly, after characterisation of dose-response and acute/chronic exposure effects on insulin secretion and cell growth stimulating effects using flow cytometric cell cycle analysis, the most potent congeners are selected. Thereafter, these compounds are investigated with microarray analysis to unravel the mechanisms underlying the observed effects. Thirdly, these results are compared to expression profiles obtained from similar exposure studies in primary pancreatic β-cells.

To evaluate if the selected congeners are able to interfere not only with insulin secretion, but also with insulin signalling, a hepatocyte cell line (H4IIE) previously shown to have a representative insulin response is exposed. Using physiological endpoints such as cell growth (flow cytometric cell cycle analysis), gluconeogenesis (repressed by insulin in H4IIE cells) and gene expression (real-time PCR analysis) of marker genes for insulin action, the potency of compounds to interfere with the response of hepatocytes to insulin is estimated.

This study evaluates the potential of pollutants to interfere with insulin metabolism. Because changes in insulin secretion and decreased insulin sensitivity resulting in insulin resistance is central in the pathology not only of diabetes, but also obesity, metabolic syndrome, etc., it is clear that evaluation of both effects on insulin secretion and insulin signalling are important steps towards a better understanding of the epidemiologically made correlation between environmental pollutants and metabolic diseases.

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Keywords: endocrine disruption, insulin, pancreas, microarray, energy metabolism

Poster

Comparison of capability of three different cell types to assess effects of chemicals on the human reproductive system

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Our aim is to develop a rapid *in vitro* method to assess the effects of chemicals on the human reproductive system, including potential endocrine disrupting compounds. For that we compared the applicability of three different cell types, focusing on both estrogen receptor mediated effects and steroidogenesis. A human cell based model has a great advantage over animal cell based models because species-specific differences can be avoided.

Three cell lines, H295R, KGN and MSF-7, were chosen to compare the effects of known chemicals. H295R is a human adreno-carcinoma cell line which expresses estrogen receptor α (ERα) and β (ERβ) and all the enzymes of the steroidogenic pathway. KGN is an ovarian granulosa carcinoma cell line which expresses mainly ERβ and is considered a useful model for exploring the regulation of steroidogenesis. The results from these two cell lines will be compared to literature data from the widely used MCF-7 cell line. The cells were exposed to well known estrogenic compounds ethinyl-estradiol (EE2), bisphenol-A, genistein and 4-nonylphenol. The selected endpoints were cell viability, proliferation as well as regulation of steroidogenesis and gene expression.

Results received so far from H295R and KGN cell tests showed only a slight increase in steroidogenesis related gene expression levels after exposure to EE2. ERα and ERβ gene expression levels were equal or below those of the reference gene in both test systems after exposure to EE2 concentrations that did not affect cell proliferation or viability. The study is ongoing.

Keywords: in vitro test, endocrine disrupters, alternatives, reproductive toxicity

Poster

Investigations towards enhanced understanding of sensitization of chemicals: implementation of phase I and II metabolism by coupling THP-1 activation with keratinocyte metabolism

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The development of alternative *in vitro* sensitization assays is necessary since European legislation requires reduction/replacement of animal testing wherever possible. Currently it is widely assumed that a multi-component tiered testing strategy is needed in order to address the key stages of the immunological mechanisms underlying the induction of skin sensitization. For some low-molecular weight chemicals (i.e. prohaptens) metabolic activation is needed to react with proteins as an initial step in skin sensitization. This metabolic activation step has been integrated in a cell-free protein binding (peptide reactivity) assay by using an enzymatic oxidation system. However, screening assays which consider phase I and II metabolism based on cell lines are still missing. Therefore, we are evaluating the metabolic competence relevant for skin sensitization in a co-culture system with primary antigen presenting cells and keratinocytes using the cell lines THP-1 and HaCaT.

Preliminary results indicate that after exposure of HaCaT cells to inducers of phase I and II enzymes they were able to bioactivate *per se* non-reactive chemicals. Thus, HaCaT cells are considered suitable for displaying the metabolic part in a cell-based co-culture assay for the prediction of the sensitization potential of prohaptens.

Keywords: coculture, keratinocyte metabolism, prohapten, skin sensitization

Poster

Comparative investigation of phototoxicity of chemical compounds using the standard BALB 3T3 NRU phototoxicity test and a three-dimensional human skin model (EST 1000)

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Where substances are intended for use in personal care products applied to the skin, an assessment of potential phototoxic hazard is required. The initial test is the measurement of a UV/visible absorption spectrum to identify absorption at relevant wavelengths, followed by an *in vitro* assay for phototoxicity, the Balb 3T3 neutral red uptake phototoxicity test (OECD 432). However, this test has its limitations, as non-hydrosoluble chemicals can be tested only at low concentrations, owing to their lack of aqueous solubility, and also many complex mixtures or formulations cannot be tested. Consequently, it does not take into account the bioavailability of test chemicals topically applied to skin and in many cases e.g. after having a phototoxic effect in this test system, such information is required.

To overcome these limitations, the use of reconstructed skin models is an interesting alternative and a useful follow-up test. In the present study the EST1000 skin model (Cell Systems, Germany) has shown its ability to correctly classify standard controls for phototoxic investigations, such as chlorpromazine,

and showed a remarkable resistance to UV radiation. Following this characterisation, different chemicals, selected based on their results in the NRU phototoxicity test and their bioavailability to skin, were investigated in the human skin model.

The obtained data indicate that the EST1000 model is a useful model for the prediction of phototoxicity and also useful as a follow-up test.

Keywords: phototoxicity, in vitro models, new methods

Lecture in Session III: Skin sensitisation and eye irritation

Assessment of chemical skin sensitizing potency by an *in vitro* assay based on human dendritic cells

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The skin sensitizing potential of chemicals is an important concern for public health and thus a significant endpoint in the hazard identification process. However, skin sensitization is not an all-or-none phenomenon and up to now the assessment of relative potency can only be derived using the *in vivo* local lymph node assay (LLNA). In this presentation we analyse the feasibility to predict the sensitizing potency, i.e. the LLNA EC3 values, of 15 skin sensitizers using *in vitro* data from our gene expression assay based on $CD34^+$ progenitor-derived dendritic cells (VITOSENS®). Hereto, we extended the gene expression dataset by an additional source of information, the concentration of the compound that causes 20% cell damage (IC_{20}). We applied a robust linear regression with both IC_{20} and expression changes of the genes CREM and CCR2 as explanatory variables. For 13 out of 15 compounds, a high linear correlation was established between the *in vitro* model and the LLNA EC3 values. The correlation holds over a range of 4 orders of magnitude, i.e. from weak to extreme sensitizers, and shows that *in vitro* data can be important in alternative potency prediction.

Reference

Lambrechts, N., Vanheel, H., Nelissen, I. et al. (2010). Assessment of chemical skin sensitizing potency by an in vitro assay based on human dendritic cells. *Toxicological Sciences*, accepted for publication.

Keywords: skin sensitizing potency, VITOSENS®, LLNA

Poster

Optimisation and application with human skin explants as an *in vitro* alternative to animal testing for skin care

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The development of *in vitro* model systems for the skin is essential for basic and applied research in skin care owing to the increased awareness of the 3Rs concepts and the European Community ban on animal testing for cosmetics. Cultured human skin explants have been used for epidermal biology and for cancer research for more than 50 years. However, many of them have poor dermal metabolic activity and their tissue architecture is compromised. The goal of our studies was to develop and validate an *in vitro* model system that best represents the physiological complexity of skin with the structural integrity of the epidermis, dermis and subcutaneous fat layers, and their metabolic activities.

Full-thickness human skin biopsies, obtained with informed consent from healthy donors undergoing abdominal surgery,

were cultured under various culture parameters to identify optimised conditions for skin explants culture. We developed human skin explants culture of all three compartments with structural integrity and metabolic activity and utilised it for studying skin biology and for evaluating dermatological agents for biological activities via both systemic and topical treatments. We have identified biomarkers for epidermal, dermal and subcutaneous adipose functions that enable a quick but thorough evaluation of the effects of agents on pigmentation, skin aging, and lipid metabolism. The skin explants system was validated using known cosmetic, skin care active agents.

In summary, a skin explants system that is viable and metabolically active was established to improve our understanding of mechanistic skin biology and to predict the efficacy of agents.

Keywords: human skin explants, in vitro alternative, cosmetics, efficacy testing

Poster

Permeability of enrofloxacin across the *in vitro* Caco-2 intestinal barrier model

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Following parenteral administration of fluoroquinolones, significant quantities of these antibiotics are found in the intestinal lumen. Although many studies described an active elimination of human use fluoroquinolones, few experiments studied the intestinal elimination of veterinary use fluoroquinolones. The objective of this study was to determine the mechanisms responsible for the trans-epithelial elimination of the enrofloxacin, a veterinary fluoroquinolone. We conducted pharmacokinetic studies on the *in vitro* intestinal barrier model, the Caco-2 monolayer. This model is commonly used to mimic the intestinal epithelial barrier in drug research centres.

Caco-2 cells were seeded in 12-well insert plates and, after 21 days confluence, bidirectional (apical to basal and basal to apical) transport experiments were conducted. Eleven concentrations of enrofloxacin (5, 10, 25, 50, 100, 250, 500 μM and 1, 2.5, 5 and 10 mM) were tested for 90 minutes. Enrofloxacin concentrations in both compartments were quantified by HPLC-UV. For both transport experiments, apparent permeability for enrofloxacin was determined as follows: Papp = (V x dC)/(dt x A x C0). Therefore, the apparent basal to apical permeability/apparent apical to basal permeability ratio was calculated (Pappb-a/Pappa-b).

The enrofloxacin concentration in the donor compartment significantly ($P < 0.001$) affected the Pappb-a/Pappa-b ratio. Indeed, compared to the lowest concentration (5 μM), the ratio was 50% ($P < 0.05$) lower at the highest concentration (10 mM). This ratio was also significantly greater than 1 ($P < 0.05$) for concentrations of 5 and 10 μM (45% and 34% respectively), whereas it was significantly lower than 1 ($P < 0.05$) for concentrations ranging from 50 μM to 10 mM, except for 250 and 500 μM concentrations. These results indicated a greater apparent basal to apical permeability (Pappb-a) compared to the apparent apical to basal permeability (Pappa-b) at low concentrations; this relationship reversed at the highest concentrations.

The differences observed being low, we could not conclude an active elimination. The intestinal elimination of enrofloxacin may mainly be due to passive diffusion, a fast saturable active transport mechanism may also exist, but with low impact.

Keywords: enrofloxacin, Caco-2 monolayer, apparent permeability

Poster

Effects of microcystin LR on the integrity of the *in vitro* Caco-2 intestinal barrier model

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Cyanobacteria are freshwater prokaryotic microorganisms among which some species have been recorded to produce toxins called cyanotoxins. Oral ingestion of contaminated food and water is a public health concern for humans. In this case, the intestine is one of the first organs exposed to cyanotoxins. The cyanotoxin microcystin LR (MC-LR) is known to be the most toxic variant of the microcystin family in rodents. However, information on its effects on the intestinal epithelium is limited.

Therefore, we investigated the effects of MC-LR on the integrity of the Caco-2 monolayer commonly used as an *in vitro* intestinal barrier model. Caco-2 cells were seeded into 12-well insert plates and, after 21-days confluence, apical exposure of the monolayer to MC-LR was conducted. Four concentrations of MC-LR (1, 10, 50 and 73 μM), a solvent control (ethanol 2.7%) and an alteration control (EDTA 10 mM) were tested. After 0.5, 1, 2, 4, 6 and 24 hours, integrity of the Caco-2 monolayer was measured both by the transepithelial electrical resistance (TEER) parameter and by the apparent permeability (Papp) of Lucifer Yellow (400 μg/ml, 3 hours incubation).

Whereas the positive control EDTA significantly ($P < 0.001$) disrupted monolayer integrity, neither MC-LR nor solvent conditions induced Caco-2 integrity alteration, even after 24h exposure.

Therefore, further experiments on intestinal permeability of MC-LR can be investigated with the Caco-2 monolayer model within the same MC-LR concentration range and up to 24 h without affecting the cell monolayer integrity.

Keywords: microcystin, Caco-2, integrity of the intestinal barrier, TEER, Lucifer Yellow

Poster

Towards the development of an *in vitro* assay to predict osteotoxicity in the developing embryo: an approach

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To assess the adverse effect of a substance on the developing embryo, animal experimentation is employed. Approximately half of the tested compounds are found to cause detrimental skeletal damage. Therefore, an *in vitro* assay with the capacity to predict developmental osteotoxicity would be a highly valuable tool to reduce animal expenditure.

Their capacity for self-renewal combined with their unique differentiation potential has led to embryonic stem cells (ESCs) becoming an attractive model system to study developmental processes. The fact that differentiating ESCs recapitulate early stages of mammalian development has made them a pivotal screening tool to identify potentially embryotoxic substances.

The ZEBET laboratory uses both murine and primate embryonic stem cells as models to study osteogenic differentiation on a molecular level. Expression profiling of marker genes/proteins is employed to identify and establish molecular endpoints indicative of skeletal damage during embryogenesis.

A detailed description of the research strategy and preliminary results will be presented at the meeting.

Keywords: in vitro test method, developmental osteotoxicity, molecular endpoints, murine embryonic stem cells, primate embryonic stem cells

Poster

Enzyme activities for xenobiotic metabolism in human reconstructed skin models

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Skin models are used to assess the irritation potential of chemicals. They may have further use in investigating tumorigenesis in the skin as well as dermal penetration and absorption of substances. The metabolic transformation of substances in the skin may be crucial for the dermal barrier function but can also facilitate adverse effects of substances in the skin.

Metabolic capacities of native rat, human and porcine skin have been reviewed by Oesch et al. (2007). There is, however, less known about the capacities for chemical transformation of skin models. The objective of this study was to investigate enzyme activities of the epidermal skin model EpiDerm® (MatTek) and the full-thickness skin model PhenionFT™ (Henkel AG) in order to specify a human skin equivalent which is appropriate for toxicological assessments.

Enzyme activities were investigated by incubation of model substrates in different concentrations within S9 fractions. Oxidising enzymes included CYP- isoforms and FMO. CYP1A, 2B and 3A were estimated by fluorescent resorufin generated from ethoxyresorufin (EROD) pentoxyresorufin (PROD) and benzyloxyresorufin (BROD), respectively. Benzydamine (BA) was used to measure the activity of FMO. The product benzydamine-N-oxide was detected by HPLC- FLD. Catalytic activities of the conjugating enzyme NAT was measured by the formation of acetylated p-aminobenzoic acid (PABA). UDP- GT activity was determined by detection of glucuronated methylumbelliferone (MUF) or 4-hydroxybiphenyl (HOBI). K_m and V_{max} for FMO and NAT were calculated using Michaelis-Menten kinetics.

In both skin models, activity of CYP-isoforms were below the detection limit. FMO activity was characterised in EpiDerm® (V_{max}= 3.15-54.69 pmol/min/mg; K_m= 300 μM) and in PhenionFT™ (V_{max}= from 3.9 to 7.2 pmol/min/mg; K_m= 2000 μM).

NAT activity was also demonstrated in both models with V_{max} =180.31-1351.92 pmol/min/mg and a K_m = 45 μM in EpiDerm® and V_{max} = 2452-2989 pmol/min/mg and a K_m= 50 μM in PhenionFT™ . Dermal U-GT activity was just above detection limit of about 200 FU/min/mg protein.

In summary, EpiDerm® and PhenionFT™ show certain metabolising capacities. Although catalytic activities differ in the skin models, the obtained results are close to data from native human tissue. Hence, EpiDerm® and PhenionFT™ represent appropriate tools to investigate xenobiotic metabolism.

We acknowledge the BMBF for funding this project (31P5324).

Keywords: skin, xenobiotic metabolism, enzyme, CYP, FMO, NAT, U-GT

Lecture in Session V: Acute and long term toxicity A

The role of the Nrf2 pathway in nephrotoxicity

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The identification of cellular stress mechanisms is fundamental to understanding the susceptibility of the kidney to chemicals and pharmaceuticals and for the development of renal biomarkers indicative of sublethal injury. Here, we utilised whole genome DNA microarrays to uncover common molecular mechanisms of response to nephrotoxin exposure.

Human renal proximal tubular cells (HK-2) were treated for 12 h and 48 h with 5 μM cadmium (Cd), 30 μM diquat dibromide (Diq) and 5 μM cyclosporine A (CsA).

Nephrotoxin treatment resulted in alteration of a total of 4608 transcripts. Ingenuity Pathways Analysis™ revealed the anti-oxidant and detoxification Nrf2 pathway as the most significantly enriched signalling pathway in the selected dataset. Activation of this transcription factor was confirmed as nuclear translocation and paralleled the temporal alterations of compound induced H_2O_2 production. Transcriptomics, Western Blot and immunofluorescence showed an induction of both HO-1 and NQO1 with Cd and Diq exposure, but not with CsA

treatment. Knockdown of Nrf2 by siRNA reduced compound induced NQO1 mRNA to basal levels and attenuated toxin induced HO-1 mRNA expression. siRNA knock down of HO-1, but not NQO1, enhanced Cd induced H_2O_2 production and Cd induced toxicity.

Using an unbiased transcriptomic approach we have identified the Nrf2 pathway as the most significant signalling response in renal epithelial cells challenged with nephrotoxins. This study highlights the importance of this pathway and particularly HO-1 in renal epithelial adaptation to oxidative stress.

Keywords: cyclosporine A, cadmium, diquat

Lecture in Session I: Legal, ethical and policy topics regarding alternatives

Alternative methods as an integral part of in-market control of cosmetics in the Czech Republic

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Since the year 2004, the Cosmetics Directive prohibits animal testing on final cosmetic products and since 2009, with exceptions, also on ingredients. This situation underlines the necessity to employ alternative techniques in cosmetic toxicology. The National Reference Centre for Cosmetics, as an authorised facility for testing of cosmetics in the Czech Republic, introduced *in vitro* alternatives as a regular part of testing required by competent authorities during in-market control already in the 1970s. In the meantime a number of alternative approaches have been validated or accepted as valid.

According to the new Regulation (EC) No 1223/2009 on cosmetic products, the safety assessment should focus in particular on local toxicity evaluation (skin and eye irritation), skin sensitisation, and in case of UV absorption on photo-induced toxicity, taking into account all significant toxicological routes of absorption.

The Neutral Red Uptake (NRU) Cytotoxicity Assay according to standard INVITTOX protocol No. 46 has been used in the past to assess the potential cytotoxic effect of individual categories of cosmetics. The highest tolerated concentration of the product in the cell culture depends on the product formulation, particularly on the content of potentially harmful ingredients. The cytotoxicity test according to ISO 10993:5 for medical devices is applied also for safety evaluation of other products, e.g. toys and children's products, for which prolonged contact with mucous membranes is expected. The effect of active ingredient is assessed by means of *in vitro* tests during new product development, aiming to find an optimal non-toxic and highly efficient concentration.

The battery of alternative methods employed comprises NRU assay, Neutral Red Release (NRR) assay, 3T3 NRU Phototoxicity Test, reconstructed human skin/corneal models EpiDerm™ and EpiOcular™ , HET-CAM and determination of skin absorption *in vitro*. The alternative methods are routinely performed on request for manufacturers as a significant part of safety assessment of their products. Furthermore, they are applied in first line in case of consumer complaints of skin/eye irritation before human tests are performed, or to prove product quality from different product batches/manufacture sites.

The results of *in vitro* tests cannot be utilised in isolation. Typically, they are used against a background of a database of previously tested or marketed cosmetic products. This relative assessment approach comprises combination of historical *in vivo* human data, *in vitro* data and knowledge of market experience. The *in vitro* test systems represent a valuable tool for safety and efficacy testing of cosmetics before skin testing in human volunteers is performed.

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Keywords: cosmetics, alternatives methods, skin irritation, eye irritation, skin absorption, phototoxicity

Poster

Phototoxicity and mutagenicity hazard of pigments frequently used in cosmetics

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A number of colorants expected to be safe are widely used in consumer products, foods and feeds. Pigments approved for cosmetics are subject to exclusive regulation in the Cosmetics Directive 76/768/EEC, Annex IV. However, the available information on their safety is largely incomplete, sometimes confusing or controversial. The extent of data frequently does not comply with requirements of the SCCP's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (2006).

Our study was focused on the crucial missing toxicological data related to local toxicity of selected colouring agents allowed in all cosmetic products, in particular those regularly incorporated in leave-on products applied on skin areas exposed to sunlight. Colorants are obvious photoabsorbers in the UV/vis region and therefore phototoxicity assessment should be considered a prerequisite for their safe use in consumer products, particularly cosmetics.

In a group of selected cosmetic colorants 4 out of 13 substances exhibited a phototoxic effect identified by means of the *in vitro* 3T3 NRU Phototoxicity Test. The positive results were confirmed by a phototoxicity assay on a reconstructed human epidermis model (EpiDerm™) reflecting the intended topical skin exposure. Substances exhibiting positive phototoxicity were subjected to further evaluation regarding skin penetration and possible mutagenic effects. The employed assays comprised evaluation of skin absorption *in vitro*, reverse mutation test using bacteria (Ames test) and an *in vitro* mammalian chromosome aberration test. DNA fragmentation after irradiation of NIH3T3 cells was studied by means of single cell gel electrophoresis *in vitro* (Comet assay). The production of reactive oxygen species (ROS) was detected in cell culture of NIH3T3 cells and investigated using molecular probe CM-H2DCFDA in combination with fluorescence microscopy/fluorimetry.

Missing toxicity data for a number of allowed cosmetic colorants should be completed so that their possible hazard can be taken into account when safety assessment of finished cosmetic formulations is performed. Although used in low concentrations, the positive findings suggest that hazardous colorants exhibiting phototoxicity and/or mutagenicity potential may contribute to adverse skin reactions and/or body systems diseases, e.g. phototoxicity, photoallergy or even photocarcinogenicity.

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Keywords: phototoxicity, mutagenicity, in vitro, cosmetics, pigments

Poster

Assuring safety without animal testing: Development of mechanistic understanding of dendritic cell activation response to chemical sensitisers

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Assuring consumer safety without the generation of new animal data is currently a considerable challenge but one that we believe is ultimately achievable. Skin allergy (sensitisation) is an important consumer safety endpoint for home and personal care products and

an endpoint where animal data (e.g. mouse local lymph node assay data) are often needed to inform risk assessments. We are investigating a number of *in vitro* approaches to model key events in the biology of skin sensitisation including dendritic cell activation.

In this study, transcriptomic analysis was used to examine the ability of a dendritic cell (DC) activation model using cultured DCs (MatTek DC-100), derived from $CD34^+$ progenitor cells, to differentiate between sensitisers and non-sensitisers. DCs, obtained from four separate individuals, were exposed to five sensitisers and five non-sensitisers for one hour, followed by 24 h recovery (established from an initial time course using the sensitiser dinitrochlorobenzene (DNCB)), at concentrations giving a cell viability of 85%. RNA was extracted, quantified and analysed using the Agilent Whole Human Genome Microarray platform. The results of this analysis were then further examined using GeneSpring and Metacore analysis software.

Principle Component Analysis (PCA) showed discrimination between sensitisers and non sensitisers on the basis of gene expression changes, and further, selectivity between non-sensitisers exhibiting reactive and non-reactive chemistry was also indicated. Approximately 1900 genes with altered expression, as measured by a greater than 1.5 fold change with parametric test (variance not assumed to be equal) value of $p<0.05$, were identified as relevant to sensitisation status. Metacore analysis was used to map altered genes to molecular pathways relevant to DC maturation (for example, cytokine-cytokine receptor interactions, complement and coagulation cascades, toll-like receptor signalling pathway). Analysis of the initial DNCB time course also showed DC maturation to be a time-dependent process.

Use of Metacore has provided molecular insights that can be used to underpin the reported cellular events (for example antigen processing and presentation, DC migration) and in addition provided insight into the model used.

These data have provided a source of potential new biomarkers to aid in future studies of DCs as part of a weight of evidence model that could be used to generate hazard characterisation data capable of informing future consumer safety risk assessment decisions in skin allergy.

Keywords: dendritic cells, sensitisation, in vitro, transcriptomics, pathway analysis

Poster

A web-based database on the main publicly available sources of toxicity data published in Russian language: contribution to REACH and 3Rs

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Valuable toxicological data published in the Russian language are difficult to access for the non-Russian audience owing to the language barrier as well as poor digitalisation of these journals. However, this information can be beneficial for the EU chemical policy REACH, where new animal tests may only be conducted as a last resort and all available existing test data on substances must be gathered and assessed first. Sufficient previous information would allow the use of non-testing information for regulatory decision making.

The EU FP6 project OSIRIS (http://www.osiris.ufz.de/) is working on integrated testing strategies (ITS) for the hazard evaluation of industrial chemicals. One of the tasks of the group of Dr. Anne Kahru (NICPB, Estonia) in OSIRIS is to create a web-based database called "E-SovTox" (http://kbfi-databases.eu/database/). This is a result of the search and selection of REACH-relevant toxicological literature from Russian language scientific journals published during the Soviet era and available in Estonian libraries as hard copies, to simplify the acquisition of toxicity data on chemicals from Russian data sources.

The main source of information – Journal *Gigiena truda i professional'nye zabolevaniia* (Industrial hygiene and occupational diseases) – involves acute but also chronic toxicity data for various industrial chemicals and preparations. Articles include mostly data on LD_{50} values, skin and eye irritation, skin sensitization and cumulation. As a rule, rats, mice, guinea pigs and rabbits were exposed via various exposure routes to derive the maximum allowable concentration values for industrial chemicals in the occupational health settings of the former Soviet Union.

As an example of the number of animals used in some experiments: 180 white rats (180-240 g), 130 white mice (18-24 g) and 40 rabbits (2-2.5 kg) were used to derive a maximum allowable concentration for benzonitrile (CAS nr 100-47-0) in the working area air (Agaev, 1977). As a rule, in addition to the LD_{50} values the articles include anatomy, histopathology, enzymatic and blood-component analysis and various additional physiological parameters – information that might be informative for industrial toxicologists and/or authorities in charge of the chemical safety dossiers for REACH.

Currently, the E-SovTox database contains data selected from more than 500 articles covering more than 600 chemicals. The user is provided with main toxicity information as well as abstracts in Russian and in English (if provided in the original paper). The search engine allows the user to search either by type of compound (inorganic or organic), compound name, CAS number or author of the data source as well as keyword.

The search by keyword is performed simultaneously in all fields of the database (incl. text of the abstracts). The demo-version of the database (http://www.kbfi-databases.eu/) will be presented.

This research is part of the EU 6th Framework Integrated Project OSIRIS (contract no. GOCE-ET-2007-037017). We dedicate this presentation to our colleague involved in this work, Prof. Henri-Charles Dubourguier, who passed away recently.

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Keywords: REACH, 3Rs, in vivo toxicity data, rodents, Russian language, databases and information sources

Lecture in Session VII: Nanotoxicology

High-to-medium-throughput evaluation of the ecotoxicity and toxicological profiling of synthetic nanoparticles

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At nano-scale materials have different or enhanced properties compared with the same materials at larger sizes owing to increased relative surface area, reactivity and the dominance of quantum effects. The changed properties may include increased bioavailability and toxicity (Nel et al., 2006). Although nanotechnological industries are rapidly developing, little is known about the potentially harmful effects and the mechanisms of nanomaterial toxicity, especially concerning environmentally relevant test organisms (Kahru and Dubourguier, 2010; Kahru and Savolainen, 2010).

We have developed a combined ecotoxicological-biosensor approach for the high-to-medium-throughput evaluation of the toxic effects and profiling of the toxicity of synthetic nanoparticles (NPs). The current presentation will focus on effects of chemical composition and size/structure of metallic NPs to various aquatic organisms. Several particle-ingesting (protozoa, daphnids) and particle-"resistant" model organisms (unicellular algae, bacteria, yeast) were chosen for the toxicity studies in acute and chronic exposure settings. ZnO, CuO, TiO_2 NPs and their respective bulk analogues as a control for size-related effects were used as model compounds as were Ag-NPs. Structural properties of particles were described by SEM and TEM, and specific surface area by BET. For explanation of the (eco)toxicological effects soluble salts of Zn^{2+}, Cu^{2+} and Ag^{+} were also analysed. Solubility of metallic NPs was evaluated by quantifying the metal ions by recombinant luminescent metal sensing bacteria (Kahru et al., 2008; Heinlaan et al., 2008; Ivask et al., 2009). With few exceptions the solubility was the key determinant of the toxicity of metallic NPs (Kahru and Dubourguier, 2010). ROS-production as a mechanism for toxic action was demonstrated for some organisms for Ag-NPs and CuO-NPs as well as for Cu-ions. We also present some data on the toxicity of organic NPs - fullerenes and PAMAM dendrimers.

This talk will summarise the nanoecotoxicological research made in my group (NICPB, Estonia) during the past five years.

We dedicate this presentation to our colleague also involved in this work, Prof. Henri-Charles Dubourguier, who passed away recently.

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Keywords: synthetic nanoparticles, hazard, toxicological properties, biotests, toxicity mechanisms

Poster

Testing of developmental protoxins in the embryonic stem cell test: simulation of metabolic conversion by treatment with defined mixtures of parent compounds and their respective metabolites

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Lack of metabolic activity is one of the drawbacks of many *in vitro* assays. It still represents a major challenge to supplement existing *in vitro* methods with biotransformation systems. To estimate the amount of parent compound that needs to be converted into its major metabolite before affecting the outcome in the embryonic stem cell test (EST) we mimicked bioactivation by testing fixed combinations of selected chemicals with their respective teratogenic metabolite. Effects of parent compound/metabolite pairs were assessed separately and in combination at ratios of 80:20 and 50:50, thus mimicking incomplete bioactivation in the range of 20 and 50%, respectively.

The EST is an *in vitro* test to predict the embryotoxic potency of test substances based on differentiation of murine embryonic stem cells (mESCs) into contracting cardiomyocytes. In addition, cytotoxic effects on stem cells and 3T3 fibroblasts are being monitored. The EST was validated in an ECVAM validation study and showed a very good correlation between *in vitro* and *in vivo* data with an overall accuracy of 79%. In this study valpromide (VPD), retinol (ROH) and albendazole (ABZ) were chosen as parent compounds together with their metabolites, i.e. valproic acid (VPA), all-trans retinoic acid (RA) and albendazole sulfoxide (ASO), respectively, and all were tested for their effects on mESC differentiation and cytotoxicity.

The active metabolite VPA was 12-times more potent to inhibit differentiation into contracting cardiomyocytes compared to its parent, VPD, while no such significant differences were detected on the half-maximal cytotoxic concentrations (IC_{50}) of these compounds. The mixtures showed an increasing potency to inhibit cardiomyocyte differentiation with increasing VPA concentration.

The well-known teratogen RA was 200- to 300-fold more potent in inhibiting cardiomyocyte differentiation and 700-fold more potent in reducing cell viability when compared to its mother compound, ROH. The 80:20 mixture was 40-times more potent than ROH alone. For this compound pair, toxicity became apparent when calculated RA concentrations of about 0.5 to 1 nM were reached in the mixture, which is in the ID_{50} range of the pure substance.

ABZ was more toxic (factor 40) in comparison to its metabolite ASO. Testing pairs of these compounds, inhibition of differentiation as well as cytotoxicity could be associated with an ABZ concentration of about 0.2-0.4 μM. At this level it was no longer possible to discriminate between mixtures and ABZ as pure test substance.

Our data show that the EST, which itself lacks any metabolic competence, can be used as readout for assessing developmental toxicity of fixed pairs of compounds and its respective metabolites. In comparison to the parent compounds VPD and ROH, their major metabolites were more toxic, while conversion of AZB into ASO represents a detoxification step. Due to the absence of any metabolic activity in mESC cultures the EST was capable of sorting out toxic potencies of parent compounds from those of their major metabolites.

Keywords: embryonic stem cell test, EST, bioactivation, in vitro method, embryotoxicity

Poster

Variability of elimination rate and cytotoxic potency of peroxides in C6 astroglioma cell cultures

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In many studies dealing with oxidative stress *in vitro* hydrogen peroxide (H_2O_2) is used as the oxidative stressor. The median cytotoxic concentrations (EC_{50}) of H_2O_2 determined *in vitro* vary widely and range from <10 μM to >1000 μM. Cultured cells are known to eliminate H_2O_2 and other peroxides from the culture medium. The concentration of cells in the *in vitro* system

thus must affect the cytotoxicity of peroxides. Using C6 glioma cell cultures we examined how the cell concentration influences elimination kinetics and cytotoxicity of H_2O_2 and the organic peroxide cumene hydroperoxide (CHP), respectively.

C6 cells were grown for 24 h in different culture plates (96-well microtiter plates, 24-well tissue culture plates, 35 mm dishes) and then exposed to various peroxide concentrations for a further 24 h in serum- and pyruvate-free DMEM medium. Cytotoxicity was assessed by the MTT assay. The cell concentration was characterised by the cell protein concentration (mg/ml) derived from the cell protein content and the medium volume in the wells or dishes during exposure to the peroxides. The cell concentration was varied either by changing the cell number or the medium volume. The concentration of the peroxides in the culture medium was measured by the ferrous oxidation xylenol orange (FOX) assay.

Depending on the culture plate and the cell concentration, the nominal cytotoxic concentrations (EC_{50}) of H_2O_2 in C6 cell cultures varied between 10 μM and 1500 μM and of CHP between 2 μM and 160 μM. The EC_{50} values increased proportionally to the cell concentration. The influence of the cell concentration on the cytotoxic potency of the peroxides almost disappeared when the amount of peroxide applied to the cell cultures was expressed as cell dose (nmol/mg cell protein) instead of as nominal concentration. From the relationship between EC_{50} values and cell protein concentration median cytotoxic cell doses (ED_{50}) in C6 cell cultures of 440 nmol/mg cell protein for H_2O_2 and of 90 nmol/mg cell protein for CHP could be calculated. The nominal peroxide concentrations applied were found to be actually initial concentrations, declining immediately after administration with a rate proportional to the cell concentration. This may explain the linear relationship between the EC_{50} values and the cell concentration. The elimination capacity of C6 cells for CHP (V_{max} = 2.6 nmol CHP/min x mg cell protein) was much lower than for H_2O_2 (V_{max} > 260 nmol H_2O_2/min x mg cell protein). This could be the cause of the higher sensitivity of the C6 cells to CHP compared to H_2O_2.

Taken together, our results indicate that the cell concentration substantially affects the rate of peroxide elimination in cell cultures and thereby also their nominal cytotoxic concentrations (EC_{50}). Therefore, it is recommended to use the cytotoxic cell dose (ED_{50}) as measure for the cytotoxic potency of peroxides. This would also improve the comparability of *in vitro* studies.

Keywords: cell dose, cell concentration, hydrogen peroxide, cumene hydroperoxide, cytotoxicity

Poster

Skin irritation *in vitro*: evaluation of long-term reproducibility and reliability of the validated EpiDerm SIT method

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In autumn 2007, an international validation study was performed to evaluate the reproducibility and confirm the predictive ability of the EpiDerm Skin Irritation Test (SIT) method. In November 2008, ECVAM endorsed the EpiDerm SIT as a full replacement method for the *in vivo* rabbit skin irritation test. As reflected in draft OECD guidelines for skin irritation testing, US and EU regulators have appropriately maintained that a test method must demonstrate reproducibility on an ongoing basis so that regulators and commercial end users are assured that the assay method is stable and continues to give valid test results over time. The purpose of the present study was to investigate the reproducibility of the EpiDerm SIT post-validation.

Over a 7-month period in 2009, 24 independent lots of EpiDerm tissue were exposed to 3 irritants (alpha terpineol, heptanal and butyl methacrylate), 3 non-irritants (benzyl benzoate, benzyl salicylate and isopropanol) and the positive control (sodium dodecyl sulphate) and negative control (Dulbecco's phosphate buffered saline) using the SIT protocol. As per the SIT method, tissue viability was determined using the validated endpoint (MTT assay) following a single, 60 min exposure and 42 h post-exposure incubation.

In all cases, the SIT method correctly identified the irritants and non-irritants. Coefficients of variation (CV) between the tests (n=24) for the tissue viability for all non-irritants were <10%, except for isopropanol (IPA) which had a CV of 11.3%. Further study of the IPA results revealed the importance of thoroughly rinsing the tissue following the 60-minute exposure (as outlined in the SIT method). These results together with quality control results for EpiDerm over the past 14 years confirm that the EpiDerm SIT method is REACH-ready and compliant with the EU 7th Amendment to the Cosmetic Directive.

Keywords: skin irritation, in vitro, EpiDerm SIT, ECVAM, validation, reproducibility, reconstructed human tissue model

Lecture in Special Session on antibody production

Recombinant antibodies. Past, present and future

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Over the past three decades, we have witnessed the emergence of synthetic biology, its roots can be traced back to the pioneering work of George Smith and peptide display on filamentous phage. In parallel Kary Mullis was developing what we now call polymerase chain reaction (PCR), Marc Better and colleagues and Arne Skera and Andreas Pluckthun were investigating antibody fragment secretion in bacteria. Over in Cambridge, Greg Winters group were developing ways to access immunoglobulin genes using PCR. The breakthrough came in 1989 when Huse and colleagues built on this body of work to effectively use PCR to capture a library of immunoglobulin chains encoding Fd and light chains and insert them into bacteriophage lambda to create the frst combinatorial antibody Fab libray. The following years McCafferty and colleagues described how a single chain Fv could be fused to the minor coat protein 3 of filamentous phage and be selected by affinity. My own work was to take this a stage further and demonstrate that it was possible to assemble a Fab on the surface of phage using phagemid and to select by binding to antigen.With all these tools in place, it has become possible to synthesise antibody libraries by chemical methods and create artificial antibody libraries and artificial scaffolds. Over the past ten years we have seen refinements and incremental advances, such as yeast, bacteria and most recently ribosomal display. I will present an overview of where we came from and where we are going in the field of animal cell free antibody discovery and development.

Keywords: recombinant antibody, synthetic receptors, diagnostic, therapeutic, in vitro selection

Poster

Selective MeHg neurotoxicity

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Methylmercury (MeHg) is an environmental neurotoxicant that affects the developing and mature nervous system (Clarkson et al., 1988). It has a high association constant (15<pKa<23) for -SH groups (Carty and Malone, 1979) and can react with any -SH group (Hughes, 1957), leading to conformational changes and thus inhibition of many enzymes. However, upon MeHg exposure a specific pattern of neurological damage is seen, such as in the glutamatergic cerebellar granule cell layer and in the GABAergic occipital cortex in human (Choi et al., 1978; Eto, 1997; Hunter and Russell, 1954; Takeuchi, 1982) and rodent brains (Nagashima, 1997). The reason for this selective degeneration of certain areas of the nervous system has not been satisfactorily explained.

We address this issue by comparing the glutamatergic C6 glial cell line with the GABAergic B35 neuronal cell line. One of the major mechanisms behind MeHg-induced toxicity is the reduction in the amount of intracellular GSH (Choi et al., 1996; Miura and Clarkson, 1993; Sarafian et al., 1994) and augmentation of ROS formation (Ali et al., 1992; Gasso et al., 2001; Sanfeliu et al., 2001; Sarafian, 1999; Shanker and Aschner, 2003; Sorg et al., 1998; Yee and Choi, 1996). MeHg induced changes in mitochondrial activity, reactive oxygen species (ROS) and glutathione (GSH) content were measured by MTT reduction and fluorescent indicators monochlorobimane (MCB) and -chloro methyl derivative of di-chloro di-hydro fluorescein diacetate (CMH2DCFDA). The intracellular MeHg content was measured by high resolution-inductively coupled plasma mass spectrometry (HR-ICPMS).

MeHg treatment resulted in a significant ($p<0.05$) decrease in GSH content and MTT activity and an increase in ROS levels in C6 as compared to the control group. However, in B35 cells only a decrease in MTT was observed. Additionally, no significant differences were noted for intercellular MeHg content in C6 and B35 cell line.

These findings provide experimental evidence that MeHg can cause cytotoxicity in certain cell types independent of ROS generation.

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Keywords: methylmercury, reactive oxygen species, glutathione, cell lines

Poster

Eye irritation hazard tested *in vitro* in a group of chemicals and final formulations

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Various cell, tissue and organotypic models have been employed with the aim of evaluating skin and eye irritation hazard using *in vitro* protocols. Special emphasis is currently given to this subject in Europe under the REACH chemical strategy and the Cosmetics Directive. The key difficulty in determining the validity of alternative *in vitro* methods is that the *in vivo* animal or human data are both scarce and often of limited utility for hazard prediction (ECETOC, 1998). Consequently, recently obtained human 4 h patch test data generated according to a standardised protocol (Jírová et al., 2008) were extended by a number of *in vitro* data related to skin and eye irritation potential. A group of selected chemicals employed in previous EU validation studies of *in vitro* methods for skin irritancy classification (Spielmann et al., 2007) and a limited number of cosmetic formulations were subjected to further *in vitro* testing. Additional *in vitro* methods (www.ecvam-dbalm.jrc.ec.europa.eu) including Hen's Egg Test

– Chorioallantoic Membrane (HET-CAM), Neutral Red Release Assay (NRR), Neutral Red Uptake Assay (NRU) and an eye irritation test using the EpiOcular™ tissue model (MatTek, USA) were performed. Human and animal skin/eye irritation hazard data (www.mattek.com) were compared with the results of the *in vitro* methods.

The study revealed that skin irritants with reference *in vivo* skin irritation data are not necessarily eye irritants. Volatile or solid materials may be misclassified due to their physicochemical characteristics. The NRR assay may provide false negative results in case of substances with a fixative effect, preventing the NR release or in case of chemicals absorbing NR not removable by standard washing procedure. Microscopic evaluation is recognised as a crucial additional endpoint for correct result assessment. HET-CAM offers valuable results related to conjunctiva. Although overpredictive, it provides the lowest false negative rate. The EpiOcular™ assay correctly identified the most aggressive cosmetic formulation, in accordance with NRU and NRR results, while HET-CAM correctly identified the mildest formulation.

Each of the *in vitro* methods is related to a separate endpoint of ocular irritation and can provide only partial information on the mode of action of the tested material. Despite good reproducibility of individual *in vitro* assays, only the weight-of-evidence approach considering physicochemical and chemical characteristics of the tested material and results of multiple selected *in vitro* tests can provide an estimation of the ocular effect *in vivo*. The *in vitro* models for testing of skin/eye hazard seem to be useful tools for the prediction of human hazard, particularly for consideration of initial concentrations for confirmatory human clinical trials to prove absence of local irritative effects and to confirm safety of consumer products, e.g. cosmetics.

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Keywords: cosmetics, chemicals, eye irritation, in vitro

Poster

In vitro differentiated adult human liver progenitor cells display mature hepatic metabolic functions: A potential tool for *in vitro* pharmaco-toxicological testing

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The potential use of stem/progenitor cells as alternative cell sources to mature hepatocytes for pharmaco-toxicological testing remains basically dependent on their ability to exhibit some, if not all, metabolic liver functions (Najimi et al., 2007).

In the current study, four major liver functions were investigated in adult derived human liver stem/progenitor cell (ADHLSCs) populations submitted to *in vitro* hepatogenic differentiation: gluconeogenesis, ammonia detoxification, and activity of phase I and phase II drug-metabolising enzymes. These acquired hepatic activities were compared to those of primary adult human hepatocytes, the standard reference. Amino acid content was also investigated after hepatogenic differentiation.

Differentiated ADHLSCs display higher *de novo* synthesis of glucose, correlated to an increased activity of glucose-6 phosphatase and mRNA expression of key related enzymes. Differentiated ADHLSCs are also able to metabolise ammonium chloride and to produce urea. This was correlated to an increase in the mRNA expression of relevant key enzymes such as arginase. With respect to drug metabolism, differentiated ADHLSCs express mRNAs of all the major cytochromes investigated, including CYP3A4 isoform (the most important drug-metabolising enzyme). Such increased expression is correlated to an enhanced phase I activity as demonstrated independently using fluorescence based assays. Phase II enzyme activity and amino

acid levels also show a significant enhancement in differentiated ADHLSCs.

In conclusion, the current study, according to data obtained independently in different labs, demonstrates that *in vitro* differentiated ADHLSCs are able to display advanced liver metabolic functions supporting the possibility to develop them as potential alternatives to primary hepatocytes for *in vitro* settings.

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Keywords: mesenchymal cells, hepatic stem cells, hepatocyte differentiation, metabolic activity, tissue-specific stem cells

Poster

Virtual reality simulators instead of animal training for physicians who treat acute stroke patients

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Stroke, the clinical manifestation of cerebrovascular disease, is the third leading cause of death in the United States and Europe. Stroke can now be treated using interventional neuroradiologic therapies, which rely on the insertion and navigation of catheters and guidewires through a complex network of arteries to restore blood flow. Because the treatment is delivered directly within the closed brain, using only image-based guidance, the dedicated skill of instrument navigation and the thorough understanding of vascular anatomy are critical to avoid devastating complications that could result from poor visualisation or poor technique.

Traditionally, basic catheterisation skills are acquired by interventional fellows while performing routine angiograms under the close supervision of an experienced mentor. The skills gained during these diagnostic procedures are directly transferable to the execution of endovascular interventions. Interventional procedures on patients or animal models constitute the normal training for interventional fellows. One solution to the conflict of clinical skill acquisition versus patient safety and avoidance of animal models is the use of virtual reality (VR) simulation. Such simulators can systematically expose the trainee to a broad variety of pathologies in a safe, reproducible environment, thereby eliminating the usual randomness of training, and allow endovascular fellows to develop basic psychomotor skills and fully acquaint themselves with the clinical environment prior to traditional patient-based mentoring.

The Christian Doppler Clinic in Salzburg uses two simulators for physician and student training: the Procedicus-VIST system (Mentice Medical Simulations, Gothenburg, Sweden) and the AngioMentor system (Simbionix Ltd, Lod, Israel), which are multidisciplinary simulators that provide hands-on practice in an extensive and completely simulated environment of interventional endovascular procedures.

We report our observations that basic psychomotor and procedural skills can be learnt using simulators. The combination of using actual clinical equipment in conjunction with future simulator systems with haptic feedback, which is under ongoing development, may be directly transferable to the clinical setting without animal trials.

Keywords: stroke, animal training, simulator, virtual reality

Poster

Culture of organotypic, 3-dimensional tissue models – use of alternative tissue culture inserts

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Highly differentiated, organotypic tissue models are being used increasingly in lieu of animals to meet regulatory testing requirements. The consistent quality of these tissue models is of prime importance so that US and EU regulators and industry users can be assured that the toxicological system is reproducible both during the validation process and afterwards. The purpose of this study was to investigate the effects of various tissue culture inserts (TCI) on the tissue morphology and reproducibility.

Tissue culture inserts (TCI) from 5 commercial manufacturers were obtained and standardised culture conditions were used to produce the skin (EpiDerm™) and ocular (EpiOcular™) organotypic tissue models. These models were then subjected to quality control (QC) tests which include histological evaluation and determination of the exposure time of a common surfactant (Triton X-100) that reduces the tissue viability to 50% (ET_{50}). In addition, the EpiOcular Eye Irritation Test (EIT) was run using 26 test articles (21 liquids, 5 solids).

Of the 5 TCI tested, histology of tissues cultured on 2 TCI was distinctly different and inferior to the standard tissues, while the histology for tissues produced on the remaining TCI were structurally equivalent to the control tissues. The average ET_{50} for EpiDerm produced with the best substitute TCI was 7.64 ±0.95 hr (n=6) and was not statistically different from that of tissues cultured on the control TCI, 7.73 ±0.56 hr ($p = 0.79$, paired student t-test); similarly, the ET_{50} for EpiOcular cultured on the best alternative TCI was 22.6 ±5.0 min (n=5), which was not statistically different from that of the control tissue, 27.2 ±5.0 min ($p = 0.18$, paired student t-test). Finally, use of the best alternative TCI in the EpiOcular Eye Irritation Test (EIT) showed no differences in irritant/non-irritant predictions for 26 organic chemicals.

The TCI is one of the crucial parameters in producing high quality, reproducible, organotypic tissue models but multiple commercially available TCI appear to have appropriate properties.

Keywords: ocular irritation, in vitro alternative, tissue model, EpiOcular

Poster

In vitro photodynamic effect study on MCF7 cell lines

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Photodynamic therapy (PDT) is an alternative treatment method for certain malign and nonmalign diseases. The cytotoxicity results from a combined effect of sensitizer, oxygen and light. During these processes the reactive oxygen species (ROS) produced are among the major agents triggering cell death by apoptosis or necrosis. We report the production of reactive oxygen species (ROS) and the phototoxicity of ClAlPcS2 assessed using MCF7 (human breast adenocarcinoma) cell lines.

Irradiation was achieved with light-emitting diodes (670 nm, 10 $mWcm^{-2}$) at total doses of 10, 25 and 50 Jcm^{-2}. Phototoxicity was evaluated by MTT test. ROS measurement was performed using 10 μM 5-(6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) producing fluorescence dye CM-DCF in the presence of ROS. Fluorescence of CM-DCF was calibrated according to the fluorescence response of the probe to the addition of external H_2O_2. The production of ROS and the distribution of ClAlPcS2 in cells were visualised with an inverted fluorescence microscope and image analysis.

Our results show ROS production in MCF7 cells is dependent on the sensitizer concentration of ClAlPcS2 and the dose of radiation. Viability studies show that the optimum phototoxic effect evaluated on MCF7 melanoma cells was achieved with the combination of light doses of 25 or 50 Jcm^{-2} and phthalocyanine

ClAlPcS2 at concentrations from 0.5 to 10 μg/ml. This combination of phthalocyanine and corresponding radiation dose was lethal for MCF7 cells.

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Keywords: photodynamic therapy, light, MCF7 cell line

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Poster

Comparison of ^{3}H-thymidine incorporation and non-radioactive endpoints for evaluating LLNA results during routine testing

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The local lymph node assay, as currently described in the OECD guideline 429, is based on measuring lymph node cell proliferation by ^{3}H-Thymidine incorporation into the lymph node cells (OECD, 2002). Alternative endpoints were evaluated recently without a final conclusion on their suitability (Basketter et al., 2008). We suggest to base the evaluation of cell proliferation on measuring the number of cells in single cell suspensions produced from the ear lymph nodes (as described by Vohr et al., 2000), which proved to be useful for evaluation of LLNA results in a multicenter study when the cut-off stimulation index (SI) for positive tests was adjusted to reflect the overall range of cell count increase (Ehling et al., 2005). The comparison of cell count and ^{3}H-Thymidine incorporation in a project examining the skin sensitizing potency of 13 epoxy resin constituents (epoxides and amines) showed very good congruence of test evaluation (Gamer et al., 2008).

Here we present additional data from routine studies with a variety of chemicals and mixtures proving the correlation of the two endpoints. When using an SI of 1.5 for cell count cut-off to predict a positive response in CBA/J mice, equivalent estimated concentrations (ECs) for the prediction of skin sensitizing potency are obtained in the majority of cases with both measurements. In addition we present the assessment of alternative, non-radioactive LLNA endpoints including cell count and immunophenotyping based on the test results of the LLNA performance standards as suggested for the updated OECD guideline 429.

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Keywords: LLNA, non-radioactive, alternative endpoints, performance standards

Poster

Estrogen receptor transcriptional activation assays: comparing HeLa cell- and yeast-based methods

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Exogenous substances may bind to the estrogen or androgen receptor; this may lead to adverse effects, which are usually categorised as endocrine disruption.

Recently, a method to study the binding of substances to the human estrogen receptor alpha was adopted by the OECD (test guideline 455). This method uses the activation of the human HeLa-9903 cell line stably transfected with human estrogen receptor alpha. In these cells the receptor-ligand complex translocates to the nucleus, where it binds specific DNA response elements and activates a firefly luciferase reporter gene, resulting in increased cellular luciferase activity, which is used for quantification of the receptor activation by luminescence measurement. The assay is used to detect agonistic effects of substances on the human estrogen receptor alpha.

In our laboratory we have optimised and validated yeast-based assays to detect agonistic and antagonistic effects of substances on the human estrogen receptor alpha (YES) and on the androgen receptor (YAS). Similar to the HeLa assay, the gene coding for the human estrogen receptor alpha has been integrated into the genome of a yeast strain. Additionally, the yeasts contain a plasmid carrying the lac Z gene, which is receptor-dependently expressed and serves as a reporter gene. The lactase activity is used to quantify the activation of the human estrogen receptor alpha. The YES was validated with 105 test compounds and the assay is currently used in screening more than 50 substances per year.

We have compared the performance of the HeLa cell- and the yeast-based assays. Both assays perform similarly, but we prefer the yeast assays for their significantly greater robustness and the option to study agonistic and antagonistic effects on human estrogen- and androgen receptors. Moreover, we successfully integrated hepatic metabolising activities into the YES and YAS.

Keywords: estrogen, transcriptional activation, yeast estrogen screening assay, yeast androgen screening assay, HeLa-9903, OECD 455

Lecture in Session V: Acute and long term toxicity A

Statistical analysis of the AcuteTox data aimed to identify an *in vitro* testing strategy for prediction of acute oral toxicity

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The ACuteTox project aims at developing a simple and robust testing strategy for prediction of human acute oral toxicity, which could replace the animal acute oral toxicity tests used nowadays for regulatory purposes. In the first phase of the ACuteTox project, 57 chemical compounds were tested in various *in vitro* test systems, resulting in more than 50 endpoints for evaluation. The main focus of the statistical evaluation was the identification of a subset of assays that are promising to classify chemical compounds into the different acute oral toxicity categories (GHS and EU). In the second phase of the project, different classifiers obtained from the first phase are challenged with data from a validation set of 32 compounds evaluated blindly in the subset of selected assays.

Statistical analysis of the concentration-response data was performed using log-logistic models and a characteristic value such as the EC_{50} was derived for each experiment. If the experiment showed no clear concentration-response relationship, or if the EC_{50} estimate exceeded the concentration range tested in the experiment, the estimate was reported as right-censored value. Often more than one concentration-response experiment was

carried out for an endpoint × compound combination. In this situation, a meaningful summary EC_{50} value had to be computed from all values estimated separately in individual experiments. The classification task for ACuteTox involves a mixture of uncensored and censored observations in the predictor variables of the classification models and, therefore, classical statistical methods for classification, such as CART (see Breiman et al., 1984) or Random Forests (see Breiman, 2001), could not be applied directly to the ACuteTox data. Appropriate modifications of these methods will be presented.

From the first phase data, 36 endpoints were chosen to be included in the second phase of the project. The rationale for endpoint choice was quality of the data reported and performance in univariate and multivariate CART analyses. The selected classifiers and the results of the challenging exercise will be presented.

One of the issues to be discussed is that the selected classification algorithm will have to be communicated to non-statisticians and regulators. Results indicate that the error rates obtained from Random Forests are more convincing than those obtained from CART. However, Random Forests cannot be translated into a simple integrated testing strategy and a user-friendly software solution would have to be implemented for its application. This is in contrast to CART, which can be easily translated into a decision tree and which can be applied by biologists without use of additional computational support given that dose-response analysis is completed.

The authors would like to acknowledge all the partners of the ACuteTox consortium who contributed to the generation of the results.

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Keywords: acute oral toxicity, testing strategy, 3Rs

Poster

Development of a partition-controlled dosing system for cell assays

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Hydrophobic and volatile chemicals have proven to be difficult to dose in cell assays. The aim of this study was to develop a partition-controlled dosing system to maintain constant concentrations of polycyclic aromatic hydrocarbons (PAHs) and chlorobenzenes in an ethoxyresorufin-O-deethylase (EROD) assay and a cytotoxicity assay with the rainbow trout cell lines RTL-W1 and RTgill-W1. Polydimethylsiloxane (PDMS) sheets were loaded with test chemicals in a spiked methanol/water solution and placed in the wells, filled with culture medium, of a 24-well culture plate. Cells were grown on inserts and were subsequently added to the wells with the PDMS sheets. The system reached equilibrium within 24 h, even for the very hydrophobic PAHs. The reservoir of test chemical in PDMS was large enough to compensate for medium concentration losses of >95%. The PDMS sheets maintained medium concentrations constant for >72 h. Median effect concentrations (EC_{50}) were significantly lower in the partition-controlled dosing systems than in conventional assays spiked using dimethyl sulfoxide (DMSO) as a carrier solvent, thus indicating a better sensitivity of the bioassay when controlled and constant exposure conditions could be assured. The EC_{50}s of the test chemicals based on freely dissolved concentrations could also be estimated in the partition-controlled dosing systems using measured PDMS-bare culture medium partition coefficients.

Keywords: fish, acute toxicity, free concentration, dosing

Poster

Modelling the free fraction of polycyclic aromatic hydrocarbons in basal cytotoxicity assays using physicochemical properties

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Only the freely dissolved concentration of a chemical is generally considered to exert a response in organisms. Thus, if *in vitro* assays are to be used to quantitatively predict the toxicity of chemicals *in vivo*, it is necessary to determine the free concentration *in vitro* such that chemical losses through binding to serum protein, sorption to plastic and evaporation can be better understood and accounted for. The aim of this study was to determine what assay components and physicochemical properties influence the free concentration of phenanthrene in a Balb/c 3T3 and RTgill-W1 MTT assay.

Solid phase microextraction (SPME) was used to measure the partition coefficients of PAHs to serum constituents, well plate plastic, cells and headspace. These partition coefficients were correlated with the chemical's Henry's law constant (H) and octanol-water partition coefficient (K_{OW}). These physicochemical properties, in turn, were used to model the free concentration of the chemical *in vitro*. The estimated free concentrations were compared to the free concentration measured in the assay using SPME.

Results indicate that the free concentrations of PAHs are significantly reduced in a typical *in vitro* set-up and that altering the *in vitro* set-up alters the measured effect concentration.

Keywords: free concentration, cytotoxicity

Poster

Alternative thinking on hazard assessment of new materials: nanomaterials and 3Rs

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Development of nanomaterials with new functionalities is increasing and facilitates a wide range of innovative applications. Along with the design of nanomaterials, the safety of these materials must be addressed, applying a valid and pragmatic testing strategy. Nanomaterials come in a wide variety of shapes and chemical origin; therefore conventional toxicity testing may be less appropriate. In order to improve testing efficiency with a minimal need for animal testing, our research focuses on relevant *in vitro* screening assays to evaluate hazard characteristics of engineered nanomaterials. As the functionality of nanomaterials is often based on their surface area, we compared effects of both nano- and micro-sized materials. In this study various silica (SiO_2) and cerium oxide (CeO_2) materials with different physical properties were studied with regard to their characteristics and evaluated in four different *in vitro* assays with respect to cytotoxicity (MTT conversion and LDH leakage), inflammatory response (cytokine release), oxidative stress (Heme-oxygenase-1 induction) and genotoxicity (Comet assay). The particles were characterised regarding surface chemistry, surface energy and particle size and shape. *In vitro* screening provided guidance for further testing and aided product development. A genomics approach was used, enabling assessment of mechanisms of toxicity and identification of cellular pathways affected by particles. In addition, methods to study the toxicity of several nanomaterials in lung models using relevant exposure routes (via air) were developed.

From the physico-chemical characterisation of the particles and the evaluation in the four *in vitro* assays, different mechanisms of (geno)toxicity were observed for the materials. The nano-sized materials behaved differently in the *in vitro* screening assays compared to their micro-sized compounds. Next to size, chemical properties appeared to play an important role in the observed *in vitro* (geno)toxicity. CeO_2 appeared less toxic than SiO_2, but induced DNA damage, particularly in nano-sized form.

Keywords: nanomaterials, (geno)toxicity, characterisation

Poster

The Doerenkamp-Zbinden Foundation's chairs for alternatives to animal experimentation in research and education: the projects

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The DZF is a Swiss-based foundation that has dedicated its activities and support to the development and promotion of alternatives to animal experimentation in biomedical research and education according to the well-known 3R principle (replacement, reduction and refinement) in the field of biomedicine. The foundation has undergone four eras of existence and development. In the last era, which started in 2004, the foundation's course was strongly streamed into the direction of promotion of replacement and reduction alone. The last era of the foundation can also be called an era of chairs on alternatives to animal experimentation. Realising the importance and the role of academic chairs on alternatives to animal experimentation the foundation has, up to now, focused on establishing endowed chairs at several universities. The first chair named "Doerenkamp-Chair for In Vitro Toxicology" was installed in 2003 at the University of Erlangen (Germany) by the foundress, H. Doerenkamp, personally. This chair was installed for a period of five years and is now in a prolongation phase of two more years. It has been mainly focused on refinement and reduction by imaging techniques in biomedical research. In 2006, a "Doerenkamp-Zbinden Chair of in-vitro Toxicology and Biomedicine" was installed at the University of Konstanz (Germany). The exceptionally good start of these chairs motivated the foundress to encourage the decision to place the emphasis of the foundation's work on the establishment of further university chairs. Since then the following chairs have been established:

In October 2008 at Utrecht University (The Netherlands) the "Doerenkamp-Zbinden Professorship for Alternative Methods in Toxicology" was installed. The chair will be financed for six years. In January 2009, in co-operation with the Egon-Naef Foundation the DZF established the "Doerenkamp-Naef-Zbinden Professorship on Alternative Methods to Animal Experimentation" at the University of Geneva (Switzerland). The chair will be financed for six years. Also in January 2009 the "Doerenkamp-Zbinden Endowed Chair for Evidence-based Toxicology" was established at Johns Hopkins University, Baltimore, USA. This chair has been permanently installed for as long as the Johns Hopkins University exists. In July 2009 a contract for five years was signed with the the Bharathidsan University, Tiruchirappalli/Tamil Nadu in India to found a "Mahatma Gandhi-Doerenkamp Center for alternatives to the use of animals in life science education" with a Gandhi-Gruber-Doerenkamp chair.

Keywords: 3R, chairs, research, education

Poster

Communication about animal experiments and alternatives to the general public in the Netherlands

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In the Netherlands, every year approximately 600,000 animals are used in biomedical experiments and in the testing of a range of products like medicines and consumer goods. They are strictly regulated under the Animal Experimental Act of 1977, which changed in 1996.

Since 1977 much has improved: the number of animals has sunken from 1.5 million to the current number, a thorough ethical review is compulsory and awareness of the 3Rs is growing. From the animal protection point of view though, much more change is needed. One of our main focal points of attention is the necessary and desirable openness about animal experiments and the vital importance of the development of 3R methods.

But openness is not an easy subject. Because animal experiments are executed behind closed doors, out of sight and without the presence of inquisitive eyes, the general public gets little information on why they are being done and which alternatives are available to reduce the number of animals used. The subject is complex and sensitive and not easily ex-

plained in a few words or even images. The lack of communication to the public has had negative effects. In the last few years the Netherlands has been the target of extremely aggressive persons attacking facilities and people, demanding an immediate stop to animal experiments. We argue though that openness is essential and a dialogue between all stakeholders and involving the general public can possibly avoid these attacks. This will in turn prevent animal experiments from disappearing behind closed doors again. To increase openness and awareness of the public we have developed several tools.

First, although every permit holder has signed a "Code of openness", it is unclear how this should be put into practice. To support them in this, we developed a special card that can be folded like a credit card and easily stored in a wallet. On one page patients, family and friends can read about animal experiments and the search for alternatives while they are waiting for treatment at the outpatient departments of the 9 academic hospitals with extensive animal laboratory facilities.

Second, to bridge the gap between researchers and the public we set up a prize, the "Guts-in-the-Lab"-prize, which is awarded to one of four or five nominees every year around "World Lab Animal Day", the 24th of April. Instead of us deciding who will be the winner, the general public can vote for a nominee on a special website. Researchers are required to explain their work in simple, ordinary words that an average interested person can understand and relate to, e.g. not to talk about "cardiovascular research" but about "heart and blood vessel diseases".

A third activity is publishing a brochure for the general public on the yearly registration of animal experiments by the Dutch government. Since 1977 researchers and permit holders are required to submit extensive information on the animal experiments they perform each year. These are published in an annual report called "*Zo doende*", freely translated as "That's why".

Keywords: alternatives, animal experiments, communication, general public

Poster

Improved cellular analysis by impedance-based real-time cell monitoring to detect compound-induced hepatic cytotoxicity

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One of the major reasons for withdrawal of pharmacological compounds from drug development is the occurrence of adverse effects such as organ toxicities. Cell-based assays are a key tool for the assessment of compound-induced toxicities at an early stage of drug development, before animal testing. Prediction of hepatic toxicity – the most frequent organ toxicity – using *in vitro* assays has been a major challenge so far. To date, cellular assays used are endpoint assays where the drug-effect is measured at a single defined time point. Thus, choosing the right time point for the *in vitro* assay is critical and some effects may remain undetected because they develop either before or after the endpoint measurement is performed.

A tool which enables measuring cellular behaviour over the entire length of the experiment therefore bears the potential of generating *in vitro* data of higher quality. Such a tool is provided by the xCELLigence Real-Time Cell Analyzer (RTCA) System. This system is based on measuring electric impedance generated by adherent cells, which interact with a microelectrode biosensor at the bottom of the well of a cell-culture plate. The readout, termed "cell index" (CI), measures increase or decrease in cell number as well as morphological alterations caused for example by drug-treatment. Thus, the technique allows label- and hands-free dynamic monitoring of cellular events, such as proliferation, cell death, adhesion and spreading in a 96-well format over an extended period of time.

In the presented work, compound-induced toxicity to hepatic cells was continuously monitored by the xCELLigence System. Data showed comprehensive compound- and concentration-dependent impedance profiles, which could be used to quantify toxicity by a time-resolved IC_{50} (half maximal inhibitory concentration of a substance).

The information obtained from impedance readouts over the entire length of the experiment allowed timed decisions for the administration of compounds as well as down-stream analysis, such as biochemical assays or gene expression analysis. In many cases, CI proved to be a highly sensitive indicator for drug-induced toxicity at low concentrations and at earlier time points as compared to established standard measurements, such as glutathione depletion, ATP content or release of lactate dehydrogenase.

Taken together, the xCELLigence technology may significantly improve the quality of toxicological *in vitro* tests as it appeared to be more sensitive compared to standard endpoints. Because the impedance measurement is non-invasive, the xCELLigence system does prevent the use of any additional readout. Impedance measurements therefore could improve the quality of *in vitro* assays widely used in preclinical development.

Keywords: in vitro toxicology, impedance, primary hepatocytes, real time cell analysis

Poster

Novel biomarkers in dendritic cells contribute to understanding the skin sensitization process

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Chemical induced skin sensitization is characterised by the recognition and incorporation of low molecular weight chemicals by resident immature dendritic cells (DC). DC migrate from the epidermis to the draining lymph nodes to stimulate the proliferation of antigen-specific T cells. The underlying molecular events leading to DC evoking this specific immune response are not yet understood.

Transcriptomic analyses revealed a set of genes that are differentially expressed in CD34-DC after exposure to skin sensitizers. Starting from the differential expression of 13 genes, a preliminary classification model (VITOSENS®) has been developed to identify chemicals as (non)sensitizing (Hooyberghs et al., 2008).

The goal of this study was to further elucidate the role of a selection of these biomarkers in the response of DC to sensitizing chemicals. We tested the hypothesis that selected genes correspond with functional proteins in DC after exposure to sensitizing compounds. Flow cytometric analysis revealed differential protein expression of the markers CCR2 and COX2 in CD34-DC exposed to three sensitizers versus three non sensitizers.

Next, the functional relevance of CCR2 and COX2 in DC activation was investigated by pharmacologically counteracting these molecules in CD34-DC exposed to a model sensitizer. Evaluation of surface DC activation markers showed that CD86 was significantly down-regulated due to COX2 inhibition, whereas expression of HLA-DR was significantly reduced when an agonist of CCR2 was applied to sensitizer-exposed CD34-DC.

The observations in this research reveal that a selection of the novel biomarker genes for skin sensitization are differentially translated after sensitizing versus non-sensitizing exposure and furthermore they appear to be functionally relevant in sensitizer-induced DC activation. Therefore, this research contributes to a deeper understanding of the role of these novel biomarkers in the skin sensitization process and increases acceptance of the *in vitro* assay in which they function.

Parts of these results have been published in Toxicology Letters (2010): doi:10.1016/j.toxlet.2010.04.003

Reference

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Keywords: skin sensitization, VITOSENS, mechanistic insight

Lecture in Session VII: Nanotoxicology

The use of alternative methods for toxicity testing of nanomaterials

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The engineering of nanomaterials offers extraordinary opportunities in various technological fields. Nano-structures can change the properties of materials and also alter their biological effects. Hence their toxicity can differ from that of larger-scale materials; but as of today, no new quality of a general nano-specific toxic effect has been observed. Therefore the established

testing methods are capable of detecting potential effects of nanomaterials.

It is, however, rather difficult to apply the nanomaterials (Landsiedel et al., 2010) to *in vitro* test systems, since it is the nature of these materials to change their surface properties and agglomeration state in different environments. Additionally, the kinetics of nanomaterials in the body may have a critical influence. This presents two complications in applying *in vitro* models to nanomaterials (both for the nature and the dose of the nanomaterial).

While the methods for topical effects may be used for nanomaterials without further modifications, the *in vitro* methods for genotoxicity testing require the dispersion of the nanomaterial in the culture medium. The use of efficient, reproducible and well-documented dispersion protocols is *de rigueur*. For most materials, proteins (e.g. albumin or serum) were found to be efficient as stabilising agents. As important as the dispersion is the characterisation of the particle size distribution; different methods gave results that varied over two orders of magnitude; fractionation methods are generally considered most appropriate for polydisperse suspensions (Schulze et al., 2008). Reviews of published genotoxicity studies on nanomaterials led to the conclusion that there is no nano-genotoxicity *per se* and that consequently individual nanomaterials have to be tested (Landsiedel et al., 2009).

Moreover, it is necessary to learn about the mechanism and the relevance of the results obtained *in vitro* by correlation to *in vivo* studies. Modern toxicology is based on insight into the toxic pathways. For nanomaterials a testing strategy should include testing for their primary effects (which might be only a handful: particle effects, catalysing the formation of reactive molecules and ion release) and their uptake, distribution and clearance. The use of *in vitro* dermal penetration studies for the risk assessment of sunscreen nanomaterials has been demonstrated. *In vitro* methods for specific primary effects are currently awaiting validation (for both chemicals/molecules as well as nanomaterials).

In the meantime alternative short-term *in vivo* studies with optimised biological readouts can deliver information on the toxic pathways as well as the biokinetics and dose-response relation of nanomaterials in the body. A short-term inhalation test for nanomaterials has already been used successfully (Ma-Hock et al., 2008).

A testing strategy based on those methods engages fewer animals and provides more significant data than classical testing. Moreover, data from these methods will serve as a benchmark and a validation for the *in vitro* models under evaluation. In due course it shall be possible to link the toxicity of nanomaterials to their physico-chemical properties. Alternative methods will be crucial in defining groups of nanomaterials with similar biological effects as well as assigning new materials to these groups.

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Keywords: nanomaterials, test methods, inhalation, dermal penetration

Poster

A novel human small intestinal functional cell model and its application in gut toxicology

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In vitro functional cell models are becoming increasingly important since animal experiments do not always provide data that is relevant for humans. Moreover, they are expensive and inhumane. In this spirit, the EU has banned all repeated dose animal experimentation by 2013, which raises a profound need for efficient alternative methods.

We developed a novel human small intestinal functional cell model that could be applied to various studies in the human gut. The model consists of the non-carcinogenic small intestinal epithelial cell line H4cl.1 growing on a microporous membrane, co-cultured with the monocyte/macrophage cell line TLT. When the epithelial cell line grows on the membrane, cells polarise and form a tightly packed monolayer with measurable transepithelial resistance and potential, similar to the *in vivo* situation.

This experimental set-up was applied to evaluate the bioavailability and toxicity of some common toxic compounds, which

can be found in food (heavy metals and polycyclic aromatic hydrocarbons). Additionally, we estimated the transport of the model food intoxicants through the epithelial layer of H4 cells. Intoxicants were applied to the apical side of the membrane and after incubation concentrations were measured in the apical and basolateral compartment as well as intracellularly. Results were compared with the Caco-2 cell line, which is usually applied in such studies. Our results proved that our human intestinal model H4/TLT is more relevant for toxicological and bioavailability studies than the carcinogenic colon cell model of Caco-2 and can be, upon validation, proposed as a standardised method for gut toxicology.

Keywords: novel, gut, small intestine, non-carcinogenic, functional cell model, bioavailability, toxicology

Poster

Toxicity of redox-active and electrophilic organic compounds in the *Tetrahymena pyriformis* bioassay

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In the context of integrated testing strategies (ITS) for REACH, *in silico* methods are used to assess the hazard risk of chemical compounds according to their mode of action. While quantitative toxicity predictions are feasible for narcotics, a correspondingly general approach is still missing for excess-toxic compounds. For the latter, however, structural alerts have been developed to discriminate them from narcosis-level toxicants (Von der Ohe, 2005). The aim of this study is to characterise the excess toxicity of reactive and specifically acting compounds in the bioassay with the ciliates *Tetrahymena pyriformis* and to trace back the observed toxicities to underlying mechanisms.

A compound is called "excess toxic" if its experimental EC_{50} is (significantly) smaller than the baseline-narcosis EC_{50} value predicted from its hydrophobicity, which in turn is quantified in terms of K_{ow} (octanol/water partition coefficient). The ratio between narcosis-level and observed toxicity is called toxicity enhancement (Te). With log Te = log EC_{50} (narcosis-level) – log EC_{50} (exp.), large positive log Te values indicate correspondingly large excess toxicities. Reactive toxicity typically results from formation of covalent bonds with nucleophilic compounds such as proteins and DNA. The toxicity of redox active organic compounds appears to involve the mode of action as redox cycler, forming superoxide anion, which leads to the generation of hydrogen peroxide and hydroxyl radicals. Ongoing studies indicate that these different mechanisms of action are associated with different Te values and thus can be discriminated when employing the *Tetrahymena pyriformis* assay.

In the present investigation, the toxicity of quinone derivatives and nitropyridine compounds was determined by measuring concentration-effect curves in terms of 24 h 50% growth inhibition concentration values (log EC_{50}, effective concentration 50%). Growth inhibition was quantified through cell counting. Calculated log Kow values were used for predicting baseline-narcosis EC_{50} values, and comparison of the latter with the experimentally observed EC_{50} values resulted in Te values quantifying the toxicity enhancement as compared to the narcosis level.

The observed log Te values suggest that for most of the quinone and nitropyridine derivatives, toxicity is driven by redox cycling, and that for some compounds electrophilic reactivity is also involved. The discussion includes comparison with Te data associated with further reactive compound classes.

The study is supported by the EU project OSIRIS (contract No.: 037017) and kindly supported by Helmholtz Impulse and Networking Fund through Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE).

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Keywords: Tetrahymena pyriformis, EC_{50}, narcosis baseline, excess toxicity

Poster

Clinical trial to evaluate ocular discomfort by shampoos and the relation to the Slug Mucosal Irritation (SMI) assay

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Sight is one of our most important senses. Our eyes are very sensitive, and stinging sensations induced by normal use of a shampoo may occur. A screening method for ocular discomfort would be very helpful in the development and refinement of formulations. In the past, the Slug Mucosal Irritation (SMI) test demonstrated a relation between an increase in mucus production (MP) in slugs and an elevated incidence of stinging, itching and burning (SIB) in human eyes. The aim of this study is to compare subjective ocular discomfort caused by shampoos evaluated in volunteers with results obtained with the SMI-test.

The stinging potency of one artificial tear and ten shampoos (5% and 10% dilution) was evaluated with the SMI-test by placing three slugs per treatment group three times on 100 μl of the test item. After each 15 min contact period, MP was measured. Evaluation of the results is based upon the total MP (expressed as % body weight) during three repeated contact periods. Experiments were repeated three times.

A Human Eye Irritation test was set up with the same test items (12-period cross-over study with 24 volunteers, study approved by an independent Commission for Medical Ethics, associated with University Hospital Ghent, Belgium). Ten μl of a 5% or 10% shampoo dilution in water or the artificial tear is dripped into one eye while 10 μl of water is administered to the other eye. The evaluation of the tested substances is done both by the participant (no to severe stinging: score 0 to 5) and the ophthalmologist at several time points (30 sec to 30 min).

At this moment, half of the study is finished, while the other part will be completed by the end of June 2010. Preliminary data analyses reveal some interesting results. Scores of shampoos are clearly higher than the artificial tear and water. Among the different shampoos, shampoo C is best tolerated by the majority of the volunteers (n=14), while shampoo F is ranked as causing most discomfort by 8, and as second most by 5 participants. The results for shampoo A, D, and E are more variable; they are ranked dissimilarly by the volunteers. In general, the artificial tear was best tolerated, 14 of the 24 participants never felt discomfort. Ten others reported only very slight stinging sensations after administration, but the feeling disappeared immediately.

The ranking according to the SMI-test was: artificial tear (total MP <1.5%) << shampoo B ≈ C ≈ D (total MP: 5.3% to 8.9%) < shampoo F (total MP: 8.2% to 12.1%). Shampoo A also resulted in more variation in the SMI assay (total MP varied between 1.6% and 6.4%).

With the obtained results we are able to improve the newly developed protocol and examine the predictability of the SMI-test with reference to non- and mildly irritating formulations in humans. Although it is too soon to draw final conclusions, results of the clinical trial already indicate that the SIB-procedure is a good tool to predict clinical ocular discomfort.

Keywords: ocular clinical discomfort, shampoos, clinical trial, Slug Mucosal Irritation assay

Poster

Predicting clinical discomfort with the SMI assay: The effect of tonicity, pH, cooling and warming agents

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Some chemicals, medicines and cosmetics cause stinging, itching, and/or burning (SIB) sensations. These discomforts often remain undetected until the clinical phase, since neither animal tests nor *in vitro* models have been able to detect these sensations. Slugs however, react on these stimuli. Consequently, a new variant of the Slug Mucosal Irritation (SMI) assay was developed, focusing specifically on the prediction of short-term SIB potential of test items.

The effect of tonicity (osmolality 280 to 2900 mOsm; NaCl 0.9-10%,), pH (1.7 to 7.4; lactic acid 0.1-10%), warming agents (capsaicin 0.001%-1%) and cooling agents (menthol 0.01-5%) was evaluated by placing three slugs per treatment group three times on 100 μl (liquids) or 100 mg (semi-solids) of the test item. After each 15 min contact period (CP), mucus production (MP) was measured (expressed as % body weight) and the slugs were transferred to a fresh Petri dish for a one hour rest period. Evaluation of the results is based upon the total mucus produced during three repeated CPs. Compounds are predicted into four classes of SIB potency: total MP < 3% (no SIB), 3% to 8% (slight), 8% to 15% (moderate) and total MP > 15% (severe). Additionally, the nasal tolerance of two isotonic (0.9%) and two hypertonic saline solutions (2.2% and 2.3%) was evaluated in 20 volunteers (study approved by an independent Commission for Medical Ethics, associated with the University Hospital Ghent, Belgium).

In the SMI-test the total MP increased with tonicity: isotonic solutions induced a total MP < 1%, while exposure to hypertonic solutions resulted in an increased MP (≈ 4% for 650 and 725 mOsm). In the clinical trial, the majority of the participants reported no to a very mild effect when administering the isotonic solutions. Any sensations disappeared within 15 min. For the hypertonic solutions, the majority reported very mild to mild effects. In a few cases even moderate discomfort was observed.

Concerning neurosensory discomfort, a clear concentration-response (CR) effect was observed for lactic acid: 0.5% caused an increased MP (> 3%); 10% resulted in very high MP (> 40%). The latter result may be explained by the combined effect of increased osmolality (1400 mOsm), a pH of 1.7 and the effect of the compound itself. Menthol also resulted in a CR-effect: a concentration of 0.01% already induced mild discomfort (total MP > 3%), but the effect was less pronounced compared to lactic acid. Results for capsaicin were not straightforward. A slight increase of MP was observed in all tested concentrations in comparison with the negative control, but MP did not increase proportionally with the concentration.

The new one day protocol of the SMI assay appeared to be a good tool in predicting clinical discomfort caused by nasal sprays with different tonicity: the higher the osmolality, the stronger the observed reaction in both humans and slugs. Investigating the ability to predict neurosensory discomfort, results for lactic acid and menthol agreed with literature, but capsaicin showed some anomalies.

Keywords: stinging-itching-burning, neurosensory discomfort, tonicity, pH, slug mucosal irritation assay

Lecture in Session VII: Nanotoxicology

In vitro model of inflammatory bowel disease for drug formulation testing and screening

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Evaluation and screening of novel drugs and formulations targeted for the treatment of inflammatory bowel diseases (IBD) so far have been conducted in rodent IBD models. Most of these models, such as the DSS and TNBS colitis model, induce an acute inflammatory reaction in the animal's GIT. The animals suffer from hematochezia, body weight loss, shortening of the intestine, mucosal ulcers and high mortality. The scientific value of these models is questionable, as the acute injury induced in this model poorly represents the chronic inflammatory condition observed in humans. To replace this scientifically and ethically questionable *in vivo* approach an *in vitro* model representing the pathophysiological changes in IBD is needed for medium to high throughput screening of anti-inflammatory drugs and formulations.

An *in vitro* co-culture model of inflamed intestinal mucosa was developed and established in our laboratory, consisting of Caco-2 intestinal epithelial cells and human blood-derived macrophages and dendritic cells (Leonard, 2010). An inflammatory reaction is triggered by the addition of the pro-inflammatory cytokine interleukin-1β. The model responds to the stimulation with an increased release of pro-inflammatory marker interleukin-8 and a rearrangement of tight junctional proteins accompanied by a reduction of barrier function and an increased mucus production. In this inflamed setup various budesonide formulations (free budesonide solution, 220 nm sized budesonide loaded PLGA nanoparticles, or 190 nm sized budesonide loaded liposomes) were applied to the model and IL-8 release of the cells was measured for inflammation monitoring. Transepithelial electrical resistance (TEER) was monitored to observe recovery in epithelial barrier function. Furthermore, particle deposition was observed using confocal laser scanning microscopy.

Free budesonide solution and PLGA budesonide nanoparticles were effective *in vitro* in treating the inflammation, as a decrease in IL-8 protein release as well as an increase in TEER value were observed within the first 24 h of treatment. Liposomal budesonide seemed to impair the barrier function, as we found an immediate decrease of TEER value and higher amount of IL-8 release compared to the non-treated inflamed control. The model was also able to differentiate between different levels of effectiveness in treating the inflammation: while IL-8 release increased again 48 h after treatment in cells incubated with free budesonide solution, the PLGA budesonide nanoparticles maintained their effect both with regard to IL-8 release as well

as TEER, confirming previous observations of prolonged availability of drug encapsulated in nanoparticulate formulations (Lamprecht, 2001). Nanoparticle formulations were applied to different compartments of the *in vitro* model. Liposomes were applied in the basolateral side of the system, co-localised with macrophages and dendritic cells. PLGA nanoparticles mostly accumulated intercellularly between epithelial cells, hinting at an intestinal enhanced permeability and retention effect. Thus the model also provides mechanistic insights.

In conclusion, the *in vitro* model of the inflamed intestinal mucosa is an adequate tool for the screening of novel IBD drugs and formulations, combining the convenience of *in vitro* studies with the complexity of pathophysiological changes of inflamed mucosal tissue. PLGA nanoparticles were the most effective formulation, while the liposomal formulation still needs further improvement in order to achieve better anti-inflammatory effects.

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Keywords: Caco-2, inflammatory bowel disease, pathophysiological, co-culture, nanoparticle, drug formulation

Poster

Comparison of the sensitivity of fibroblasts, renal epithelial cells and hepatoma cells to selected pharmaceuticals

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In the "alternatives" field, *in vitro* cell culture technology is promoted as the major contender for the refinement, reduction and replacement of animal use for human safety assessment of chemicals and pharmaceuticals. Indeed, cell culture technologies have many advantages, such as potential for high throughput, ease of use and also their relative cheapness. In addition, the fact that they are ideally suited to "omic" approaches has led to their use in many large EU projects as the basis to develop more predictive toxicity models. However, an issue that has not been extensively addressed is the specificity of these cell systems to target organ toxicity. To this end, we investigated the effect of long term repeat dose exposures of four nephrotoxins, three hepatotoxins and one CNS toxin to human renal proximal tubular cells (RPTEC/TERT1), human hepatoma cells (HepaRG), mouse embryonic fibroblasts (BALB - 3T3) and human renal interstitial fibroblasts (TK173).

HepaRG and RPTEC/TERT1 cells were time-differentiated for two weeks before treatment. All cell lines were treated daily for 14 days with five concentrations of the nephrotoxins cadmium chloride ($CdCl_2$), cyclosporine A (CsA), cephaloridine and cidofovir, the hepatotoxins troglitazone, diclofenac and amiodarone and the CNS toxin buflomedil. At days 1, 3, 7 and 14, lactate and LDH levels were measured in the supernatant medium. At day 14, cells were assayed for resazurin reduction capability and subsequently lysed in methanol. ATP level was measured in the methanol lysates.

In both HepaRG and RPTEC/TERT1 cells, a consistent finding was that lactate production increased prior to toxicity, demonstrating that these cell types can increase glycolysis in stress situations. Surprisingly however, RPTEC/TERT1 cells were the least sensitive to the chosen nephrotoxins and HepaRG cells were the least sensitive to the hepatotoxins. In general, the fibroblast cell lines were the most sensitive to the panel of toxins. There were also differences in 3T3 and TK173 cells responsiveness, for example CdCl2 was very toxic to 3T3 cells but not at all to TK173 cells.

The results demonstrate that fibroblasts are more sensitive to nephrotoxins and hepatotoxins than renal epithelial cells and liver cells. This is a somewhat surprising finding and the interpretation is not trivial. Fibroblasts may be more sensitive simply due to their undifferentiated nature and proliferative status. They may be unable to adequately increase stress related proteins, to extrude the compound via p-glycoprotein or to metabolise the compounds. Further work will be required to interpret this data more completely.

Keywords: target-organ toxicity, sensitivity, pharmaceutical

Lecture in Session VI: Acute and long term toxicity B

In vitro gastrointestinal mucosal surfaces for studying host-pathogen interactions at the mucosal interphase

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The mucosal tissues of the gastrointestinal tract present an enormous surface area to the exterior environment and therefore represent a site of infection or route of access for bacteria that cause human disease. Mucin glycoproteins are secreted in large quantities and cell surface mucins are a prominent feature of the apical glycocalyx of all mucosal epithelia. Cell-surface mucins play an important role in mucosal defence, since they may provide both a barrier and a reporting function. Bacteria have evolved to overcome this mucin barrier and understanding these interactions is vital to understanding and blocking bacterial invasion. The differences in pathogenicity observed between the human host and the animal model of disease may well be due to existing species-specific differences in the glycosylation patterns of mucins.

We have shown that several pathogens, including the gastric pathogen *Helicobacter pylori*, interact with mucins and that development and severity of disease depend on this interaction. Therefore, an *in vitro* cell culture system expressing the human mucin layer is a vital step in replacing and reducing animal experimentation in this field. The cell cultures that are currently used rely on transformed cells, which are sometimes of non-human origin and are suboptimal, because they are very different from an *in vivo* mucosal surface: they are often not polarised, lack important components of the glycocalyx and lack the mucus layer. We are developing gastrointestinal artificial mucosal surfaces optimal for studying host-pathogen interactions that are relevant for studying human disease at the mucosal interphase. The cell lines Caco-2, LS513, LS174TL, LS174TL8, HT29, T84, MKN1, MKN7, MKN28, AGS, NUG-4 and MKN45 were cultivated under a range of conditions and subjected to chemical as well as mechanical stimuli. Ussing chamber methodology was used to determine trans-epithelial resistance, membrane current and mucus secretion in response to stimuli. The samples were fixed using a special method to retain the mucus layer and stained (using immunohistochemistry) for the gastrointestinal mucins MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC6, MUC13 and MUC17. The cell lines varied significantly with regard to what mucins they express, what compartment of the gastrointestinal tract they most resemble and how they grow: some form 3-dimensional structures, some grow as a single cell layer, some are firmly adherent and polarised whereas others are loosely adherent and disorganised. We aim to develop both mucosal surfaces representing "pure" cell types (i.e. enterocytes, goblet cells, gastric surface mucus cells, etc.) as well as models representing mucosa containing mixed cell populations, such as colon and small intestine. We have promising results with regard to the cell compartment of the *in vitro* model of the mucosal surface where we have developed culture methods allowing some of the cell lines to differentiate into polarised cultures with growth patterns resembling those of gastric and intestinal mucosa. However, further work is needed for the development of the mucus layer mimicking the firm and loose mucus layer present *in vivo*.

Keywords: mucosal surface, epithelium, host-pathogen interactions, mucin, mucus

Poster

Cell viability and hepatocyte-typic functions in the HepaRG and LX-2 cell co-culture

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The metabolic activity of isolated hepatocytes decreases when cultured for longer times. The maintenance of hepatocyte-specific functions is an important challenge in the attempt to develop a high-fidelity human liver cell model that could be applied as an alternative testing model to the animal for long-time drug metabolism and hepatotoxicity studies. Heterotypic cell-to-cell interactions between hepatocytes and human stellate cells (HSC) are thought to be of benefit for the maintenance of hepatocyte-specific functions. *In vivo*, HSC are involved not only in liver injury but also in hepatic development, regenera-

tion and xenobiotic responses. One prospect is that cell-to-cell contacts with HSC are essential for hepatic progenitor cell amplification and differentiation, because HSC have several important functions, such as vitamin A metabolism, extracellular matrix reorganisation, production of various growth factors and cytokines. To explore the role of HSC on the hepatocyte-typic functions of proliferative and pre-differentiated HepaRG cells, we co-cultivated with the immortalised cell line (LX-2), which represents partially activated HSC (Xu et al., 2005).

Proliferating or differentiated (treated with 1% DMSO for 14 days) HepaRG cells (first human hepatoma cell line able to differentiate *in vitro* into hepatocyte-like cells) were co-cultivated with human LX-2 for 1, 7 or 14 days. Cell proliferation (MTT assay) and cell cytotoxicity (LDH assay) were determined. CYP-P450 expression was analysed by qRT-PCR. Albumin secretion as metabolic functionality was measured using ELISA assay.

Both HepaRG-LX-2 co-culture models led to a significant cytotoxicity against HepaRG monoculture after 14 days co-cultivation time but not after 1 or 7 days. The results of LDH release were in accordance with the morphological appearance that was observed using light microscopy analysis. The co-cultivation of proliferating or differentiated HepaRG cells with LX-2 did not improve CYP1A2 and 3A4 mRNA expression versus HepaRG monoculture over time. The albumin secretion in the differentiated HepaRG with LX-2 increased and stayed at the same level over time.

Under the described conditions co-cultivation of differentiated HepaRG cells with LX-2 could ameliorate some liver metabolic functions compared to the proliferating HepaRG-LX-2 co-culture, but not in comparison to the differentiated HepaRG cells in monoculture. Furthermore, both co-cultivation models did not maintain cell functionality over 14 days. Therefore, further optimisation of culture conditions is necessary to get a reliable HepaRG and LX-2 cell co-culture model for the long-term study of liver function in drug metabolism and hepatotoxicity.

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Keywords: HepaRG, HSC, LX-2, co-cultivation, liver, 3R

Poster

Effect of serum addition on cytochrome P450 dependent substance metabolism in primary human hepatocytes cultivated in a perfused 3D four-compartment bioreactor system

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Primary human hepatocytes are widely used as a reliable model for pre-clinical drug metabolism and toxicity testing. Fetal calf serum (FCS), commonly used to support hepatocyte maintenance *in vitro*, makes standardisation of culture models difficult, since it contains a large number of undefined factors. In addition, FCS affects analytical methods, such as proteomics and metabolomics, and could influence the bioavailability of lipophilic substances. Therefore it would be desirable to omit FCS in culture media for *in vitro* hepatocyte cultures.

Based on a four-compartment membrane capillary bioreactor technology for clinical bioartificial liver support, we developed a new miniaturised bioreactor prototype that allows 3D perfusion culture of liver cells in a down-scaled design for reduced cell numbers.

In this study we investigated i) the potential influence of serum addition on the adsorption of model substances with different binding properties to membrane surfaces within the cell-free bioreactor system and ii) the quality of primary human hepatocytes cultured in the bioreactor with or without FCS (2.5 %) supplementation over a culture time of 2 weeks. A culture medium specifically conceived for high-density perfusion culture of primary liver cells (Heparmed, Biochrom, Germany) was used. The quality and metabolic performance in hepatocyte cultures was determined by means of cytochrome P450 activities

(CYP1A2, CYP2B6, CYP2C9, CYP3A4/5 and CYP2D6) and further metabolic parameters.

Studies on drug adsorption performed with diclofenac, used as an example of low-binding drugs, showed the expected concentration in the medium circuit in the presence of FCS, while in the FCS-free group a slight loss of the drug was observed. In contrast, verapamil and amiodarone revealed high binding rates (up to 97% and >99%, respectively) with no obvious differences due to FCS supplementation. Hepatocytes maintained in the bioreactor system with or without addition of serum showed comparable metabolic performance of most parameters (albumin, urea, transaminase release) in both groups. CYP1A2 and CYP2D6 activities were also similar in both groups, while a tendency towards higher activity of CYP2B6, CYP2C9 and CYP3A4 was observed in the absence of FCS during the assayed culture period.

The results from drug binding studies show that FCS can influence the bioavailability of xenobiotics, indicating that an exact determination of the free concentration of substances is essential prior to drug testing studies. Furthermore, the findings from CYP activity studies in primary human hepatocyte bioreactors suggest that the use of serum is dispensable in bioreactor cultures maintained with a medium adapted for high-density perfusion culture. Thus, the miniaturised bioreactor represents an innovative tool for performance of drug metabolism studies under serum-free conditions.

Keywords: bioreactor technology, cytochrome P450, fetal calf serum, metabolic performance

Poster

Assuring consumer safety without animal testing: skin allergy case study

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Assuring consumer safety without the generation of new animal data is currently a considerable challenge. Skin allergy (sensitisation) is an important consumer safety endpoint for home and personal care products and an endpoint where *in vivo* assay data such as the mouse local lymph node assay are often needed to inform risk assessments. A conceptual framework for skin sensitisation risk assessment is currently under evaluation that does not require the generation of new animal data (Fentem et al., 2008; Maxwell et al., 2008).

The biology of skin sensitisation is complex but the majority of the key initiating events are understood: ingress of the ingredient into the epidermis, potential activation of the chemical by metabolism, covalent modification of proteins and cytokine-mediated activation of Langerhans' cells, which subsequently migrate to the lymph node and initiate a specific T-cell response. It is expected that information on more than one of these processes will be required in order to characterise hazard for risk assessment (Jowsey et al., 2006).

A number of non-animal approaches are currently under investigation that have the potential to provide information on the key process as described above, specifically: assessing epidermal bioavailability of an ingredient with *ex vivo* human skin; determining the reactivity profile of an ingredient; *in vitro* models to study the activation of Langerhans' cells and the inflammatory response from keratinocytes following ingredient exposure and approaches to assess the potential for skin metabolism of an ingredient.

The predictive capability of these non-animal approaches is under investigation and our recent work in this area is presented here. Various empirical modelling approaches have been applied, compared and contrasted to data both in isolation and in combination in order to predict the hazard potential and potency of ingredients. Particular emphasis has been on probabilistic approaches applied to various *in vitro* and *in silico* data for hazard characterisation. Our progress in evaluating the predictive capacity of these approaches and the underlying data for use in consumer safety risk assessment is presented.

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Keywords: alternatives, risk assessment, skin allergy, probabilistic, modelling

Lecture in Session AXLR8 '21st Century Toxicology' Info Forum

The EU FP6 project "VITROCELLOMICS"

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see late abstracts

Poster

CO_2-euthanasia of laboratory mice is strongly criticised. Are inhalant anaesthetics a better choice?

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Carbon dioxide (CO_2) is commonly used for euthanasia in laboratory rodents, but its use is discussed controversially from the animal welfare point of view. Existing recommendations and previous studies cannot provide clear information on the use of CO_2 and its impact on welfare. Alternative agents have not been well characterised yet.

It is our aim to assess the degree of stress caused by exposure to these anaesthetic gases in an objective and multilayered way in order to provide basic information for the development of euthanasia recommendations in laboratory rodents.

In male and female NMRI mice we compared the effects of common euthanasia methods using different filling rates of 100% CO_2, i.e. 20% (CO_2 20), 60% (CO_2 60), and 100% (CO_2 100) of chamber volume/min and an overdose of the volatile anaesthetics isoflurane 5% (Iso 5%) and sevoflurane 8% (Sevo 8%). Control animals were exposed to airflow with 20% of chamber volume/min. We investigated multiple types of biological responses to stress due to euthanasia by anaesthetic gases only during induction of anaesthesia until the point of loss of consciousness, hereby focusing on the period of conscious perception of stress and pain.

During the induction of anaesthesia, we recorded:
– behaviour,
– vocalisations in the audible and ultrasound range,
– course and changes of breathing motions.

After loss of righting and pedal reflexes and after 3 min of air exposure respectively, mice were decapitated and the following procedures conducted:
– gross examination of ocular and respiratory mucous membranes and of organs of the respiratory tract,
– measurement of blood glucose, plasma epinephrine and norepinephrine,
– histopathological examination of trachea and lung tissue.

Preliminary data revealed that during the induction of narcosis no vocalisations in the audible or in the ultrasound range could be detected. The loss of pedal reflexes tended to be reached within the shortest period after exposure to CO_2 100 < CO_2 60 < Iso 5% < Sevo 8%. CO_2 20 did not reliably induce unconsciousness within 5 min of exposure, so this group was excluded from further analysis. In the remaining anaesthesia groups, blood glucose levels were slightly increased in comparison to the control animals.

The present data suggests that CO_2 60 and CO_2 100 induce narcosis in a much shorter time period than isoflurane and sevoflurane. However, the remaining data need to be analysed to substantiate this assumption.

The elaboration of this project has been supported by a Dahlem Research School scholarship and by the Innovationsfonds 2009 of the *Freie Universität Berlin*. The project has been submitted for support by ZEBET (*Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch*) in Berlin.

Keywords: carbon dioxide, euthanasia, isoflurane, mice, sevoflurane

Poster

How to use available alternative methods to predict skin sensitization: lessons from a benchmark exercise on preservatives

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Allergic disease resulting from industrial or environmental exposure to sensitizers is the most common manifestation of immunotoxicity in humans. Allergen risk assessment of chemicals so far relies on animal assays. In the context of the 7th Amendment to the Cosmetic Directive and the REACH-legislation on chemicals, the cosmetic industry is concerned by the challenge of finding non-animal approaches to assess the sensitizing potential of chemicals.

Among the *in vitro* methods so far available for pre-validation studies, we developed the Myeloid U937 Skin Sensitization Test (MUSST) based on the induction of CD86 on U937 cells. Years of in-house experience with this assay led us to identify its limits and to develop further methods (apoptosis assay) and further models (including 3D-models) to overcome these limits. During the past two years, we enlarged our set of cell-based assays by incorporating the well-described direct peptide reactivity assay (DPRA). Currently, we are addressing the question of the integration of *in vitro* data that we are now able to produce, together with *in silico* data, in order to predict skin sensitization potency of cosmetic ingredients. While the generation and the detailed analysis of complete data on a large set of raw materials is ongoing, we present here the analysis of the results obtained on a benchmark of 25 preservatives.

This study allowed us, in a very pragmatic approach, to gain insight into the feasibility, but also the actual limitations of using *in vitro* methods for the screening and/or the evaluation of new chemicals for skin sensitization potential.

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Keywords: skin sensitization, in vitro methods, MUSST, DPRA, preservatives

Poster

AquaSpec™ MIR analytics as a broad and fast screening-method to classify toxic effects of agents on cells

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Since the REACH regulation came into force in June 2007, the risks posed by all chemicals have to be evaluated. With respect to the 3R strategy and the large number of chemicals which have to be tested, new methods and rapid and widely applicable screening tests are required. The AquaSpec™ MIR (mid infrared) technology is a simple, fast and cost-effective solution for investigating the effects of chemicals on cells. Our toxicity assay scans the toxin-induced changes in cell metabolism by detecting specific spectral patterns in the culture media and cell lysates. Furthermore, details of the effects of chemicals on cells, i.e. influences on metabolism and the break down of the applied chemicals can be tracked down. Due to its sensitivity to all chemical bonds and functional groups in molecules, the AquaSpec™ technique is suitable to create a biochemical snapshot of all events that reflect the status of what is going on in the cell. Moreover, the concentration of compounds, their kinetics and even the decay of substrates can be quantified. That way, it can be visualised whether a substance is taken up and metabolised by the cells and if so, to which products it is converted. For example, the metabolism of glucose and the release of CO_2 are followed in order to get information about the cellular respiration capacity.

In an experiment, liver cells were treated with agents of known toxic characteristics, such as toxic to cell metabolism, genotoxic and mutagenic. For control, harmless reference chemicals were applied. Samples of culture media were taken and characterised by multivariate data analysis. Abnormal reactions, for example stress reactions which are triggered by the toxic agents,

were classified in a cluster model. Within less than 6 hours, substances could be classified as toxic and non-toxic. Additionally differentiation into toxic subclasses was possible after less than 12 hours of cell cultivation.

In current studies a screening-assay of toxic compounds based on the experimental model eukaryote yeast is investigated. Yeast cell culture testing offers the possibility of more robustness and easier handling as compared to human cell culture testing. First experiments are designed to verify whether the results obtained on human cells are reproduced on yeast cells.

In summary the AquaSpec™ Technology is characterised by a high accuracy of measurement and reproducibility. Compared to alternative techniques, which are based on cell culture testing, the native state of the cell cultures can be investigated with almost no artificial sample preparation. In the future the technology can most likely be an important implement in the evaluation of chemicals on living cells due to its wide applicability, its speed and its cost-efficiency. That way, a classification of the impact of the substances on cells into toxic, genotoxic, mutagenic, eco-toxic or even teratogenic seems to be possible. In conclusion, our toxicity-assay is characterised by speed, easy handling, and the generation of reliable results.

Keywords: IR-spectroscopy, screening, cell culture testing, toxicity assay

Poster

New pieces to the puzzle: is the LLNA overestimating the sensitization potential of surfactants?

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An integral part of hazard and safety assessments for consumer and occupational health is the estimation of a chemical's potential to cause sensitization. Both the LLNA (OECD 429) and guinea pig based tests (OECD 406) are accepted test methods. As a 3R method, the LLNA has become the preferred method for sensitization testing under REACH. Comparative test data resulting from LLNAs and GPMTs reveal that there is a disproportionately large number of discordant results when testing certain substance classes, e.g. surfactants and fatty acids (Kreilingeret al., 2008; Mehling et al., 2008). In these cases, the results from LLNAs generally indicate a sensitization potential that warrants classification whereas the GPMT results do not. In this context it should be noted that the surfactant sodium lauryl sulphate is considered to be the classic "false positive" in the LLNA (Basketter et al., 2009). To decide whether the GPMT or the LLNA result correctly predicts the sensitization potential in these cases, it has been proposed to use a weight-of-evidence approach (Basketter et al., 2009). In order to gain more insight into the responses induced by exemplary surfactants, a battery of *in vivo* and *in vitro* tests was therefore conducted using exactly the same batch of chemicals. These included LLNAs (with B220 cell counting), GPMTs, and various *in vitro* tests including LC-MS based peptide reactivity assays and anti-oxidant response element (ARE)-assays (KeratinoSens, Emter et al., 2010). Results from these tests indicate that the LLNA may be overestimating the sensitization potential of these test substances: All the surfactants were negative in the GPMTs and KeratinoSens assay and did not form covalent adducts with test peptides, thus confirming the GPMT result. As results obtained from LLNAs are increasingly being used as the gold standard for the development of new non-animal alternative test methods, results such as these highlight the necessity to carefully evaluate test results and the applicability domains of different tests methods. It is also important to stress that an accurate database is needed to develop and validate satisfactory non-animal alternatives for sensitization testing.

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Keywords: sensitization, LLNA, GPMT, KeratinoSens, peptide reactivity

Poster

In vitro assessment of skin and mucosal tolerance of cosmetic products

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Today validated alternatives for acute skin irritation, skin corrosivity, photo-toxicity and dermal absorption are available and implemented for REACH registrations and to define the toxicological profile of ingredients. Based on specific protocols, several epidermal 3D models are the biological systems of validated alternatives for skin corrosion (OECD 431), skin irritation (draft OECD, 2009) and human corneal epithelium (HCE). The latter is currently in the pre-validation process for acute eye irritation testing. Because of their unique advantages (multilayered structure, barrier function allowing application of doses relevant for *in vivo* applications, living and metabolically active tissue) they are suitable systems to assess finished product safety for internal risk assessment and as a screening tool during product development. Cosmetic industry has invested in the application of established *in vitro* parameters and protocols for ingredients in order to assure a more accurate, sensitive and predictive safety assessment of finished products in order to comply with the incoming European cosmetic regulation asking for deeper and robust testing of finished products. On the basis of the modified Multiple Endpoints Analysis (MEA) the aim of this work was to assess *in vitro* skin and mucosal tolerance on a series of prototypes and commercially available cosmetic formulations. Many of them had been previously classified by the results of the *in vivo* patch test on humans or by clinical testing.

The SkinEthic reconstructed human epidermis (RHE) and human vaginal epithelium (RHV) models were used as biological systems. 31 cosmetic products (13 leave-on creams, 13 rinse-off formulations and 5 intimate hygiene products) were tested compared to positive and negative controls for skin and mucosal tolerance using the following testing parameters: cellular viability by MTT test method, histomorphological evaluation by H&E, transepithelial electrical resistance measurement (TEER), IL-1α release by ELISA and a transcriptomics approach based on quantitative mRNA quantification of two early bio-markers, i.e. occludin and TNFα, by Taqman technology (qRT-PCR).

The following exposure times based on product type specific protocols after acute exposure were used: 1 h followed by washing and 16 h recovery for rinse-off (for skin and mucosa) and 24 h and/or 24 h + 24 h recovery for leave-on (for skin).

All parameters tested were shown to be relevant for the assessment of the irritation potential: a deeper analysis of this complementary information allows better discrimination between products and the definition of specific prediction models. Finally a good correlation between *in vitro* and *in vivo* results was found.

Keywords: skin irritation potential, MEA, transcriptomic approach

Poster

New approach for chemical sensitizing potential assessment using THP-1 and NCTC 2544 co-culture

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The skin is continuously challenged by environmental antigens (xenobiotics, allergens, chemicals) that may evoke protective or detrimental immune responses. Haptens penetrate the skin and become sensitizing entities by binding to epidermal proteins. These hapten-carrier complexes are immunogenic and can elicit the development of allergic contact dermatitis (ACD), one of the most frequent allergic skin diseases. Dendritic cells (DC), as key players in the initial process of skin sensitization, have been considered in the development of *in vitro* alternatives for detection of contact allergens.

In order to assess the sensitization potential of different chemical sensitizers, a KC-DC surrogate co-culture (NCTC-2544 hu-

man keratinocytes and THP-1 cell line) has been used as a relevant model to study the cross-talk between keratinocytes and dendritic cell after contact with the sensitizer, allowing objective and reproducible quantification of complementary testing parameters. This loose-fit co-culture develops into an allergen-sensitive system consisting of keratinocytes (KC) and of mobile DC-related cells.

Recently, several studies demonstrated that human myeloid cell lines, such as THP-1, are good surrogates for DC, and this alternative method was validated in Japan. The property of the monocytic cell line THP-1 (h-CLAT) to discriminate between skin sensitizers and non-sensitizers is currently under evaluation by Colipa and undergoing the prevalidation/validation process at ECVAM.

The THP-1 cell line was used to find the sub-cytotoxic concentration of products to be evaluated for skin sensitization potential. MTT assay was performed after short and long term exposure. Changes in THP-1 transcriptional activity following direct contact at the defined concentration were evaluated. CD86, CD54, CCR7 were chosen as skin sensitization biomarkers, β-actin was used as endogenous control to normalise mRNA levels.

The nuances of sensitization potential can be strong, moderate, weak, or non-sensitizing. The following positive control chemicals were used for each category: chloro-dinitrobenzene, hydroquinone, ethylendiamine, hexylcinnamaldehyde and lactic acid.

The results reported in this study show that this loose-fit co-culture model was able to discriminate between skin sensitizers and non-sensitizers. The new approach, studying the transcriptional modification of the DC cell line co-cultured with the KC cell line following contact with a chemical, provides a complementary alternative method to existing assays.

Keywords: skin sensitization, THP-1, h-CLAT

Poster

A leading case for the (dis-)approval of applications for animal experiments

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On the 7th of October 2009, after three years of litigation, the Federal Supreme Court of Switzerland put an end to the legal dispute regarding two animal experiments on primates. The "Zurich monkey experiments", which had been approved by the canton's veterinary office despite a contrary recommendation by the advisory committee on animal experimentation, leading to appeals by the commission, may definitively not be performed. Even though this judgement does not generally preclude experiments on non-human primates, it is a milestone success in the struggle against animal experiments. It certainly will influence the future approval practice.

The Supreme Court judgement clarifies four points that are of a general nature for animal protection and so have implications over and above the two cases in question: 1) Some animal experiments are apparently approved wrongly. 2) In the approval process the suffering caused and the violation of the animals' dignity is, to some degree, not given sufficient weight. 3) In turn, the expected research results and their use for humans are, to some degree, given too much weight. 4) The weighing of interests therefore produces an unfair result in which the suffering of the animals in comparison to the expected gain of useful knowledge appears defensible when in fact it is not.

Among other points, the justification of the judgement highlights the importance of the advisory committee, which is of great value for its legitimisation. The justification explains that the law demands that animal experiments be limited to an "indispensable measure" and that it is one of the committee's tasks to interpret such vague legal terms. A deviation from their recommendation therefore would only be acceptable with very good justification.

The judges laid great importance on the weighing of interests. They emphasised that the gain of rudimentary knowledge about human physiology cannot justify severe suffering of experimental animals. In both cases in question the Federal Supreme Court considered the burden for the animals to be disproportionate. This makes the judgement a leading case.

The judges also stressed the value of the animals' dignity, which was given a high status in the Swiss Animal Protection Law in 2005. Dignity carries special weight in the case of non-human primates on account of their strong genetic and sensory similarity with humans. The expected gain of knowledge would thus have to be far higher than in the "Zurich monkey experiments" to justify the animals' distress and the violation of their dignity associated with the proposed experiments.

This time a number of primates were lucky to be saved by the Federal Supreme Court's judgement. Hopefully this judgement will have further consequences: The right of the advisory committee to appeal against approvals that have already been issued is currently only applicable in the canton of Zurich. It has proven to be an additional and important instrument to prevent illegal animal experiments. This right of appeal must therefore be awarded to all advisory committees in Switzerland and should also be demanded for committees abroad.

Judgements and justifications of the Supreme Court (only in German) http://www.bger.ch/index/juridiction/jurisdiction-inherit-template/jurisdiction-recht/jurisdiction-recht-urteile2000.htm (click on "*Staats- und Verwaltungsrecht*" and put "*Primaten*" as keyword for the search).

Keywords: primates, ban, Supreme Court

Poster

Nimesulide dynamics in membrane model systems: can membrane effects determine activity?

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Nimesulide is a common NSAID, widely used despite some reported secondary effects that include hepatotoxicity and interference with mitochondrial physiology. It acts by inhibiting COX, but its proneness to induce alterations in biophysical properties of membranes has been proposed as an additional effect contributing to its anti-inflammatory action. Therefore, in this study we carried out experiments in order to detect nimesulide binding to membranes and its effects on the biophysical properties of different membrane model systems by using differential scanning calorimetry and fluorescent probes.

Nimesulide promotes a decrease in membrane order, but it does not seem to promote lateral phase separation in a less stable binary lipid system consisting of DPPE and TOCL (7:3). The consequences of increased fluidity on membrane permeability were studied by performing calcein release studies, either in a simple monomeric model (DPPC) or in a ternary one that mimics the mitochondrial membrane (1 DOPC: 1 DOPE: 1 TOCL). These studies revealed a moderate permeabilising effect of nimesulide. 31P NMR studies were also performed with the same trimeric model, pointing out that nimesulide is a stabiliser of lamellar arrangements, increasing the lamellar/HII transition temperature.

Nimesulide quenching of the fluorescent probes DPH and DPH-PA was used to investigate the compound distribution across the bilayer thickness in both the single and the ternary lipid system, in the latter case at both 25 and 70ºC.

Finally, these same fluorescent probes were used to monitor membrane dynamics in the different lipid systems by means of anisotropy studies, determining if lipid composition and membrane structure may affect the activity of nimesulide in such lipid environments.

The present study reinforces that nimesulide is a membrane-active compound capable of interfering with membrane biophysical properties and dynamics, and therefore with physiological processes that are membrane-mediated/modulated, as may be the case of the activity of inflammatory enzymes. The relevance of these findings is also discussed in terms of the reported nimesulide interference with mitochondrial physiology.

Keywords: nimesulide, membrane model system

Poster

Cultivation of primary human hepatocytes in a 3D membrane bioreactor as an alternative to 2D culture systems for improved hepatotoxicity testing

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Three-dimensional cell cultivation represents an important step towards the *in vivo* situation. Hollow-fibre bioreactors, which support the formation of tissue-like structures, are promising tools in this approach. The cultivation of primary human hepatocytes and its improvement has been a major field of research over the last decades, resulting in alternative cell cultivation systems that exhibit good cytochrome P450 activity profiles. This is a prerequisite for good hepatotoxicity prediction.

In this study, which is part of the BMBF-HepaTox project, we present a 3D-hollow fibre bioreactor system for the cultivation of human hepatic cells. The bioreactor was designed for analytical purposes, leading to the possibility of in-depth characterisation of the system. Using primary human hepatocytes, we analysed both general parameters, such as membrane integrity, oxygen uptake and substrate consumption, as well as liver-specific functions, like drug-metabolising capacities and production of albumin.

Our results demonstrate that long-term maintenance of primary human hepatocytes within the 3D-bioreactor system is possible. Oxygen uptake, substrate consumption and albumin production were stable for over 14 days of incubation. Cytochrome P450 activity was first assessed on cultivation day 5 and could still be detected even after 18 days of cultivation.

This study represents an overall description of the 3D-bioreactor system including newly developed modifications. Maintenance of cytochrome P450 activity *in vitro* for longer periods is the bottleneck in their use for chronic toxicity testing. 3D cultivation of primary human hepatocytes in the system presented here is promising, not only for drug screening and acute toxicity studies but also for chronic hepatotoxicity studies.

Keywords: 3D-bioreactor cultivation, primary human hepatocytes, chronic toxicity studies

Poster

Sensitive assessment of drug-induced changes in primary human hepatocyte metabolome using a combination of GC-MS and multivariate statistics

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Long-term, chronic cellular changes and idiosyncratic drug effects are difficult to detect using common endpoint toxicity assays. Nowadays, sensitive and non-invasive methods using human relevant cell systems are an intense focus of research. Metabolomic approaches are more sensitive using analytical methods such as gas-chromatography coupled to mass spectrometry. Multivariate statistics could lead to an optimised overall interpretation of the chromatographic data.

In this study, as a part of the BMBF-HepaTox project, we tested therapeutic concentrations of well-known reference drugs on 2D-cultures of primary human hepatocytes, which are still the "gold standard" concerning human hepatotoxicity studies.

Supernatant samples were analysed using gas chromatography time-of-flight mass spectrometry (GC-TOFMS) combined with principal component analysis (PCA).

Over 100 peaks were found per sample in the chromatogram and identified using standard reference libraries. Biologically relevant metabolites were normalised to internal standard, autoscaled and applied to PCA. Changes in the metabolome of primary human hepatocytes in response to exposure to therapeutic concentrations of the drugs were detected using this approach. Cell viability remained unaffected compared to the untreated control during the experiments.

This is a novel method to assess drug-induced changes in cellular metabolism at therapeutic concentrations. It can be adapted to any other alternative testing system based on *in vitro* models, such as human cell lines, stem cells or stem cell derived cells instead of animals, and to other cell cultivation setups, such as 3D cell cultures.

Keywords: GC-TOFMS, principal component analysis, metabolomics, hepatotoxicity

Poster

The cytotoxic effects of fumonisin B1 and ochratoxin A on human and pig lymphocytes using methyl thiazol tetrazolium (MTT) assay

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In this study the effect of two mycotoxins, fumonisin B1 (FB1) and ochratoxin A (OTA), both singly and in combination, on isolated human and pig lymphocytes was investigated in the methyl thiazol tetrazolium (MTT) cytotoxicity test. Although a previous study on cells had shown the potential synergistic effects of this combination (Creppy et al. 2004), it was anticipated that comparable effects on pig lymphocytes would provide a more accessible source of cells for cytotoxicity testing.

Lymphocytes were isolated from venous blood of a healthy human donor and weaned pigs of Hungaro-Seghers genotype. The MTT assay was performed using phytohaematoglutinin (PHA) as a cell stimulant. Various concentrations (10, 20 μg/ml) of FB1, OTA and both mycotoxins combined were added. Cytotoxicity was determined after 48, 72 and 96 h incubation. The optical density (OD) was read and cell proliferation was determined as percentage viability of the control: % viability = (ODM/ODC) x 100 (M = mycotoxin-treated cells; C = control cells).

The results showed a progressive decrease in lymphocyte viability with time of exposure. Cell viability decreased on average by 3.5 and 11.3% after treatment with FB1 at 5 and 20 μg/ml, respectively; and 10.8 and 14.0% for OTA at 5 and 20 μg/ml, respectively, after 24 h of cell exposure to the mycotoxins. With further incubation, the viability decreased further. After 96 h viability had decreased by 16.3 and 23.5% for FB1 at 5 and 20 μg/ml, respectively, and 21.2 and 39.3% for OTA at 5 and 20 μg/ml, respectively. Pig lymphocytes showed a decrease in cell viability by 8.8 and 13.0%, respectively, for FB1 and 8.8 and 16.7% for OTA at 5 and 20 μg/ml, respectively, after 24 h of cell exposure. After 96 h of exposure to FB1, the viability of pig lymphocytes decreased by 20.3% and 29.1% and for OTA by 22.2 and 41.5% at 5 and 20 μg/ml, respectively.

The cell viability decrease was 53.4 after 24 h and 70.0% after 96 h of cell exposure to both FB1 and OTA (5 μg/ml of each mycotoxin) on pig lymphocytes, and 56.4 after 24 h and 76.3% after 96 h exposure to 20 µg/ml of each mycotoxin. Human lymphocytes showed a lesser but significant decrease of cell viability by 47.7 (24 h) to 33.5% (96 h) at 5 μg/ml exposure, and 53.8 to 69.6% at 20 μg/ml (after 24 and 96 h, respectively) as compared to results obtained with pig cells.

In summary, FB1 as compared to OTA had a lower cytotoxicity on both human and pig lymphocytes. A synergistic decrease of cell viability in both human and pig lymphocytes was observed, with pig lymphocytes showing a greater sensitivity to both mycotoxins. The MTT assay can be used for the determination of cytotoxicity of mycotoxins using animal, and in particular pig, lymphocytes, which avoids the use of human donors.

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Keywords: mycotoxins, cytotoxicity, synergistic, lymphocytes, methyl thiazol tetrazolium

Lecture in Session III: Skin sensitisation and eye irritation

Prediction of skin sensitizers by combining a high-throughput keratinocyte-based reporter gene assay with peptide reactivity measurements

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Skin sensitization is caused by protein-reactive chemicals (Gerberick et al., 2008), which modify endogenous proteins in the skin, thus rendering them immunogenic. Skin sensitizers then activate the immune system, leading to the proliferation of T-cell clones specific to these modified proteins. This process thus involves a chemical and a biological step. The biological part involves activation of early innate signals and later on the specific proliferation of T-cell clones. Progress has been made to model the early innate signals with an *in vitro* assay. The Nrf2-pathway is a toxicity pathway generally activated by skin sensitizers (Natsch, 2010).

It is widely accepted, that certain complex toxicological endpoints such as skin sensitization may only be predicted with an integrated testing strategy based on a battery of *in vitro* assays. Here we thus present our approach of combining a biological and a chemical assay: (i) The KeratinoSens assay is a new, highly standardised assay that measures Nrf2-dependent gene activation and cytotoxicity of chemicals in parallel in a high throughput format. For each chemical full dose-response curves are measured. We show that sensitizer-induced gene activity is not directly related to cytotoxicity, as gene induction by skin sensitizers occurs at subtoxic concentrations. (ii) In parallel, an LC-MS based peptide reactivity measurement monitors peptide depletion and peptide-adduct formation to characterise the potential for covalent protein binding (Natsch and Gfeller, 2008). We summarise results in both assays on a list of 67 reference chemicals. By combining positive evidence from both assays an overall accuracy of 89.6% is achieved. In addition, we present data on the use of this approach for specific applicability domains: We show that (a) terpenes acting as pre-haptens (i.e. chemicals becoming skin sensitizers upon air oxidation) can reliably be identified with an oxidation step prior to the assay and that (b) photosensitizers absorbing in the UVA-range activate the reporter gene if an irradiation step is included in the assay.

Finally, this approach has already been integrated into our chemical discovery process for an early prediction of the sensitization risk without the use of animals, and we present case studies from this industrial application of a novel approach in *in vitro* toxicology.

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Keywords: skin sensitization, reporter gene, high-throughput assay, Nrf2, antioxidant response element, peptide reactivity

Poster

The proliferation of fibroblasts in human serum as alternative for fetal calf serum

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Cell culture is an important tool in biotechnological research. However, cells in culture are still universally supplemented with fetal calf serum (FCS), a complex mixture that contains growth factors required to maintain cell function and proliferation. This form of supplementation is far from ideal, as its quality varies from batch to batch, its composition is not completely known, and it may contain virus and prion contamination and may also cause immunologic complications. Ethical questions concern-

ing the procurement and economical factors are also of great relevance. Owing to these problems, there is a worldwide effort to find alternatives to the use of xenobiotic elements in cell cultures.

In this study we assayed human serum as a replacement for FCS in human fibroblast culture. Human serum was obtained from blood of 10 healthy volunteers submitted to serological evaluation. Fibroblasts were obtained from samples of healthy skin obtained from the tissue bank of the Hospital das Clinicas donated for research purposes. Fibroblasts were cultivated in multiwell plates with 10% FCS or 10% human serum in DMEM. After 24, 48, 72 and 96 h cells were counted in a hemocytometric chamber. Results were expressed as mean ± standard deviation to obtain the proliferation cell curve. There was no statistical difference between the two proliferation curves.

Human serum fully supported growth and proliferation of human fibroblasts, showing its high potential as a substitute for FCS in cell culture.

Keywords: cell culture media, human serum, fetal calf serum

Poster

Toxicity and mode of action evaluation of commonly used adjuvants on the basis of bacterial gene expression profiles

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Adjuvants are compounds that modify the effects of other compounds (mostly active ingredients) without having any direct effect themselves. They are added to the active ingredients to enhance performance or handling. This large and heterogeneous group of chemicals is used in pesticides, detergents, personal care and many other products. Despite their widespread use, very little toxicological information and no clear legislation exist at the moment.

In this study we use a bacterial reporter assay to gain insight into the toxicity and toxicological mode of action of several adjuvants (alcohol ethoxylates, alkyl phenol ethoxylates, alkyl amine ethoxylates, organosilicone surfactants, solvents, etc.). The *Escherichia coli* based reporter assay consists of a selection of genetically modified strains responding to different types of stress, like DNA damage, oxidative stress, protein denaturation, membrane damage, osmotic stress, general cellular stress and heavy metal presence. The results of this *in vitro* alternative method were compared with classical LC_{50} values from literature where available.

Keywords: bacterial reporter assay, stress genes

Poster

3D organoid like culture of Hep G2 using alginate matrix for hepatotoxicity assessment

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A wide range of *in vitro* test systems and methods have been developed for the assessment of hepatotoxicity. Two-dimensional (2D) cell cultivation systems (using primary hepatocytes, microsomes or cell lines) are commonly used, but these do not offer the possibility of long-term studies for chronic toxicity and, very importantly, do not represent the *in vivo* situation.

In this study, we used alginate matrix for three-dimensional (3D) cultivation and maintenance of Hep G2 cells. 3D cultures of Hep G2 cells were prepared using alginate matrix. Metabolic parameters such as glucose consumption and urea production were determined. Moreover, the storage of glycogen was assessed and compared to 2D cultures. Finally, dose-response

curves of well-studied reference drugs were assessed and compared to 2D-systems.

Long-term cultivation over two weeks was successfully performed, whereby 3D organoid formation was microscopically observed. Glucose consumption and urea production were observed over the whole cultivation time. The storage of glycogen was significantly greater in 3D-cultures compared to 2D-maintained cells. Toxicity of well-studied reference drugs was assessed and EC_{50} values were calculated.

The results show that 3D alginate cultures are not only suited for long-term liver cell cultivation but can also be applied in preclinical drug screening. The system offers an alternative to both conventional *in vitro* cell systems and animal test models for long term toxicity assessment. Furthermore, it can be adapted to other cell types, such as primary liver cells or stem-cell derived cells.

Keywords: 3D cultivation, alginate cultures, HepG2

Poster

Evaluation of a biochip technology for the prediction of metabolism-mediated toxicity

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The aim of the present work was to assess the ability of a MetaChip/DataChip technology to predict metabolism-mediated toxicity.

A set of 67 industrial chemicals was assessed using diverse combinations of mammalian organ-specific cells and enzyme mixtures. Cytotoxicity measurements were performed for the entire set and the analysis of the data generated confirmed the capability of the technology to reveal metabolism-mediated toxicity and to differentiate phase I from phase II enzyme activities. The findings also suggested that the platform could be used to detect species and tissue-specificity.

The second stage of this study focused on selecting the combination of metabolising enzymes that would be suitable for predicting rat *in vivo* acute toxicity. For this purpose, test substances were categorised as toxic or non-toxic on the basis of an arbitrarily defined LD_{50} threshold of 500 mg/kg. A decision tree that considered the responses in human and rat hepatoma cell lines was constructed.

The application of the logic sequence proposed resulted in good performances (sensitivity and specificity of 85% and 78%), indicating that the biochip concept can be used, not to predict all possible *in vivo* toxic effects, but rather to estimate the risk of failure in conventional long-term *in vivo* studies.

Keywords: metabolic toxicity, systemic toxicity, biochip technology

Poster

Real-time and label-free monitoring of bioenergetics and cell impedance/adhesion for long and short term cell-based functional assays *in vitro*

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Advanced *in vitro* methods may help to identify pharmacodynamic properties of substances and prediction of compound toxicity in early phases of development. Two major aspects are mostly relevant for pharmaceutical studies. The first one is the investigation of short-term effects involving e.g. membrane transporter mechanisms or receptor signalling. The second one deals with long-term effects mediated by toxins and xenobiotics. Therefore, the functional analysis of living cells in a physiologically controlled environment, which provides information about pharmacological and toxicological as well as metabolic

properties, may serve as an alternative to animal experiments.

Bionas® analysing systems detect physiological parameters of cell lines and primary cells in label-free and non-invasive assays. The multi-parametric sensor chip continuously measures the oxygen consumption and the extracellular acidification, which both determine the acute rates of cellular energy metabolism. In addition, cell impedance/adhesion measurement detects alterations in the cellular adhesion and morphology of cells (Ehret et al., 1997, 1998). A perfusion system guarantees highly defined cell environmental conditions and allows parameter recordings in long and short-term studies.

By generating profiles of bioenergetics (glycolysis and mitochondrial respiration) and cellular impedance, the systems monitor the action of substances and their cytotoxic effects, including potential regeneration of cells in the area of toxicology, oncology and drug discovery, respectively. Thus, specific data from the drug/cell interaction can be determined (Thedinga et al., 2007). These data may either be generated by long-term studies over hours to days or via short-term detection of cellular responses. In the field of drug development, membrane transporter-mediated mechanism and receptor interaction studies are the major focus.

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Keywords: acidification, respiration, cell impedance, metabolism, toxicity, regeneration

Poster

Silencing of neuropathy target esterase (NTE) causes changes in gene expression during the early stages of differentiation

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Neuropathy Target Esterase (NTE) was initially identified as the primary target of some organophosphorus compounds that cause delayed neuropathy. Some *in vivo* studies suggest that this protein may also perform a critical function in embryonic development and therefore also in cell differentiation. An interfering RNA approach has been used in order to investigate the influence of NTE on the proliferation and differentiation of D3 mouse embryonic stem cells. The silencing of NTE reached a maximum effect 48 h after the treatment. In this point the expression of the gene was reduced to 80% of control. Although their expression levels were fully recovered a day later, our studies have shown that this inhibition is sufficient to cause important changes in the process of differentiation. We have studied the expression of several genes after 48 h and 168 h of NTE silencing. Patterns of expression of different gene structural markers have been altered in samples where NTE was previously silenced. The most relevant change was found for the expression of Nestin, a neuroendothelial gene marker, which was decreased to 60% of control 96 h after silencing (when NTE was totally recovered from its silencing). Our results suggest that NTE might play an important role in cell differentiation since its silencing alters the gene expression of neuroectoderm.

This study was supported by the Ministry of the Environment of the Government of Spain (Grant A051/2007/3-14.4).

Keywords: NTE, stem cells, differentiation, gene expression, embryonic development

Poster

Resveratrol may protect proteins from toxic effects of oxidative *stress in vitro*

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Proteins are vulnerable to oxidative stress. Reactive oxygen/nitrogen species (ROS/RNS) can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages and oxidation of the protein backbone resulting in protein fragmentation ultimately malfunctioned, which may lead to premature aging and related pathological events. Present work is done to evaluate the protective effect of resveratrol, a polyphenolic compound occurs naturally in the grapes and red-wine on plasma proteins form the toxic exposure of oxidative stress in humans.

Study was carried out on 39 normal healthy subjects of both the sexes. *In vitro* oxidative stress was induced in plasma with incubating tert-butylhydroperoxide (t-BHP) (10-5 mol L-1). Effect of resveratrol was studied on markers of protein oxidation; protein carbonyls (PCO), advanced oxidation protein products (AOPPs) and total thiols.

Resveratrol provided a strong protection on plasma proteins oxidation as evidenced by protecting the depletion of thiols and formation of PCO and AOPPs during oxidative stress. Effect of resveratrol was dose as well time dependent, a significant effect was observed on only 15 min of incubation.

Resveratrol elicits a potent and fast antioxidative property. We assume that administration of resveratrol/resveratrol rich diet may give protection towards development and progression of many degenerative diseases where oxidative stress is prominent.

Keywords: resveratrol, oxidative stress, proteins

Poster

Characterisation of cell death induced by palytoxin on an *in vitro* keratinocyte cell model

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Palytoxin (PLTX), produced by Palythoa zoanthid corals and Ostreopsis dinoflagellates, is one of the most poisonous compounds known. In the last two years, dermatological problems have been associated with cutaneous exposure to PLTX (Deeds and Schwartz, 2009; Durando et al., 2007; Hoffmann et al., 2008; Nordt et al., 2009). Nevertheless, little is known about its cutaneous toxicity. Recently, we performed a study on HaCaT keratinocytes as an *in vitro* predictive model for evaluating human skin toxicity (Gibbs, 2009), defining the potential toxicity of PLTX on human skin (Pelin et al., 2009). The aim of this study was to investigate the mechanisms leading to cell death induced by PLTX.

Appearance of cell necrosis was evaluated investigating lactate dehydrogenase (LDH) release (Tox-7 kit, Sigma-Aldrich) and propidium iodide (PI) uptake in the cells. Apoptosis was investigated by evaluating caspase 3/7 activation (Sensolyte™ Caspase Sampler kit, Anaspec) and DNA staining with 4',6-diamidino-2-phenylindole (DAPI). ROS production was investigated by the NBT reduction assay in cells exposed to increasing concentrations of PLTX for 1 h. Cell morphology was evaluated by confocal microscopy after staining of the plasma membrane with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiL).

After 1 h exposure, PLTX induced a concentration-dependent increase of superoxide anion production, which was inhibited by pretreatment with 10 μM ouabain. A 4 h exposure to PLTX caused a concentration-dependent leakage of LDH (EC_{50} of 18.5 ±1.1 nM), indicating the loss of plasma membrane integrity. PLTX-induced cell death was confirmed by PI uptake studies that demonstrated the presence of the probe inside the nuclei. Conversely, no activation of caspase 3/7 was detected and nuclear staining with DAPI did not revealed the presence of apoptotic bodies after 4-8 h exposure to 1 and 10 nM PLTX. Immunocytochemical analysis revealed dramatic morphological modifications with plasma membrane rupture and leakage of cytoplasmic material, already evident after 1 h of contact with 1 nM PLTX.

In a previous study we characterised the cytotoxic effects of PLTX on the *in vitro* skin irritation model HaCaT keratinocytes.

In these cells, 4 h exposure to PLTX reduced cell viability with an EC_{50} value of 0.47 ±0.09 nM (Pelin et al., 2009). In the present study, we demonstrate the ability of PLTX to generate a concentration-dependent production of superoxide anions. The PLTX-induced oxidative stress is possibly a consequence of the ionic imbalance caused by the interaction of the toxin with the Na^+,K^+-ATPase. Indeed, PLTX-dependent superoxide generation was inhibited by ouabain, a pump blocker. Sustained oxidative stress may result in either apoptotic or necrotic cell death. Therefore, we investigated which mechanism was involved in PLTX-induced cytotoxicity. We found no evidence of apoptotic event induction. Indeed, caspases, effectors of apoptosis, were not activated and no apoptotic bodies were detected. Conversely, signs of necrotic damage were found, like LDH leakage and PI uptake, indicating the occurrence of plasma membrane rupture. These findings were confirmed by morphological analysis by confocal microscopy.

These results, showing the ability of PLTX to induce necrosis *in vitro* on the HaCaT cell line, are consistent with the dermatological lesion reported in humans after contact with the toxin.

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Keywords: Palytoxin, HaCaT keratinocytes, ROS, apoptosis, necrosis

Poster

The role of endocrine-disrupting environmental chemicals in the global epidemic of obesity

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Obesity is considered one of the most important diseases of the 21st century and is associated with a reduced life expectancy and/or increased health problems. Recently an association of endocrine disrupting chemicals (EDCs) with the development of obesity has been proposed by different independent laboratories. *In vitro* studies using 3T3-L1 mouse fibroblasts, a well established model for adipogenesis, have already indicated the potential impact of EDCs such as TBT, bisphenol A, nonylphenol and genistein on adipocyte differentiation. These findings urge the need for an evaluation of the accumulation of these compounds in human tissues, such as adipose tissue, as well as in depth mechanistic research of the effects of EDCs on fat.

This study is part of a larger project which aims to investigate the role of EDCs in the pandemic of obesity and the mechanisms through which they influence the adipose tissue. Therefore, it consists of two complementary parts: one part using human fat samples and an *in vitro* part using the 3T3-L1 cells. In the human fat samples EDC concentrations as well as RNA expression of important nuclear receptors and adipocytokines will be measured and the correlation between them will be studied. However, results presented today relate to the *in vitro* part of the project using 3T3-L1 cells as model for adipogenesis.

In a first step, the influence of EDCs on the differentiation of 3T3-L1 fibroblasts into mature adipocytes will be investigated. We were able to differentiate 3T3-L1 fibroblasts into mature adipocytes by stimulation of two days post confluency cells (day0) with MDI medium (medium containing IBMX, dexamethasone and insulin) followed by stimulation with insulin-containing medium from day 2 until day 8. During terminal differentiation, the fibroblasts undergo a series of morphological and biochemical changes to eventually accumulate lipid droplets. These lipid droplets were visualised during differentiation using Oil Red O staining. The differentiation grade could be spectrophotometrically quantified by extracting the Oil Red O dye. To study the influence of EDCs on the differentiation of adipocytes, 3T3-L1 cells were exposed to a series of EDCs (mainly lipophilic compounds, e.g. musks, brominated flame retardants and phthalates) during eight days and compared to the positive control cells (MDI+insulin).

First results indicate that this Oil Red O staining method allows a quick and low cost screening of a broad group of compounds and concentrations. The potential obesogenic character of selected compounds was further confirmed by expression analysis of important differentiation marker genes, such as adipocyte specific fatty acid binding protein (aP2) and peroxisome proliferator-activated receptor gamma 2 (PPAR[gamma]2).

In a second step of the study, the influence of EDCs on proliferation and fattening of mature adipocytes will be studied. The

combination of these two endpoints will give us important information for the selection of interesting EDCs influencing adipogenesis for further mode of action research using microarray techniques. This mechanistic information can in the end lead to identification of interesting biomarker genes to test the hypothesis of a mechanistic relation between EDCs accumulation in fat tissues and the prevalence of obesity.

Keywords: endocrine disrupting compounds, in vitro, 3T3-L1, obesity

Lecture in Session AXLR8 '21st Century Toxicology' Info Forum

The EU FP7 Project "PREDICT-IV"

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no abstract submitted

Lecture in Session V: Acute and long term toxicity A

Testing of the toxicity of volatile compounds on human lung cells using the Air/Liquid Interface (ALI) culturing and exposure technique: a prevalidation study

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Testing requirements under the new European chemicals policy (REACH) are expected to trigger a large number of toxicological studies. For environmentally and occupationally relevant substances which are taken up by inhalation this will prompt extensive efforts in terms of toxicological assessments unlikely to be realised through *in vivo* testing alone. The aim of the present study was to perform a multi-laboratory evaluation of an Air/Liquid Interface (ALI) culturing and exposure technique for testing the cytotoxic and genotoxic effects of inhalable substances (gases) on human lung cells.

Human lung cells of the alveolar cell line A549 were grown on microporous membranes at the air/liquid interface and were exposed to test atmospheres consisting of different mixtures of synthetic air supplemented with the test gas (i.e. NO_2, SO_2, formaldehyde or ozone). Gas-mediated cytotoxicity and genotoxicity were assessed via electronic cell counting (CASY technology) and the COMET assay, respectively. Logistic regression and hierarchical modelling were employed to determine the intra- and interlaboratory variabilities in dose-response relationships. A linear-regression prediction model was developed for the cytotoxic endpoint.

Analyses of dose-response relationships for the endpoint cell number showed a good repeatability within and reproducibility between the laboratories for all four gases. Comparison of the derived EC_{50} values with published LC_{50} values for mice and rats revealed a tight quantitative relationship between *in vitro* cytotoxicity and *in vivo* lethality. Further, analyses of the tail-moments obtained from the COMET assay demonstrated clear and reproducible dose-response relationships for SO_2 and formaldehyde, being indicative for dose-dependent DNA strand breaks (SO_2) and DNA-protein crosslinks (formaldehyde). No such dose-dependent effects could be observed for NO_2 and ozone.

The results of the present prevalidation study are promising with respect to the reliability and relevance of the proposed *in vitro* method for inhalation toxicity testing. Before entering a formal validation stage, extended prevalidation will be necessary to establish a tested training set of compounds sufficiently large to allow for optimisation of the prediction model.

Keywords: in vitro test method, inhalation toxicity, cytotoxic and genotoxic effects, human lung cells, A549, chemical testing

Poster

Sterile production of platelet lysate as substitute for fetal calf serum – a concept

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For supporting proliferation, the use of fetal calf serum (FCS) as a medium supplement is still the gold standard in cell culture. However, the production of FCS is cost intensive and accompanied by ethical concerns. Additionally, FCS bears a potential risk for carrying diseases. Therefore, an alternative culture supplement without animal components would be advantageous.

Human thrombocytes contain growth factors at high concentrations and can be processed to platelet lysate (PL). Since platelets are regularly substituted in trauma and oncologic patients with insufficient blood coagulation, they are highly available after their 5 days expiry. The proliferative potential of human adipose derived stem cells (ASCs) expanded in PL in comparison to FCS was evaluated together with the question whether they retain their ability to differentiate towards the osteogenic, adipogenic and chondrogenic lineage.

Growth factors contained in platelets are also known to stimulate and accelerate soft tissue and bone healing. Hence, platelet derived products have already been used in surgery for several years. Aiming to apply PL in cosmetic surgery as well, a process for the production of autologous PL in a closed bag system was developed and validated.

Now, we are aiming to combine these two approaches and develop a sterile production process in a closed bag system for PL as an FCS substitute as well.

Keywords: platelet lysate, fetal calf serum

Poster

Effects of natural antioxidants vitamin C, vitamin E, quercetin and pterostilbene on beauvericin-induced cytotoxicity in CHO-K1 cells

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Toxic effects of mycotoxins in food are a human health concern. Mycotoxins are capable of influencing various cellular processes including inhibition of cell proliferation, oxidative stress and lipid peroxidation (Ferrer, 2009). Mycotoxins may differentially interfere with the antioxidant agents α-tocopherol and ascorbic acid, resulting in cytotoxic effects. The two vitamins commonly found in our diet are two of the best known efficient biological antioxidants (Rietjens, 2002). Moreover, vitamins C and E are presently marketed as an antioxidant supplement, and are claimed to increase resistance to diseases and oxidative stress. Quercetin, one of the most widely distributed flavonoids in plants, is a natural antioxidant (Rietjens, 2002). Pterostilbene, a naturally occurring dimethyl ether analogue of resveratrol, is commonly found in various plants, including grapes, berries and peanuts; moreover, it is a constituent of nutraceuticals owing to its anti-oxidative activity (Chakraborty, 2010).

The hypothesis that vitamin C, vitamin E, quercetin and pterostibene can prevent mycotoxin-induced cell death in Chinese hamster ovary (CHO-K1) cells was investigated. CHO-K1 cells exposed to the beauvericin mycotoxin (0.53 and 1.05 μM for 24 h) were simultaneously treated or pre-treated (24 h) with vitamin C, vitamin E, quercetin and pterostilbene (2.5-100 μM). The MTT assay was performed as a measure of cell viability.

Beauvericin was able to induce cytotoxicity in CHO-K1 cells in a dose-dependent manner. CHO-K1 cells co-treated with vitamins and flavonoids showed a greatly decreased cytotoxic response to beauvericin in comparison with 24 h antioxidant pretreatment. Vitamin E was the most active antioxidant among the compounds tested, with an estimated percentage protection over 100% of control. According to the protective capacity of these

antioxidants, the percentage of protection of vitamin E, vitamin C, quercetin and pterostilbene in CHO-K1 cells related to control increased from 10 to 106%, 16 to 91%, 20 to 80% and 16 to 30%, respectively.

In conclusion, vitamin C, vitamin E, quercetin and pterostilbene are able to prevent beauvericin-induced cytotoxic damage in CHO-K1 cells in a dose dependent manner. Moreover, the optimal time and dose for vitamins and flavonoids to display protection seem to be the co-treatment with antioxidants and beauvericin. Furthermore, the best antioxidant protection was observed with 25 μM vitamin C, quercetin and pterostibene, and 50 μM vitamin E.

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Keywords: beauvericin, vitamins, quercetin, pterostilbene, CHO-K1 cells, cytotoxicity

Lecture in Session VI: Acute and long term toxicity B

In vitro cell transformation assays – their use in safety and risk assessment

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The prediction and assessment of carcinogenicity of chemical compounds is an essential step in their development. The 2-year bioassay, the standard method for carcinogen detection, is time and resource intensive. Many short-term tests, especially genotoxicity tests, have been developed to aid in the identification of potential carcinogens. However, the endpoint of these systems is genotoxicity, the concordance between rodent bioassays is only about 60% and a battery of short-term genotoxicity tests cannot improve the overall concordance.

In vitro cell transformation tests using SHE-, Balb/c3T3-, Bhas 42- cells simulate the process of animal two-stage carcinogenesis. These tests are suited for the *in vitro* detection of a carcinogenic potential of test compounds in safety and risk assessment. Results from cell transformation assays can provide information that, in combination with data from other testing methods, are useful for identifying the carcinogenic potential of chemical compounds. The results of these assays, in combination with other information such as genotoxicity data, structure activity analysis, *in vivo* toxicity data and pharmaco-/toxicokinetic information, can facilitate a relatively comprehensive assessment of the carcinogenic potential of a chemical. They are used in order to gain additional information when the biological significance of the bioassay results is uncertain, for clarification of the meaning of positive results from genotoxicity assays in the weight-of-evidence assessment, for compound classes where genotoxicity data have only limited predictive capacity, for investigation of compounds with structural alerts for carcinogenicity, to demonstrate differences or similarities across a chemical category and for the investigation of the tumour-promoting activity of chemical compounds.

New technologies will contribute to a better understanding of the mechanism underlying these assays and to a more objective evaluation of transformed foci and colonies. It is time that cell transformation assays be accepted as a useful short-term *in vitro* tool for assessing the carcinogenic potential of chemical compounds. In this presentation examples will be provided on how to use these promising assays in safety and risk assessment.

Keywords: in vitro cell transformation, genotoxicity, carcinogenicity

Poster

Prevalidation of the ACuteTox *in vitro* testing strategy for the prediction of human acute oral toxicity

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The EU FP6 project ACuteTox represents the first attempt to create an integrated testing strategy based solely on *in vitro* and *in silico* methods, with the purpose of replacing animal testing for predicting human acute oral systemic toxicity. The optimisation phase of the project resulted in the selection of test methods considered to be potential candidates for building blocks of the final tiered testing strategy. The test methods were selected according to their reproducibility and reliability, as well as their potential to classify chemicals into the official acute oral toxicity categories (GHS, EU). The last part of the ACuteTox project consisted in a prevalidation exercise aimed to assess the predictive capacity of the proposed tiered testing strategies and the identification of the combination of test methods that gives the best prediction.

The test methods that entered the prevalidation were 1) the 3T3 neutral red uptake cytotoxicity assay, 2) the cytokine release assay using human whole blood, 3) the inhibition of colony forming unit efficiency in human cord blood-derived cells, 4) gene expression and uridine incorporation in primary rat brain aggregate cultures, 5) cytomic panels measuring oxidative stress and cytotoxicity screening in HepG2, SH-SY5Y and A.704 human cell lines, 6) the MTT assay using primary rat hepatocytes, 7) kinetic parameters (volume of distribution, protein binding, clearance and oral absorption using Caco-2 cells) for the estimation of the oral dose from the effective concentration observed *in vitro* and 8) the estimation of compound passage through the blood-brain barrier using neuronal networks (for neurotoxicity assays). Eight laboratories were involved in the study. A new set of 32 chemicals were selected and tested blindly. All raw data were collected using a unique standard template and analysed independently by biostatisticians.

During the optimisation phase the statisticians assisted in the development of a preliminary tiered testing strategy by assessing at least three different combinations of the selected assays. The proposed combinations have been challenged retrospectively with the new data generated during the testing phase of the prevalidation in order to identify the best predictive strategy. The use of reference compounds, which were not included in the "training set", was necessary in order to properly assess the predictive capacity of the tiered testing strategy.

This presentation will illustrate the activities carried out during the prevalidation exercise with particular emphasis on the different methods selected and the generation of the data used to assess the predictive capacity of the proposed testing strategy retrospectively.

The authors would like to acknowledge all the partners of the ACuteTox consortium who contributed to the generation of the results.

Keywords: acute oral toxicity, testing strategy, 3Rs, prevalidation

Poster

Comparison of tissue accumulation of HCB and PeCB, and action on steroidogenesis in small ovarian follicles

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Hexachlorobenzene (HCB), an organochlorine fungicide widely used in agriculture, has been one of the most frequently detected pollutants in human (Jarrell et al., 1993) and farm animal follicular fluid (Kamarianos et al., 2003). Information concerning the presence of pentachlorobenzene (HCB metabolite) in tissue samples and body fluids is limited. Moreover, there are no data concerning the action of environmentally relevant doses of HCB and PeCB on ovarian follicular function. The aim of the presented data was to test the accumulation of HCB and PeCB in the follicular wall and as a consequence its action on follicular steroidogenesis.

The small (SF) porcine follicles were incubated in M199 medium +10% FBS at 37˚C with 1 μg/ml HCB or PeCB added each

day for four days. HCB and PeCB concentrations in the follicular wall and medium were quantified by a capillary column gas chromatograph equipped with an electron capture detector (GC-ECD: Hewlett Packard 5890 Series II) (Falandysz et al., 2001). For steroid analysis, follicles were incubated with 0.02, 0.2 and 2 ng/ml HCB or PeCB for 3 days. Medium was changed every day and new reagents were added to the medium. Progesterone (P4), testosterone (T) and estradiol (E2) levels were evaluated by ELISA (DRG Diagnostic GmbH, Germany).

Almost all of the PeCB accumulated in the follicular wall (97.2% of the total dose). 57.5% of the total HCB dose accumulated. There was a difference in the action on steroid secretion.

HCB had no effect on P4 secretion, while an inhibitory effect on T and E2 secretion was observed. PeCB stimulated the secretion of all investigated steroids strongly.

The presented data showed that HCB is metabolised locally in the follicular wall, whereas PeCB accumulates fully. HCB in all doses decreased T and E2 secretion with no action on P4 secretion, suggesting antiestrogenic properties of HCB. This is in agreement with Alvarez et al., (Alvarez et al., 2000), who found that HCB treatment reduced circulating levels of estradiol without an effect on serum concentrations of progesterone in female rats. On the other hand, Foster et al. (Foster et al., 1993) also observed no effect on E2 but increased P4 levels in superovulated rats exposed to HCB. Additionally we showed that PeCB increased the secretion of all investigated steroids. To our knowledge there are no prior data concerning action of PeCB as an endocrine disruptor in the ovary. Based on the presented preliminary results we suggest estrogenic properties of PeCB, probably by action on the mobilisation of cholesterol, which serves as a substrate for progesterone, testosterone and then estradiol synthesis. The next step is to elucidate the mechanism of action, i.e. activation of enzymes involved in steroidogenesis from progesterone (CYP11A1, 3βHSD) to testosterone (CYP 17) and estradiol (CYP 19).

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Keywords: ovarian follicles, hexachlorobenzene, pentachlorobenzene, accumulation, steroid secretion

Poster

Information needs for regulatory purposes – activities of the Environment Agency Austria

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The Environment Agency Austria (EAA, *Umweltbundesamt GmbH*) is the expert authority of the federal government in Austria for environmental protection and environmental control. It comprises a wide field of environmental and health related issues. The aims of the EAA regarding regulatory testing will be presented: In order to ensure the safe use of chemicals, biocides and nanomaterials and the ensuing benefits, it is necessary to lay the legal basis and develop and adapt methodologies for evaluating exposure and effects of these substances. Knowledge gaps concerning their human and ecological toxicity must be closed. On the other hand potential benefits of nanotechnologies should not be overlooked. Information needs of the public, researchers and other relevant stakeholders have to be addressed in a target specific manner.

The activities of the EAA are summarised and will include the following aspects:

– The work of the EU National Coordinator for Testing Methods
– Participation in working groups regarding REACH and the Biocides Directive
– Participation in OECD working groups

The authors believe that by informing scientists of the work of the EAA, future collaborations will be made possible, which will help to close knowledge gaps between what regulators need and what scientists have.

Keywords: information needs, regulatory testing, methodology, environment agency

Lecture in Session IX: New developments and novel methods

Establishment of a 3D intestinal tissue equivalent *in vitro*

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The small intestine is the organ where the greatest part of the absorption of orally administered drugs takes place (Derendorf et al., 2002). Active ingredients are transported over the intestinal epithelia by several transport mechanisms and released into the adjacent circulation. Many potential drug candidates fail in phase II and III clinical trials because of their low solubility, low permeability and/or a high first-pass metabolism in gut and liver (Ungell, 2002). Therefore a detailed knowledge of the absorption and oral bioavailability of new drug candidates must be available at the early stage of preclinical trials to minimise costs and use of valuable resources. The testing of new candidates takes place predominantly in preclinical trials in the form of cell and animal experiments.

Due to their oversimplified construction, monolayer cultures reflect the physiology of the small intestine insufficiently and the transfer of generated data from animal experiments to the human organism is sometimes critical from the scientific point of view due to species-specific differences (Le Ferrec, 2001). Furthermore, not only because of ethical aspects, animal testing should be reduced in terms of the 3R principle to a minimal necessary degree by the use of meaningful alternative test methods (Lahl, 2005).

A 3D dynamic intestinal tissue equivalent, which reflects the micro environment of the small intestine, has been developed at the Fraunhofer IGB. Caco-2 cells and primary microvascular endothelial cells are co-cultivated in a 2-chamber bioreactor. The endothelial barrier simulates the *in vivo* gut-blood barrier for the systemic uptake of substances in the body. Contrary to static diffusion cells (e.g. Franz chamber), the bioreactor provides an apical and basolateral flow of culture media. By use of a decellularised intestinal collagen scaffold and a dynamic bioreactor system, the structure of the villi and the morphology of highly prismatic epithelial cells could be recreated. Due to the remaining 3D structures of the former mucosa, the scaffold would protect the reseeded cells against high shear stress and could therefore enable the regeneration of the epithelial layer and multiple testing in the future.

Under dynamic culture conditions the Caco-2 cells grow in a highly prismatic manner similar to enterocytes *in vivo*. The 3D dynamic co-culture model could be validated so far with substances that simulate high permeability, low permeability and efflux transport. Preliminary studies showed an increasing paracellular permeability of the epithelial cells under dynamic culture conditions in comparison to the standardised insert model. Therefore an improved correlation of the generated transport data with the human organism could be proven by the use of substances of low permeability (Fluorescein & Desmopressin). In addition, the accomplishment of directed transport studies for the investigation of the transport protein p-gp by the use of Rhodamine 123 was possible and correlated with the data of the conventional Caco-2 assay (Troutman and Thakker, 2003).

Our test system should provide a basis for ADMET studies and the characterisation of different substances. The results should be validated to predict systemic effects and for *in vivo* classification. Ongoing studies concentrate on the exchange of Caco-2 cells with primary enterocytes.

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Keywords: small intestine, absorption, physiological test system, oral bioavailability

Poster

Wound healing model: comparison of contraction induced by cultured fibroblasts from normal skin and from hypertrophic scar

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Hypertrophic scars are common problems in clinical daily practice, but there is still a lack of knowledge of the mechanism of their formation and of possibilities to prevent or treat them. (Bell et al., 1979) have showed that culturing fibroblasts in hydrated collagen gels induced gel reorganisation and led to gel contraction.

The present study aimed to investigate differences in reorganisation of the matrix with the presence of normal and hypertrophic scar fibroblasts. Fibroblasts were obtained from full thickness normal human skin (named normal human fibroblasts – NHF) (n=10) and from hypertrophic scars (named hypertrophic scar human fibroblast – HSHF) (n=10). Cultured cells were seeded in a collagen lattice and then assayed for gel contraction. After 24 h and 48 h dishes were photographed and measured using image analyser software. Obtained values were statistically analysed using generalised estimating equations.

There was a significant increase in lattice contraction in the hypertrophic scar group when compared to the normal skin group. At 24 h, HSHF exhibited a 33% reduction in the area while NHF-FPCL was reduced in 26% of the original area. After 48 h, although the rate of contraction was 37% for HSHF and 30% for NHF, there was no statistic difference in lattice contraction ($p < 0.0001$).

In the present study we observed that the maximum contraction of the gel occurred during the first 24 h in both NFH and HSHF populated gels but contraction of HSHF-FPCL was statistically higher than NHF-FPCL. The data obtained in these experiments indicate that the behaviour of hypertrophic scar fibroblasts is different from normal skin fibroblasts and that this model can be used in hypertrophic scar studies.

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Keywords: fibroblast culture, collagen, three-dimensional contraction model, hypertrophic scars, wound healing

Poster

Update on recent EU activities and initiatives

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Over the past year there have been a number of activities and initiatives in the EU with an increased focus on the 3Rs. Agreement on the revision of Directive 86/609/EEC, the EU Animal Experimentation Directive, may not have incorporated all the possible improvements necessary for better animal welfare, but this European law now aims to harmonise legal protection for animals and calls for the use of alternative tests where available.

Furthermore, alternative methods are increasingly being taken up in the development of new and revised legislation, as there are many which now include the obligation to keep animal testing to the absolute minimum and testing requirements are more

in line with actual requirements, with alternatives used where possible. In addition to references in the 7th amendment to the Cosmetics Directive and REACH, the EU chemicals policy, these now include pesticides, i.e. plant protection products and biocidal products, and food and feed testing.

International harmonisation is key! 2010 saw the launch of the Transatlantic Animal Welfare Council (TAWC), a platform that aims to strengthen the cooperation between organisations in the field of animal welfare in Europe and the USA in order to achieve the highest standards of animal welfare. Furthermore, 2010 saw the launch of CAAT-EU, a collaboration between the Johns Hopkins Bloomberg School of Public Health and the University of Konstanz, which will coordinate transatlantic activities to promote humane science and its education, and will participate in publicly and privately funded European projects.

Additional initiatives were under renewal in 2010, where we saw the European Partnership for Alternative Approaches to Animal Testing (EPAA) commit to continue its work on alternative methods. Launched in November 2005 with a 5 year action plan, this joint initiative of the European Commission and industry and trade federations works to promote the development of new 3R methods as modern alternative approaches to safety testing. Also under renewal is the European Action Plan on the Welfare and Protection of Animals, originally released in January 2006 with a 5 year strategy, with a wide-reaching scope to improve animal welfare in the EU and internationally, including the objective to apply the principles of the 3Rs to experiments involving animals.

Possibilities for advancement of the 3Rs remain in place for the future, with commitment from industry and EU institutions and future resources made available through new requirements incorporated into the EU Framework Programmes and Community Environment Action Programmes.

Keywords: animal testing, 3Rs, legislation

Lecture in Session IX: New developments and novel methods

The hen´s egg micronucleus test (HET-MN): successful intra- and interlaboratory reproducibility study

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The hen's egg test for micronucleus induction (HET-MN) has been studied for several years and is intended to serve as an alternative test system to the *in vivo* micronucleus test (OECD guideline 474). In order to verify the applicability of the HET-MN, an intra- and interlaboratory reproducibility study was carried out at the University of Osnabrück and the Henkel AG & Co. KGaA.

At first Henkel, which served as naive test laboratory, tested a range of compounds that had been pre-examined at the University of Osnabrück to show the successful transfer of the methodology and to adjust the existing standard operating procedure (SOP): the genotoxic chemicals cyclophosphamide (CP), dimethylbenzanthrazene (DMBA), both used as positive controls during the next phase, as well as methotrexate (MTX), acrylamide (AC), azorubin (E122), N-nitroso-dimethylamine (NDMA), mitomycin C (MMC), and the non-genotoxic compounds Orange G (OG) and isopropylmyristate (IPM).

During the subsequent interlaboratory trial, compounds not tested in any of the laboratories before were examined for the induction of micronuclei: (i) the non-genotoxic compound ampicillin (APS); (ii) the clastogenic p-chloroaniline (PCA); (iii) the two aneugens carbendazime (MBC) and vinorelbine (VNR); (iv) isophorone (TCT), malathion (BDP), and 2,4-dichlorophenol (DCP), which are considered irrelevant positive (positive in standard *in vitro* tests but negative *in vivo*). Based on these results a prediction model was established.

15 of the 16 examined compounds were predicted correctly. Remarkably, the results for the *in vitro* irrelevant positive compounds correspond to the published data of the *in vivo* MNT, which are considered biologically more relevant than results from the *in vitro* assays.

Due to the high rate (approx. 95%) of correctly identified mutagenic and non-mutagenic compounds we consider the HET-MN to represent a promising tool to supplement existing test batteries. The commonly established SOP and prediction model will be challenged in a prevalidation study starting in summer 2010. It will be funded by the German Ministry of Research and Education (BMBF), as the reproducibility study was.

Keywords: in vitro, genotoxicity, HET-MN

Poster

Dermal xenobiotic metabolism: a comparison between human native skin, four *in vitro* test systems and a liver system

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The skin is the first site of contact to external stimuli. It protects the body in several ways. Its barrier function determines the local and systemic bioavailability of topically applied substances. Dermal xenobiotic metabolism contributes to the potential toxicity of substances by converting compounds that have penetrated the barrier into harmless or toxic metabolites. Consequently, growing efforts are put into species- and organ-specific safety assessment of topically applied compounds: corrosion, irritation, genotoxicity, and sensitization. However, in comparison to the liver, little is known about xenobiotic metabolism of the skin and appropriate *in vitro* systems.

To select an *in vitro* system that is most similar to the *in vivo* situation, human native skin was compared to several *in vitro* models of different physiological complexity: (1) the Phenion® Full Thickness Skin Model comprising dermis and epidermis, (2) an open source epidermal model (OS-REP), and (3+4) monolayer cultures of fibroblasts or keratinocytes.

The four *in vitro* models were produced in-house with biopsy cells from the same donor to exclude donor variability. First, the basal gene expression of phase I and II enzymes of three different donors were investigated by quantitative RT-PCR. Second, protein expression and enzyme activity were compared between the *in vitro* systems.

The results demonstrate that *in vitro* skin models with rising physiological complexity mirror the native situation more realistically: of 14 analysed genes only two display reduced expression in epidermal models but twelve and nine in keratinocytes or fibroblast monolayer cultures, respectively.

In addition, the dermal *in vitro* models were compared to cells (cultivated as monolayers and spheroids) mirroring the liver system. The analysis of gene expression and enzyme activity revealed that phase I enzymes are much more prominent in the liver system. In contrast, phase II enzymes exhibited higher values regarding gene expression and enzyme activity in the dermal systems.

Keywords: skin, liver, xenobiotic metabolism

Poster

In vitro model for the identification of respiratory sensitizing chemicals

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Respiratory allergens may be an underlying cause of adult-onset asthma, which is the major occupational respiratory disease in industrialised countries. Despite international regulatory requirements there is no established *in vitro* protocol for the identification of chemicals that cause respiratory sensitization. This study investigated the alterations in gene expression of human bronchial epithelial (BEAS-2B) cells after exposure to respiratory sensitizing and non-sensitizing chemicals. The aim is to identify gene transcripts that are able to discriminate between the two groups of chemicals.

BEAS-2B cells were exposed during 6, 10, and 24 h of exposure to a panel of nine respiratory sensitizing (i.e. 7-aminocephalosporanic acid, ammonium hexachloroplatinate IV, hexamethylene diisocyanate, diphenylmethane diisocyanate, glutaraldehyde, maleic anhydride, piperazine, styrene, and trimellitic anhydride) and 9 respiratory non-sensitizing chemicals, (i.e. five skin sensitizers (2-bromo-2bromomethyl glutaronitrile, 1-chloro-2,4-dinitrobenzene, cinnamaldehyde, 2-mercaptobenzothiazole, and tetramethylthiuram disulfide) and four irritants (i.e. acrolein, chloropicrin, epichlorohydrin, and methyl salicylate)). Overall changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide arrays. Fisher Linear Discriminant Analysis was used to obtain a ranking of genes that reflects their potential

to discriminate between respiratory sensitizing and respiratory non-sensitizing chemicals.

The results of an initial study (after the exposure of BEAS-2B to three respiratory sensitizing and three respiratory non-sensitizing chemicals) are indicative that the BEAS-2B cell line can be used as an alternative cell model to screen chemical compounds for their respiratory sensitizing potential. These results have been published in Toxicology 255 (2009) 151–159. The incorporation of gene profiles after exposure to 12 additional chemicals will result in the identification of potential gene expression markers that can discriminate respiratory sensitizing from respiratory non-sensitizing chemicals.

This work was partly funded by the EU FP6 Integrated Project Sens-it-iv (LSHB-CT-2005-018861) aiming at the development of novel strategies for in vitro assessment of allergens (www.sens-it-iv.eu).

Keywords: respiratory sensitization, genetic markers, in vitro assay, human bronchial epithelial cells (BEAS-2B)

Poster

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

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Photosensitivity is a delayed type IV hypersensitivity induced by a broad spectrum of active components and concurrent exposure of skin to ultraviolet radiation. Due to a high frequency of applied substances in toiletry and clinical therapy there is a need for predictive methods in order to determine/classify potential photosensitizers before applying them to cosmetics or pharmaceuticals. Up to now no adequate *in vitro* alternatives are available. Our intention was to provide an *in vitro* photosensitivity assay to detect photosensitizers. Since dendritic cells play a key role in the induction of contact allergies, this *in vitro* assay is based on the established monocyte derived dendritic cell (MoDC) assay.

$CD1a^-/CD14^+$ monocytes were positively selected from human peripheral blood and differentiated with IL-4 and GM-CSF supplementation for 5 days. Test substances were preincubated with MoDCs prior to UVA radiation followed by 48 h incubation. CD86, HLA-DR and CD83 were measured by FACS.

Known chemicals were chosen for evaluation, including chlorpromazine, olaquindox (phototoxic and photoallergen), muskambrette (photoallergen and allergen), 2,4-dinitrochlorobenzene and nickelsulphate (allergen only). Results obtained from the *in vitro* assay were consistent with previously described photosensitizing potentials for all chemicals tested.

Thus, this assay allows the evaluation of the photoallergic potential of substances. Moreover the assessment of their allergic, phototoxic and toxic potential in this single assay is an additional benefit. To improve validity additional substances are being tested at present.

The method presented here provides a promising assay for assessing photoallergic and, in addition, phototoxic potential of relevant substances.

Keywords: in vitro toxicology, photosensitization, CD86, dendritic cell, alternative method

Poster

Teratogenicity of valproic acid derivatives: evaluation of structure-activity relationships using the embryonic stem cell test

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Testing for reproductive and developmental toxicity of drugs and other chemical compounds *in vitro* is an attractive alternative procedure to time-consuming and expensive *in vivo* or *ex vivo* experiments. The embryonic stem cell test (EST) represents a scientifically validated method for the detection and classification of compounds according to their teratogenic potency. However, more work is required to assess its applicability domain and to improve its predictive capacity before gaining full regulatory acceptance.

We picked valproic acid (VPA) as a model compound to evaluate the suitability of the EST for distinguishing between developmental toxicity potencies of substances with closely related structures. VPA is among of the most frequently used anti-epileptic drugs worldwide. Further, it is used for migraine prophylaxis and in the treatment of bipolar psychotic disorders. Two severe side effects of VPA, hepatotoxicity and teratogenicity have prompted research into derivatives of VPA. Here we investigate six closely related analogues of VPA whose teratogenic potential has been previously determined in the NMRI exencephaly mouse model.

The validated EST employs the murine embryonic stem cell line D3, which spontaneously differentiates into contracting cardiomyocytes. This morphological feature is used as an endpoint of differentiation along with the measurement of cytotoxicity on both D3 stem cells and 3T3 fibroblasts. Recently we reported on an abbreviated protocol of the EST using flow cytometry, termed FACS-EST. This protocol reduces the assay length from ten to seven days and replaces the morphological assessment with the immunofluorescence detection of structural proteins of the sarcomere apparatus.

Determining the concentration of half maximal inhibition of differentiation of VPA and its six congeners in the EST revealed a ranking similar to that found previously. Distinct embryotoxicities *in vivo* of stereoisomers, which differ only by their spatial configuration, were reproduced by the EST. Similarly, an increased *in vivo* potency correlating with longer chain length of the congener was evident as higher cytotoxicity in the EST. Comparing the validated EST with the FACS-EST demonstrated a good correlation, with the ranking of the substances being identical between methods.

Our data demonstrate that the EST is capable of differentiating among closely related molecules according to their embryotoxic potency. Both *in vitro* differentiation and cytotoxicity have to be considered to assess teratogenicity comparable to *in vivo* results. The FACS-EST is equally potent to the validated EST, and all substances were ranked in the same order and in full accordance with data obtained in the NMRI exencephaly mouse model.

Keywords: embryotoxicity, valproic acid, congener, structure-activity relationship, embryonic stem cell test, in vitro testing

Lecture in Session IX: New developments and novel methods

Multiplexing various *in vitro* toxicity assays for high throughput screening

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Recent advances in assay chemistries have made it possible to multiplex a variety of cell-based assays in an automated high throughput format using standard plate readers. We have developed methods to combine various luminescent and fluorescent cell-based assays in a homogeneous format. The possible combinations for multiplexing have been expanded by the validation of novel markers to detect cytotoxicity and the development of assay chemistries that can be used directly in wells containing viable cells. The chemistries used to perform various cell-based assays will be described along with factors to consider for building a successful multiplex combination. Examples will be presented demonstrating multiplex measurement of viable cells, necrotic cells and apoptotic cells, all in the same sample. Additional examples will be included, demonstrating multiplex measurement of viable cells with other markers relevant to cell health including: cytochrome P450 activity, GSH level as a marker of cell stress, and cell-based proteasome activity. The advantages and disadvantages of multiplexing various homogeneous *in vitro* cytotoxicity assays will be presented along with results of ongoing efforts to develop new assay technologies.

Keywords: viability, cytotoxicity, apoptosis, cell stress, high throughput

Lecture in Session IX: New developments and novel methods

Skin and respiratory sensitization: *in vitro* identification and potency determination

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Since its initiation in 2005, the FP6 project Sens-it-iv (www.sens-it-iv.eu) has collected knowledge allowing the development of tests and test systems for the identification of skin and respiratory sensitizers (Weltzien et al., 2009). The applicability domain of the selected chemicals was described in Sens-it-iv newsletter 32 (http://www.sens-it-iv.eu/files/newsletter/Sens-it-iv_Newsletter_32.pdf).

The emerging tests can be ranked according to their level of maturity. Each level has its specific requirements:

Level 1: Since (Weltzien et al., 2009), the human reconstituted skin test, the NCTC2544 test and the DC test have been refined and evaluated as tests for screening. The human reconstituted skin test and the NCTC2544 test are currently being tested as a tiered strategy, where the NCTC test identifies skin sensitizers (> 99% predictivity), while the reconstituted skin test allows to predict potency in good correlation with the LLNA. A small prevalidation study is ongoing. Genomic analysis has moved the DC based tests away from activation and maturation detection. A marker signature was identified that predicts skin sensitizers with 99.9% accuracy. Further evaluation (including respiratory sensitizers) is ongoing. For the tiered approach and the DC based test new partners bringing in new chemicals are in the process of being identified (expansion of the applicability domain).

Level 2: The second level includes a test determining DC migration. This is a functional assay that has the potential of becoming a first post-screen test. This test was capable of identifying 5 out of 5 skin sensitizers in a panel of 8 compounds including 3 controls (irritants and inert chemicals). Technology transfer is ongoing and has been successful so far.

An attempt to incorporate DC in human reconstituted skin culture was promising but must still be considered a model for studying mechanisms. A “metabolic activation test” has been developed but requires more extensive evaluation.

Level 3: A bronchial and alveolar human lung model has been identified. Both models are currently under further evaluation. Finally, a T-cell priming test has been established. This test uses primary human blood cells (DC and T-cells) and is the most complex assay of the toolbox. It is to be used as the ultimate

in vitro assessment of compounds. Promising results were obtained, but further research, optimisation and evaluation is required. Research has given us a better understanding of the role of DC and the cross-talk between DC and T-cells.

In conclusion, several mechanistically relevant and promising test strategies have been developed within the Sens-it-iv project and are being refined further. It is considered realistic that some of these assays can be refined to a stage where they can be formally evaluated and validated. However, the timing to get these new technologies accepted by regulatory and legislative bodies lies outside the influence of Sens-it-iv. In that sense an answer to the original question concerning the future safety of chemicals may depend on necessary interim solutions enforced by regulatory authorities.

Reference

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Keywords: keratinocytes, human reconstituted skin, dendritic cell, genomics, proteomics

Poster

IUCLID 5.2 completed with *in vitro* data collected in a weight-of-evidence approach

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The REACH regulation asks for toxicological data on all chemicals that are marketed in the European Union. The registration dossier can be quite extensive, depending on the tonnage level of the marketed chemicals. Recent estimates foresee up to 54 million test animals to fulfil REACH requirements.

REACH accepts the use of non validated alternative methods if the scientific validity is demonstrated. According to Annex XI of REACH, data from non validated *in vitro* assays can be readily included when the outcome result is positive and the chemical shows some toxicological activity. All negative results, which are the majority of the cases, need confirmation *in vivo*.

Another possibility to use *in vitro* methods is within a weight-of-evidence strategy, i.e. when there are several datasets coming from different sources, all demonstrating a specific property of a chemical. In this way, also negative results can be accepted.

The registration dossier must be prepared using IUCLID 5.2, which is specific software created by ECHA, the European Chemical Agency. However, IUCLID 5.2 is not designed for inserting data that was not obtained *in vivo*. *In vitro* methods have specific fields only in the area of genetic toxicity. *In vitro* test methods and results are dialled in as free text, with questionable acceptability. In fact, all submitted data must undergo the TCC (Technical Completeness Check), which fails when some IUCLID 5.2 entries are left empty, as in the case of *in vitro* data.

In spite of these difficulties this poster presents how data from a weight-of-evidence approach have been used for the registration of a UVCB flame retardant. The strategy that was adopted is explained and IUCLID 5.2 records are shown. The TCC report is also included and commented. Unfortunately, the feedback from ECHA will only be available in the coming months. Nevertheless, it will be important for the future to pave the way for the submission of *in vitro* data to foster their maximum use.

Keywords: REACH, weight-of-evidence, acceptability

Lecture in Session I: Legal, ethical and policy topics regarding alternatives

New EU Directive on the protection of laboratory animals: the animal welfare perspective

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The revision of the new EU Directive for the protection of animals used in experiments and for other scientific purposes is in its final stage. The content of the new Directive is based on a compromise text by the European Commission, the European Council and the European Parliament published in December 2009. Its adoption is expected this autumn. It will replace Directive 86/609/EEC.

The original proposal of the European Commission published in November 2008 included some promising improvements for animals in comparison with the existing legislation. However, the industry and research lobbies successfully exerted massive pressure on the European Parliament and the Council of Ministers to considerably weaken the more animal friendly proposals. As a result the new Directive will be more than disappointing: There will be no real restrictions on the use of non-human primates and even experiments on great apes will still be possible. Researchers will still be able to continue to use animals even when scientifically approved 3R methods are available. The authorisation procedure is far from being strict and transparent. For about one third of all experiments, those which are performed to satisfy regulatory requirements, Member States can adopt a simplified administrative procedure. In addition, it will not be necessary to involve external independent experts as ethicists or animal welfare specialists in the cost/benefit evaluation of animal experimental projects. And, even worse, Member States shall no longer have the right to adopt stricter rules then those set in the Directive. This could result in halting the improvement of animal welfare standards and is a major step backwards from the existing regulation.

It is very disappointing that the new Directive does not reflect public opinion. The public is increasingly demanding far greater restrictions on the use of animals and better protection for those that are used, as recent opinion pools have demonstrated. The revision of the Directive also does not fully take into account the technical and scientific progress and even falls back behind Directive 86/609. At least the Directive will improve the protection of laboratory animals in some EU Member States. Animal welfare groups will urge the best possible protection for research animals when the Directive is implemented into national law.

Keywords: Revision 86/609/EEC, animal experiments, primates, alternative methods, authorisation procedure

Poster

In vitro cytotoxic potential of the aqueous extract of *Securinega leucopyrus* (Willd.) on human hepatoma (HepG20) cells: a preliminary study

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Use of the aqueous extract of *Securinega leucopyrus* (Willd.) for cancer treatment has recently become popular among Sri Lankan traditional medicine practitioners despite lack of scientific evidence for its claimed anti-carcinogenic activity. However, the presence of broad spectrum antimicrobial activity of the plant's leaves has previously been reported, indicating its therapeutic uses against dysentery and dandruff (Bakshu et al., 2001). Therefore the present study was performed with the aim of investigating possible anti-carcinogenic effects of *Securinega leucopyrus* (Willd.).

Hot water extract was obtained from aerial parts of Securinega leucopyrus (Willd.) collected throughout the year. The extract was standardised according to HPLC profiles obtained by reverse phase linear gradient of 80% acetonitrile in methanol to 100% methanol. The cytotoxicity against human hepatoma cells (HepG2) was determined by (a) the effect on cell morphology and (b) MTT and SRB assays. Concentrations of the extract used varied between 150 μg/ml and 2400 μg/ml. The cells were treated with each of these concentrations for 24, 48 and 72 h.

Our data suggest that the aqueous extract of *Securinega leucopyrus* (Willd.) exerts a significant cytotoxic effect against HepG2 cells in a time and dose dependant manner. Doses causing 50% inhibition of cellular growth were 450 µg/ml, 300 µg/ml, 150 µg/ml at 24, 48 and 72 h time points, respectively, suggesting the presence of more stable cytotoxic compound/s in the extract.

Reference

Bakshu, L. Md., Jeevan Ram, A. and Venkata Raju, R. R. (2001). Antimicrobial activity of Securinega leucopyrus. *Fitoterapia 72*, 930-933.

Keywords: Securinega leucopyrus (Willd.), HepG2 cells, MTT assay, SRB assay

Poster

Nanotechnology and animal experimentation – for better or for worse?

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Nanotechnology is considered to be a key technology of the 21st century. In support of this new research area, the European Union, many EU Member States and Associated States have set up action plans, platforms and research funding programmes.

In June 2005, the European Commission adopted the EU Nanosciences & Nanotechnology Action Plan 2005-2009 (Commission, 2005), aiming at promoting the "integrated, safe and responsible approach" to nanotechnology (Commission, 2004). The European Commission declares its commitment to promoting nanotechnological research, to addressing citizens' expectations and concerns regarding this new technology and to ensuring the safety of products and techniques resulting from nanotechnology. A subsequent Strategic Nanotechnology Action Plan 2010-2015 is currently under consideration.

The Foundation Animalfree Research is concerned that nanotechnological research has the potential to lead to an increase in animal experiments. Many animal experiments that are currently being performed in nanotechnology are moderately and even severely distressful (Sauer, 2009).

As regards the development of new research policies, the EU Treaty of Lisbon (2008) requests the Union and the Member States to pay full regard to the welfare requirements of animals as "sentient beings". Council Directive 86/609/EEC on the Protection of Laboratory Animals (Council, 1986), which is currently under revision, prohibits animal experiments if non-animal test methods are available. It requests the Community and the Member States to encourage research into developing and validating alternative techniques. It is expected that the revised Directive will contain similar provisions requesting the development and application of alternative methods.

In line with this legal obligation to prevent animal testing, for its future nanotechnology policy the European Commission has spelled out the priority "to develop further, compare and validate *in vitro*, *in vivo* and *in silico* test methods and strategies… and to enable a gradual shift towards alternatives to animal testing"(Commission, 2009).

However, so far neither validated *in vivo* nor *in vitro* test methods are available yet for nanomaterial testing. Therefore, new test batteries have to be developed, validated and accepted. At a time when international efforts are striving for a paradigm change in toxicology towards non-animal testing strategies (CTTAEA and NRC, 2007), it would not be state-of-the-art to found new nanomaterial testing strategies on animal tests, which then have to be replaced step-by-step. Therefore, instead of aiming for a "gradual shift towards alternatives to animal testing", EU nanotechnology policies should explicitly set the goal to implement a fully non-animal testing strategy for nanomaterial testing from the beginning, lay down a target date for meeting this goal and dedicate adequate funding for this.

Regardless of the specific animal welfare concerns relating to nanotechnology, the Foundation Animalfree Research is of the opinion that nanotechnological research can serve to overcome animal experimentation if promoted adequately. Emerging *in vitro* technologies, such as microfluidics and single cell evaluations, can improve the scientific application spectrum of non-animal methods in biomedical research and safety testing. We would like to see the innovative nature of nanotechnological research being used to speed up the paradigm change from *in vivo* to *in vitro* in biomedical research and regulatory testing.

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Commission Staff Working Document accompanying the 2nd Implementation Report to the N&N action plan 2005-2009. SEC(2009)1468 of 29 October 2009. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=SEC:2009:1468:FIN:EN:PDF.

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Treaty of Lisbon (2008). Consolidated versions of the Treaty on European Union and the Treaty on the Functioning of the European Union. OJ C 115/01 of 9 May 2008. http://eur-lex.europa.eu/JOHtml.do?uri=OJ:C:2008:115:SOM:EN:HTML.

Keywords: nanobiotechnology, nanotoxicology, EU Nanotechnologies Action Plan, non-animal test methods, validation

Poster

Optimisation of culture conditions for differentiation of human intestinal Caco-2 cells: serum-free medium and substrate effects

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While representing the best and most extensively used cell culture model of absorptive enterocytes, the human Caco-2 cell line displays a high degree of heterogeneity in the expression of differentiated functions that is largely due to differences in culture procedures. Addition of foetal bovine serum (FBS) to the culture medium represents one of the major sources of variability in the performance of this differentiated cell culture model. The growth substrate can also influence morphology and expression of differentiated functions, especially for epithelial cells that perform transport functions between the internal and external environment. Plastic culture-ware, that is impermeable to the passage of cell effluents, or permeable cell culture inserts are both commonly used for the culture of Caco-2 cells and can further increase the variability in the expression of differentiated functions.

Caco-2 cells (parental Caco-2 and Caco-2/TC7, a line of clonal origin, obtained from INSERM, Paris) were grown and differentiated for up to 21 days on plastic or on permeable polycarbonate cell culture inserts and development of polarity and expression of intestinal functions were monitored by immunofluorescence and confocal microscopy, gene expression by qRT-PCR and *in situ* enzyme assays. Cells seeded on permeable inserts were also maintained in different chemically defined media containing in the basolateral compartment culture medium supplemented with insulin, transferrin, selenium and a lipid mixture (oleate, palmitate, cholesterol), or a defined mixture of growth factors and hormones (MITO+ serum extender) with or without the addition of the lipid mixture. The effects of the different serum substitutes on cell differentiation were assessed by permeability assays, apical intestinal enzyme assays and functional expression of transporters.

Growth and differentiation of Caco-2 cells on plastic produced a more heterogeneous morphology and negatively affected the expression and localisation of tight junction proteins and the activity of apical enzyme compared to cells maintained on permeable supports. Among the serumfree media tested, cells supplemented with MITO+ serum extender displayed differentiated functions that most closely resembled those of cells grown in media supplemented with 10% FBS.

In order to improve the reproducibility of the cell model we recommend the use of serum-free, chemically-defined medium for differentiation of Caco-2 cells (van der Valk et al., 2010). The use of plastic supports should be restricted to cell propagation, while permeable supports should be used for Caco-2 differentiation.

Work supported by EU FP6 Project "LIINTOP" Contract number STREP-037499

Reference

van der Valk, J., Brunner, D., De Smet, K. et al. (2010). Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. *Toxicology in Vitro*, in press.

Keywords: defined media, permeable filters, plastic supports

Poster

Development of a 3D-liver cell culture model for drug metabolism and hepatotoxicity studies

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The European chemical legislation REACH requires additional safety evaluation of chemical components going along with an increase in the number of animal experiments. Recently ECHA called for the use of alternatives to animal testing, thereby indicating a strong request for *in vitro* systems.

The aim of the study is to develop a reliable and powerful cellular model for drug metabolism and hepatotoxicity studies. The HepaRG cell line, a human progenitor cell line able to differentiate into hepatocyte-like cells, was cultured in a porous polystyrene matrix to reach more physiological culture conditions than in a 2D cell culture model. We report on a three-dimensional (3D) liver cell model for drug studies.

HepaRG cell morphology and settling were investigated using haematoxylin staining, formazan formation, light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM) analysis. Cell proliferation (MTT assay) and cell cytotoxicity (LDH assay) were determined. Differentiation of HepaRG cells was induced by dimethyl sulphoxide. Then CYP450 expression was analysed by qRT-PCR and activity by fluorescence based assays. Metabolic functionalities (albumin synthesis, urea formation) were measured over time.

Cell staining and formazan formation revealed a uniform distribution of HepaRG cells in the PS matrix. Ultrastructure analysis showed HepaRG cells in monolayers tight-fitting to the PS matrix. Analysis of cell viability, cytotoxicity and cell proliferation proved that PS scaffolds are well tolerated by HepaRG cells. Differentiation of HepaRG cells within PS scaffolds resulted in significantly higher mRNA expression and activity levels of important biotransformation phase I CYP450 genes compared to a conventional 2D cell culture model.

The differentiated HepaRG cells in the porous polystyrene matrix might be a promising system for *in vitro* test applications to study liver function in drug metabolism and hepatotoxicity and might help to reduce animal use for testing.

Keywords: HepaRG, 3D-cell culture, scaffold, liver, polystyrene, 3R

Poster

Development of an *in vitro* sensitization assay based on monocyte-derived dendritic cells

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Dendritic cells, including Langerhans cells, forming a sentinel network for pathogen detection, are the most abundant antigen-presenting cells in the skin. Through their ability to take up, process and present hapten to T-cells they play a critical role in the induction of contact allergies. In this process dendritic cells undergo fundamental changes, e.g. surface marker expression. Their observance marks a potential endpoint in the experimental set-up of a predictive *in vitro* skin sensitization assay. Therefore we developed an *in vitro* sensitization assay based on monocyte-derived dendritic cells and the detection of surface marker expression changes.

$CD1a^-/CD14^+$ peripheral blood monocytes from healthy donors were isolated from buffy coats by Ficoll density centrifugation and further purified by positive selection of anti-CD14-Ig coupled magnetic microbeads. $CD1a^-/CD14^+$ monocytes were differentiated into immature dendritic cells by 5 day culture in

the presence of IL-4 and GM-CSF. Substance treatment for 48 h was followed by FACS analysis of HLA-DR; CD86; CD80; CD14; CD1a and CD83.

The expression changes of the surface marker CD86 most clearly correlated with the concentration of sensitizing substance tested.

In this assay all strong sensitizers tested, as well as non-sensitizers, were identified correctly with regard to their allergic potential in the LLNA, whereas moderate sensitizers (according to the LLNA) showed surface marker changes only close to cytotoxic concentrations. Limitations, e.g. donor variability and work intensiveness, are widely discussed. However, this assay leads to results that reflect the reaction of a healthy donor population in contrast to single individuals of cell lines. Moreover, after interpretation of comprehensive investigation we assume that a classification of the sensitizing potential of substances may be possible, marking a clear advantage over the all-or-none interpretation of cell line based assays already established.

Therefore this assay provides a basic application in assessing the allergic potential of active components.

Keywords: in vitro toxicology, sensitization, dendritic cell, CD86, alternative method

Lecture in Session IX: New developments and novel methods

Application of human dopaminergic neurons for toxicity testing

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According to the new paradigm of 21st century toxicology, we introduce a cellular *in vitro* model of human neurons that allows not only phenotypic observations but also mechanistic insights into the pathways involved in neurodegeneration. When model systems composed of only one cell type are used, no information on interactions between neurons and other brain cell types, such as astrocytes or microglia, can be observed. Most chronic neurodegenerative diseases are accompanied by pro-inflammatory conditions and glial cell contribution. Another essential field in neurotoxicology focuses on the impact of compounds on neurites and requires better *in vitro* models.

The human cell line LUHMES (Schildknecht et al., 2009) was tested in co-culture systems with glial cells. With this model it is possible to investigate cell-cell interactions as exemplified by the conversion of the parkinsonian toxin MPTP by astrocytes into the active form MPP+, which subsequently leads to a selective degeneration of LUHMES. Also, signalling molecules, such as nitric oxide, superoxide or cytokines, and pharmacological interventions were studied. As an alternative to transgenic animal models, LUHMES were transfected for overexpression of the Parkinson's Disease related protein α-synuclein and its respective mutants, thus allowing studies on posttranslational modifications (tyrosine nitration, methionine sulphoxidation). For investigations on neurites, the differentiation process into fully mature neurons was performed in the presence of test compounds. Neurite mass was detected by a newly developed automated system as the readout.

Reference

Schildknecht, S., Pöltl, D., Nagel, D. M. et al. (2009). Requirement of a dopaminergic neuronal phenotype for toxicity of low concentrations of 1-methyl-4-phenylpyridinium to human cells. *Toxicol. Appl. Pharmacol. 241(1),* 23-35.

Keywords: dopaminergic neurons, MPP+, neurite degeneration, glia

Poster

Current activities of the Foundation Animalfree Research (AfR)

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The Foundation Animalfree Research (AfR), founded in Zurich in 1976 as FFVFF, has been focussing on nanotechnology and its implications on animal testing and welfare in 2009 and 2010.

In 2008, AfR financed a literature study aiming at providing information about number, type and severity of animal experiments in the field of nanotechnology. This showed that nanotechnology has a high potential to significantly increase the number of research animals. In 2009, based on these results, Dr. Ursula G. Sauer evaluated how animal welfare issues are taken into account in political activities and research projects in Europe. Political action plans do address the 3Rs principle, at least to some extent. Likewise, funding programmes promote the development of non-animal test methods; however the proportion of *in vitro* funding does not seem sufficient to achieve a paradigm change towards non-animal testing strategies. Ethical deliberations on nanotechnology, which influence future policies, did not cover animal welfare issues. Overall, the political activities do not go far enough to pay full regard to animal welfare issues as requested in the Treaty of Lisbon. As a result of these observations, AfR has been addressing political and scientific responsible persons to draw their attention to the animal welfare implications of nanotechnology and to promote non-animal test methods in this rapidly developing area. In this context AfR has organised a Special Session for the present congress, entitled: "Towards non-animal testing strategies in nanotechnology – what needs to be done and who will do it?"

With the financial help of AfR it was possible to develop a triple-cell coculture system in order to study the interactions of synthetic nanoparticles with the lung and to better understand the potential risk for human health. This project culminated in a PhD thesis in 2010 and resulted in several follow-up projects. A poster on this topic by Dr. Christina Brandenberger can be viewed at this congress.

In 2009, AfR supported the development of an inhalation chamber that is suited to study cell-particle interactions in a controlled and life-like environment at the Helmholtz Institute in Munich. The chamber is applicable for various research areas and the results were published in 2009.

In 2009 and 2010, AfR partially supports a project which establishes an animalfree method for the safety testing of tetanus vaccines. After inactivation, these vaccines have to be tested for residual toxoid activity in guinea pigs, who are then examined for symptoms of tetanus. At Paul-Ehrlich Institute an *in vitro* system is being developed which can measure active tetanus neurotoxin.

In November 2009, AfR hosted a discussion forum focussing on the recently introduced concept of animal dignity, which has been part of Swiss animal welfare legislation since September 2008. The practical implications for the public understanding of animal welfare and the possible consequences for jurisdiction and research were then discussed in a panel of experts. A compilation of the contributions (German language only) can be ordered for 10 Swiss Francs at info@animalfree-research.org.

Keywords: alternative methods, animalfree research foundation, project funding

Lecture in Session III: Skin sensitisation and eye irritation

Identification of novel *in vitro* test systems for the determination of glucocorticoid receptor ligand induced skin atrophy

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Topical glucocorticoids (GCs) are highly effective anti-inflammatory agents but are limited by their side effect potential, with skin atrophy being the most prominent one. Thus, determining the atrophogenic potential of novel compounds is important. The aim of this study was to establish an *in vitro* skin atrophy model.

A screening cascade was applied and GCs with a known atrophogenic potential were used as tool compounds. Five rodent and human cutaneous cell types / cell lines and two human skin equivalents were tested. Known and suspected atrophy markers related to collagen metabolism and epidermal thickness were measured. Altogether a compilation of seven different cellular assays with up to 16 markers each was investigated.

A reproducible, more than 2-fold, regulation of the candidate markers by dexamethasone or clobetasol was found for: i) *MMP1, -2, -3* and *-9* expression in human keratinocytes, ii) *COL1A1* and *COL3A1* expression in 3T3 fibroblasts and iii) epidermal thickness, collagen and MMP synthesis in the full-thickness skin model FTSM. These three models were further investigated with a panel of 4-5 GCs, demonstrating dose-dependence and correlation with the atrophogenic potential of the tool compounds, qualifying the models as potentially suitable. Finally, the predictability of these models for the *in vivo* situation was analysed by testing a novel selective GC receptor agonist (SEGRA) in comparison to clobetasol. The results from the *in vitro* models suggested less atrophogenic activity for the SEGRA compound, which indeed was confirmed in the hr/hr rat skin atrophy model.

In conclusion, a combination of three *in vitro* models based on 3T3 cells, human keratinocytes and FTSM with several readouts is recommended to determine atrophogenicity of GC receptor ligands. Further experiments are necessary to explore the possibility of reducing this panel and to demonstrate the true predictability for the clinic.

Keywords: 3T3 mouse fibroblast, NHEK, collagens, MMPs, skin atrophy, glucocorticoids

Poster

The Bovine Corneal Opacity and Permeability (BCOP) test for routine ocular irritation testing and its improvements within the limits of OECD test guideline 437

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Data on eye irritation are generally needed for hazard identification of chemicals. As the bovine corneal opacity and permeability (BCOP) test is accepted for the identification of corrosive and severe ocular irritants since September 2009 (OECD test guideline, TG437), we established and evaluated this alternative method for our routine testing including a broad variety of chemicals and formulations.

We proved our technical proficiency by testing the ten reference standards recommended in the TG437 and a further 21 substances with published BCOP and *in vivo* data. Our results matched the published *in vitro* data very well, but with some intentionally selected false negatives (FN) and positives (FP), the concordance was 77% (24/31) with FN and FP rates of 20% (2/10) and 24% (5/21). Additionally, we tested 21 in-house substances, showing the utility for our own substance panel.

As it was suggested to use histopathological evaluation as an additional endpoint to help identify FN of low opacity and permeability, we also examined cross sections of treated corneas after the BCOP test. This endpoint corrected the classification of some FN, but at the same time increased the number of FP.

In parallel, we evaluated three new opacitometer models by comparing them with the current standard device. Hence, we recommend the use of our improved opacitometer with certified components and electronic data storage, resulting in excellent sensitivity, stability and reproducibility.

Taken together, we successfully established the BCOP test, which is suitable to identify ocular corrosives and severe irritants in our routine testing and for this purpose we developed a reliable opacitometer, the improvements of which are in line with TG437.

Keywords: BCOP, OECD 437, improvements

Poster

Toxicity of small heterocycles and their structural alerts developed by means of an *in vitro* test with *Tetrahymena pyriformis*

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Electrophiles are known for their potential to be significantly more toxic than baseline narcotics. This excess toxicity is caused primarily by covalent reactions with nucleophilic amino acid side chains, resulting in an impairment of protein functions. So far, there is no general model for predicting the level of reactive toxicity across different electrophilic compound classes. In this context, a way forward is to combine chemoassay data about chemical reactivity with bioassay data about excess toxicity in order to develop structural rules for discriminating excess-toxic compounds from narcosis-level toxicants.

In this work we present bioassay results for small heterocycles with three or four ring atoms. To this end, the well-known growth inhibition assay with the ciliates *Tetrahymena pyriformis* was used in a slightly modified way. Comparison of observed toxicities with associated baseline narcosis predictions (which in turn were derived from a respective regression model calibrated with a separate set of narcotics) led to the quantification of the reactivity-driven toxicity enhancement (Te). The latter is defined as ratio between (predicted) narcosis level and observed toxicity. Accordingly, large Te values indicate a large Te and thus a correspondingly large excess toxicity. Analysis of the observed Te values reveals systematic relationships with the heterocyclic ring size and with substituent effects. The resultant structural alerts can be used to screen respective heterocyclic compounds for their potential to exert excess toxicity.

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Keywords: Tetrahymena pyriformis, heterocycles, toxicity enhancement, EC_{50}, structural alert

Poster

Epigenetic modification improves hepatic differentiation of human adipose-derived mesenchymal stem cells (Ad-MSCs) for *in vitro* toxicity

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A major hurdle in preclinical drug testing is the lack of suitable *in vitro* models that allow the prediction of potentially toxic effects in humans. Furthermore, the availability of primary human hepatocytes for toxicity studies is limited by donor organ scarcity. There is general agreement that human-relevant data on hepatic drug toxicity can only be generated with cell systems that exhibit the full range of human liver-specific functions. Our group aims to generate hepatocyte-like cells from mesenchymal stem cells derived from human adipose tissue (Ad-MSCs).

For hepatic differentiation several supplement combinations of 5-azacytidine, FGF-4, dexamethasone, nicotinamide, ITS, HGF and EGF were used for 18 days. Generated hepatocyte-like cells were characterised regarding expression and activity of 8 different phase I and II drug metabolising enzymes. Primary human Heps were used as gold standard for every test.

After differentiation, Ad-MSCs showed morphological features similar to primary human hepatocytes and also gained the ability to accumulate glycogen and express glucose-6-phophatase. To

investigate their metabolic ability urea- and glucose metabolism were analysed. Basal urea- and glucose production as well as NH_4Cl metabolism rate were comparable to those of phHeps. After differentiation hepatocyte-like cells expressed phase I and II drug metabolising enzymes shown by Western blot and RT-PCR. Furthermore, fluorescence-based screening of cytochrome P450 (CYP) isoforms shows comparable activities to phHeps. We could show inhibition of CYP1A1/2 and CYP3A4 by Verapamil, Quercitin, and Nifedipin in both hepatocyte-like cells and phHeps. Both cell types also responded with cell death to DMSO and mercury (II) chloride and displayed metabolic-induced cell death upon caffeine, diclofenac and verapamil exposure.

With our differentiation protocol we are able to generate a sufficient amount of Ad-MSC-derived hepatocyte-like cells. Furthermore, our cells express a large number of phase I and II drug metabolising enzymes with an activity of up to 80% of phHeps. From the data obtained from the inhibition study and toxicity tests we propose that these Ad-MSC-derived hepatocyte-like cells are well suited for alternative *in vitro* toxicity tests. Therefore this test system provides a commercially and economically effective *in vitro* alternative to preclinical drug testing in animals, enabling more predictive studies on hepatotoxicity in humans.

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Keywords: Ad-MSCs, AZA, phHeps, CYPs

Poster

Epigenetic changes improve differentiation of adipose-derived mesenchymal stem cells (Ad-MSCs) to hepatocyte-like cells: possible use as an *in vitro* toxicity test system

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Primary human hepatocytes (Heps) are used as gold standard for *in vitro* toxicity tests and drug screenings. Their availability is limited by donor organ scarcity and this raises the urgent need to find alternative cell sources. The aim of this study was to generate, with the help of epigenetic changes, Ad-MSC-derived hepatocyte-like cells with a high metabolic potential and to investigate their possible use as an *in vitro* toxicity test system.

Human MSCs were isolated from abdominal adipose tissue from patients according to the ethical guideline of the MRI. After treatment with 5-azacytidine, epigenetic changes of the cells were analysed by measuring the global methylation status. Furthermore, expression of pluripotency genes Klf4, Nanog, c-Myc and Oct4 was assessed by real-time PCR. For hepatic differentiation, cells were treated with combinations of FGF-4, dexamethasone, nicotinamide, ITS, HGF and EGF over a period of 17 days. The expression and activity of different phase I and II drug metabolising enzymes was analysed by RT-PCR, Western blot and fluorescence-based activity assays. For hepatic characterisation different markers like albumin, α-fetoprotein and CYP3A4 were analysed by RT-PCR. We measured the toxicity of different substances, e.g. DMSO, verapamil and caffeine on the generated hepatocyte-like cells with the help of Alamar Blue and sulforhodamine B staining assay. Heps were used as gold standard.

Use of 5-azacytidine decreased the global methylation status of the cells in a concentration-dependent manner. This led to an increased expression of the pluripotency genes up to two-fold. Throughout differentiation, Ad-MSC-derived hepatocyte-like cells gained the ability to accumulate glycogen and express glucose-6-phophatase. Their basal metabolic activity, such as

urea and glucose production, was comparable to Heps. Hepatic markers like albumin and α-fetoprotein were detected in the hepatocyte-like cells at mRNA level. Moreover, a high expression of CYP3A7 and a low expression of CYP3A4 were detectable. The differentiated hepatocyte-like cells expressed the investigated phase I and II drug metabolising enzymes. Thereby the expressions of CYP2E1, CYP2A6 and CYP2B6 reached up to 100% in comparison to Heps. Furthermore there was an enzymatic activity of CYP1A1/2 of up to 60%. The investigated phase II enzymatic activity of the CHC conjugation was comparable to Heps and reached up to 90%. Sufficient resorufin, AHMC and HFC conjugation could also be detected. Direct toxicity with the help of treatment with DMSO or $HgCl_2$ and indirect metabolite-dependent toxicity with caffeine, verapamil and diclofenac generated EC_{50} values that were not significantly different to those gained with Heps.

Our work shows that inhibition of DNA-methyltransferase leads to an initial cell dedifferentiation, which contributes to a better hepatic differentiation of Ad-MSCs. Generated hepatocyte-like cells express a large number of phase I and II drug metabolising enzymes with activities up to 80% of Heps. Several hepatic markers could also be detected. The data obtained from the different toxicity tests propose that our cells are well suited for *in vitro* toxicity tests.

Keywords: epigenetics, adipose-derived mesenchymal stem cells, human hepatocytes, in vitro toxicity

Lecture in Session V: Acute and long term toxicity A

Peroxide-induced oxidative stress and cell death in C6 astroglioma cells

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Oxidative stress can cause oxidative damage and this may trigger cell death if it is severe enough. In many *in vitro* studies oxidative stress is induced by using peroxides. The objective of the studies presented here was to characterise the relationship between oxidative stress and cell death in C6 glioma cells, especially regarding the role of concentration and exposure time when different peroxides are used. Furthermore, the influence of selected antioxidants should be investigated. Finally, the effects of bolus applications of H_2O_2 should be compared with those caused by continuous exposure by means of an H_2O_2 generating system.

C6 astroglioma cells were exposed to peroxides (hydrogen peroxide, cumene hydroperoxide (CHP) and tert-butyl hydroperoxide (t-BuOOH) in serum-free DMEM. The following endpoints were studied: peroxide clearance (FOX assay), radical generation (H2DCF-DA), lipid peroxidation (TBARs, HPLC), total cell count (cell protein), apoptotic/necrotic cells (nuclear morphology), caspase 3-activity.

All peroxides disappeared from the culture medium starting immediately at the beginning of exposure. The clearance kinetics depended on exposure conditions (e.g. cell concentration) and the peroxide studied. Concomitantly, cellular radical generation was induced by all peroxides, leading to a continuous accumulation of ROS as indicated by the fluorescent H2DCF-DA. In the case of the organic peroxides CHP and t-BuOOH, lipid peroxidation was initiated in parallel to radical generation. In contrast, H_2O_2 did not induce any lipid peroxidation. With a certain time delay, these early events were followed by a concentration and time-dependent induction of cell death by all peroxides, with H_2O_2 being less potent than organic peroxides after prolonged exposure times (≥ 24 h). Analysing nuclear morphology (fluorescence microscopy) and caspase 3-activity revealed that peroxide-induced oxidative stress caused predominantly necrotic death. In a narrow window of low concentrations H_2O_2 induced apoptosis in a small fraction of the cell population (5 to 10%), while in case of the organic peroxides this fraction was even lower and hardly significant. The first apoptotic cells occur after 24 h. When oxidative stress was induced by continuous generation of H_2O_2 in the culture medium, essentially no increase in apoptotic cells could be observed. Instead, starting at a certain rate of H_2O_2 production, all cells died by necrosis. Antioxidants, like quercetin prevented the C6 cells from necrotic cell death induced by all peroxides without any effect on the fraction of apoptotic cells. In contrast, α-tocopherol was cytoprotective only against necrosis induction by the organic peroxides, not by H_2O_2. Concomitantly, it increased the fraction of cells dying by apoptosis. In other words, in a certain population of cells, α-tocopherol was able to switch the mode of peroxide-induced death from necrotic to apoptotic.

Taken together, peroxides, in general, induce oxidative stress in C6 cells. However, the stress-induced effects differ between the peroxides and are quantitatively and qualitatively dependent on the concentration and exposure time. In general, at least in C6 cells, oxidative stress induced by peroxides causes predominantly necrotic death. The relatively small fraction of cells undergoing apoptosis can be increased by antioxidants like α-tocopherol.

Keywords: radical generation, lipid peroxidation, apoptosis, necrosis, antioxidants

Poster

A first approach to distinguish cytotoxic effects of diarrheic and non-diarrheic marine toxins using two human intestinal cell lines

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Human poisoning due to ingestion of seafood contaminated by algal toxins is a worldwide problem. The monitoring of phytoplankton and phycotoxins is required in shellfish production sites to avoid food-safety problems. In Europe, the mouse bioassay is the official reference method for lipophilic biotoxins, but alternative methods based on analytical and *in vitro* approaches have been developed for a few years. Okadaic acid (OA), dinophysistoxins (DTXs) and azaspiracids (AZAs) are diarrheic shellfish toxins, whereas pectenotoxins (PTXs) and yessotoxins (YTXs) have been recently excluded from this group due to the absence of diarrheic effects *in vivo* (Aune, 2002; Rossini and Hess, 2010). In this study, we investigated whether diarrheic versus non-diarrheic lipophilic toxins can be detected by a cell-based assay.

Cytotoxicity of four phycotoxins (OA, AZA1, PTX2 and YTX) was investigated in two intestinal cell models (enterocyte-like Caco2 cells and mucus-secreting-like HT29-MTX cells; Lesuffleur, 1990) with the neutral red uptake assay. Undifferentiated and differentiated cells were exposed for 48 h before IC_{50} determination.

The cell differentiation induced a decrease in the global sensibility to phycotoxins and HT29-MTX appeared more sensitive than Caco2. Whereas IC_{50} values for OA obtained on undifferentiated and differentiated Caco2 were significantly different ($p<0.001$) (45 ±17 nM and 294 ±49 nM respectively), similar cytotoxicity was obtained on HT29-MTX (IC_{50} = 55 ±6 nM and 98 ±38nM respectively). Important differences in the cytotoxic response to AZA1 were observed between the two *in vitro* models. In Caco2 cultures, AZA1 induced morphological alterations but without or with only a slight effect on cytotoxicity, as previously described by Hess et al. (2007). However, important cytotoxicity was observed after HT29-MTX exposure to AZA1 (IC_{50} = 3 ±0.4 nM for undifferentiated cells and 14 ±4 nM for differentiated cells). Cytotoxic effects of PTX2 were similar on undifferentiated cells whatever the cell type: HT29-MTX (IC_{50} = 152 ±26 nM) and Caco2 (IC_{50} = 118 ±17 nM). These values increased (x2 to 3) for differentiated cells (324 ±45 nM for HT29-MTX and 386 ±190 nM for Caco2). Exposure to YTX induced few cytotoxic effects in Caco2; even if calculated IC_{50} were low (5 ±3 nM and 26 ±15 nM on undifferentiated and differentiated Caco2), only half of the population responded to YTX. No cytotoxicity was obtained on differentiated HT29-MTX cells exposed to YTX compared to undifferentiated cells (IC_{50} = 140 ±68nM). All these results indicate that mucus-secreting HT29-MTX are more sensitive to diarrheic phycotoxins (OA + AZA1) than to "non-diarrheic" phycotoxins (PTX2+YTX), whereas no difference in cytotoxicity between the two types of toxins could be detected with Caco2. Further investigations will be performed to study the cellular response considering markers involved in diarrhoea such as mucins and interleukins, as previously published with bacterial lipopolysaccharide (Huang et al., 2003; Smirnova et al., 2003).

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Keywords: marine toxins, cell-based assay, enterocyte-like Caco2 cells, mucus-secreting-like HT29-MTX cells, neutral red uptake

Lecture in Session AXLR8 '21st Century Toxicology' Info Forum

Contribution of the US EPA to US project on "Toxicity Testing in the 21st Century"

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no abstract submitted

Poster

Toxicological information on REACH-relevant chemicals published in Russian language

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The system for creating and publishing chemical safety information was well developed in the former Soviet Union. Despite that, valuable toxicological data published in the Russian language have remained non-cited in recent scientific papers, except when these were the only information sources available concerning a particular compound. Currently, the access to this information is limited due to the language barrier as well as the low level of digitalisation of respective journals and books.

As this information can be beneficial for the EU chemical policy REACH, we have mapped the main publicly available sources of toxicity data published in the Russian language. The search was performed mainly via publicly available internet sites and Russian peer-reviewed literature (books, journals, PhD dissertations, scientific reviews). More detailed information can be found in Sihtmäe et al. (2009). As a result, a new OpenAccess online database E-SovTox (http://kbfi-databases.eu/database/) was established, which provides toxicological data for REACH-relevant chemicals. Registered users can check the original abstract and full text of the original published work, as well as an English translation of the abstract and validate the data. The database has been created using php and SQL and it shall be opened to the public in 2010.

This research is part of the EU 6th Framework Integrated Project OSIRIS (contract no. GOCE-ET-2007-037017). We dedicate this presentation to our colleague involved in this work, Prof. Henri-Charles Dubourguier, who passed away recently.

Reference

Sihtmäe, M., Dubourguier, H-C. and Kahru, A. (2009). Toxicological information on chemicals published in the Russian language: Contribution to REACH and 3Rs. Toxicology 262, 27-37.

Keywords: safety, toxicity, REACH, Russia

Poster

Toxicological endpoints to assess developmental bone toxicity *in vitro*

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To predict the toxic potential of industrial chemicals and pharmaceutical products on human bone development, rodent and non-rodent (e.g. rabbits) *in vivo* models are commonly used. The assessment of osteogenic toxicity in the embryo is integrated into the OECD testing guideline #414, which covers prenatal developmental toxicity. In experiments using rodents, one half of the litter (approximately 450 animals per test substance) is sacrificed for the examination of skeletal damage. In addi-

tion to the high number of laboratory animals required, these *in vivo* studies are costly and time-consuming. Until today, there is no validated *in vitro* test to assess developmental bone toxicity. Therefore, current work at ZEBET in the frame of a joint project, funded by the German Ministry for Education and Research (BMBF), focuses on the development of a robust assay with the capacity to predict osteotoxicity in the embryo.

Embryonic stem cells are pluripotent cells which can differentiate into a multitude of diverse cell types. Their capacity for unlimited self-renewal together with their ability to faithfully recapitulate early developmental programmes in the embryo *in vitro* makes them an extremely attractive model for embryonic toxicity studies.

Crucial stages during osteogenesis involve the sequential expression of a tightly regulated set of molecular markers and the mineralisation of the extracellular matrix. We are currently assessing whether any of these markers can serve as a predictive endpoint to assess bone toxicity using a diverse range of molecular biological methods, e.g. real-time PCR, Western blot, flow cytometry, Fourier transform infrared spectroscopy (FTIR) and cytochemical staining. Mouse embryonic stem cells (line D3) are currently employed as a model system to study osteogenesis. As a next step this approach will be expanded to other cellular systems including stem cells from rhesus monkey, human iPS cells as well as human mesenchymal stem cells.

The applicability of the different molecular markers and the morphological approach depending on the method is discussed regarding their potential as toxicological endpoints.

Keywords: bone development, embryonic stem cells, in vitro test method, toxicological endpoints

Lecture in Session VIII: Reproductive toxicology and stem cells

Optimised 1D and 3D isolation and expansion of multipotent human adipose-derived stem cells

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In the adult human body a number of mesenchymal stem/progenitor cell (MSC) niches are present, all showing a unique self-renewing and multipotent behaviour. The best-characterised human source is the bone marrow. Due to a low isolation yield of multipotent cells and a risky isolation procedure, alternative supplies have been sought. Promising and readily available resources are human adipose tissue stem cells (hADSC), which can be easily obtained in large quantities from plastic surgery waste material. Current hurdles, however, are the lack of standardised isolation protocols and large-scaled growth procedures. Indeed, classical isolation protocols, and particularly those based on the adhesive character of MSC, result in heterogeneous cell populations of MSC, fibroblasts and other cell types, all undergoing growth-dependent senescence.

In the present study, we therefore searched for efficient isolation and expansion protocols in terms of differentiation efficiency and up-scalability. Hereto, novel 1D and 3D techniques were developed. A rating scale was assessed by comparison of growth characteristics (growth rate, homogeneity/heterogeneity) and phenotypic profiling of both undifferentiated and meso-, endo- and ectodermal-directed cells. The trilineage differentiation potential was investigated upon exposure to various (non-) commercially available differentiation formulations, i.e. two commercial neural media (Lonza, Stem Cell technologies), five commercial keratinocyte media (CellnTec, Epilife), three fine-tuned commercial adipocyte media and second "in house" prepared sequential hepatic differentiation media. Special attention was paid to their efficiency to induce lineage-specific differentiation and maturation.

This work was supported by grants from the Fund for Scientific Research (FWO) Vlaanderen, Belgium; BruSTEM, Brussels, Belgium; the European Union Sixth Framework Program (STREP project no. 037499 LIINTOP); and the European Union Seventh Framework Program (IP project no. FP7-201619 ESNATS).

Keywords: adult stem cells, in vitro model, multipotency, upscaled technology

Lecture in Session VIII: Reproductive toxicology and stem cells

The way forward in reproductive/developmental toxicity testing

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The use of experimental animals in reproductive toxicity testing is critically reviewed on the occasion of the 50th anniversary of the publication of the Three Rs concept by Russell and Burch, since there is major concern that the use of experimental animals will significantly increase due to the requirements of the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) system. A comparison of the test guidelines for drugs, agrochemicals and industrial chemicals shows that, for historical reasons, significantly different testing strategies are applied.

The current status of development and validation of *in vitro* tests in reproductive toxicology is also critically evaluated. The mouse embryonic stem cell test (mEST) is the most advanced and promising of the *in vitro* tests. Although it has not yet been accepted for regulatory purposes, its use in preclinical drug development is well established. Moreover, promising molecular endpoints have been established in the mEST, including proteomic and toxicogenomic endpoints. To date only preliminary results have been obtained with a human EST (hEST).

In addition, an overview will be given on new *in vitro* reproductive toxicity tests that are currently being developed in relation to the EU FP6 project ReProTect, since the ReProTect test battery, which covers the essential steps of female and male fertility, implantation and embryotoxicity, holds promise for use as a screening assay for reproductive toxicity testing according to the EU REACH legislation. However, since validated *in vitro* methods will not be available in the short term, opportunities for the refinement of the standard *in vivo* tests are discussed in order to reduce the number of animals used in reproductive toxicity testing.

Finally, recommendations for toxicity testing in the 21st century call for the harmonisation of test methods across all areas of regulatory testing as a first step. Since the REACH system testing framework for industrial chemicals is driven by the reproductive safety testing requirements of agrochemicals, a shift is proposed to exposure-driven testing of industrial chemicals. In particular, the implementation of a new "extended one-generation reproductive toxicity study" (EOGRTS), which includes triggers for additional testing for fertility, developmental neurotoxicity and immunotoxicity, would significantly reduce test animal numbers.

It is concluded that *in vitro* methods hold great promise for reproductive toxicity testing in the 21st century, e.g. the ReProTect *in vitro* battery and embryonic stem cell (ESC) technology focusing on molecular endpoints in both the mEST and the hEST.

Keywords: reproductive toxicology, in vitro tests, EST, ReProTect, fertility, extended 1-generation study

Lecture in Session AXLR8 '21st Century Toxicology' Info Forum

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

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Conventional approaches to toxicity testing and risk assessment are often decades old, costly and low-throughput, and of dubious relevance to humans. These factors have prompted leading scientific bodies to call for a transition to a 21st century paradigm, including a move away from apical outcomes at high doses in whole animals and toward a mechanistic understanding of the source-to-outcome continuum between xenobiotic exposure and adverse health effects. Such a shift will require a robust understanding of the cellular response/toxicity pathways that can lead to adverse effects when perturbed, appropriate *in vitro*

systems to study chemical interactions at key targets along a pathway, and computational systems biology models to describe the "circuitry" underlying each pathway as a basis for creating biologically realistic dose-response models.

The AXLR8 project aims to support the transition to a toxicity pathway-based paradigm for quantitative risk assessment and will: 1) organise a series of annual workshops to map research progress, gaps and needs in the FP6/FP7 programme on alternative testing strategies; 2) provide a range of tools and opportunities for enhanced interdisciplinary and international communication, coordination and collaboration in order to maximise the impact of available resources; 3) work to streamline regulatory acceptance procedures to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems as they become available and 4) produce annual progress reports on the state of the science, including recommendations on priority research and funding targets in order to ensure a prominent role for European science in this rapidly developing global research area.

Keywords: AXLR8, EU DG RTD funding, in vitro toxicology, toxicity testing in the 21st century

Poster

Improving the sensitivity of organotypic *in vitro* assays for eye irritation testing: the Ex Vivo Eye Irritation Test (EVEIT)

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Regulative acceptance of organotypic *in vitro* assays for eye irritation testing was an important step to reduce animal tests applied for evaluating the ocular irritation and corrosion potential for regulatory purposes. Today, the use of the accepted methods – namely the Bovine Corneal Opacity and Permeability (BCOP) assay as well as the Isolated Chicken Eye (ICE) test – is limited to the identification of ocular corrosives and severe irritants within a tiered-testing strategy. Negative results still have to be confirmed by animal tests. Therefore, improving the sensitivity of the organotypic *in vitro* assays for eye irritation testing will directly result in further reduction of the severity of harm to laboratory animals and the number of necessary time- and cost-intensive animal tests by reducing false negative results within the preceding *in vitro* test.

In order to achieve this goal, the here introduced Ex Vivo Eye Irritation Test (EVEIT) pursues two objectives in striving for higher sensitivity: First, enucleated eyes are replaced by corneal cultures and second, process monitoring is enhanced from static endpoint analysis to in-time dynamic observation using optical coherence tomography.

The EVEIT is based on living rabbit corneas from animals slaughtered for food production in long term culture (Frentz et al., 2008). This allows for *in vitro* observation of the metabolic stability of the living organ over several days, including the evaluation of recovery after e.g. chemical trauma. Optical coherence tomography enables *in situ* monitoring of the area and depth of structural damage and the progress of healing over time (Spöler et al., 2008). It also allows detection of the amount of endothelial damage and is therefore an ideal analytical tool for the intended test platform. Additionally, endpoints covered by the established organotypic *in vitro* assays, i.e. corneal opacity and corneal swelling, can be quantitatively analysed by OCT. Epithelial permeability is accessed within the EVEIT by fluorescein staining.

First experiments using the EVEIT to evaluate ocular irritation of a wide range of chemical substances are presented. These experiments demonstrate that a detailed analysis of structural damage and the progress of healing during several days is a sensitive tool to identify ocular irritants that are not accessible using organotypic *in vitro* assays based on freshly enucleated eyes.

Thus, these results suggest that the EVEIT is a promising tool to improve eye irritation testing with a considerably reduced need for animal experiments.

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Keywords: eye irritation, in vitro model, EVEIT

Lecture in Session VIII: Reproductive toxicology and stem cells

The coupling of a metabolic activation system (primary hepatocytes) to the murine embryonic stem cell test expands its use in reproductive toxicity testing

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The murine embryonic stem cell test (EST) represents a validated *in vitro* method to test the embryotoxic potential of chemicals. A limitation of the classic EST is that compounds needing to be metabolically activated in order to be embryotoxic are not detected as such. In the present study a metabolic activation system was coupled to the EST with the aim of improving its performance, and the two compounds tested in a first step were the well-known proteratogens cyclophosphamide (CPA) and valpromide (VPD).

Metabolic activation was performed by first incubating the compound of interest with freshly isolated murine or human hepatocytes. Thereafter, the supernatants were included in the EST, whereby a cell culture medium that could be used for the incubation step as well as for the EST was developed. The quantification of the substances prior to and after incubation was performed by gas chromatography (GC) coupled with mass spectrometry (MS).

It could be shown that CPA led to a concentration-dependent increase in embryotoxicity with and without a preceding metabolic activation, whereby CPA was 70 times more potent if it was metabolically activated. GC/MS analysis revealed that the degree of CPA metabolisation by murine hepatocytes paralleled the increase in the magnitude of the embryotoxic effect of CPA. In contrast, VPD was negative when tested in the EST, whereby the preceding incubation of VPD with murine hepatocytes had no influence on the outcome of the EST. In accordance with this result a subsequent GC/MS analysis showed that hardly any VPD metabolites were formed when incubating VPD with murine hepatocytes. On the other hand, the incubation of VPD with human hepatocytes led to a significant conversion of VPD into the teratogenic metabolite valproic acid. An EST with the supernatant derived from the incubation of VPD with human hepatocytes remains to be performed.

In the present study the successful combination of the conventional EST with a metabolic activation system has been described for the first time. In a first step two compounds were tested. In the next step a validation of the presented approach by testing a greater number of test chemicals will have to be performed. Due to species differences in the bioactivation of a number of proteratogens, further optimisation of the test system taking human metabolism into account is needed. Furthermore, a modification of the EST protocol in terms of generating embryonic bodies other than by the classic "hanging drop" method or exposure towards the test compounds from day 3 to 10 would make a direct co-cultivation of metabolically competent and ES cells possible. Hence, although not all difficulties have been circumvented, the developed test system shows that the coupling of a metabolic activation system to the EST is principally possible.

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Keywords: cyclophosphamide, embryotoxicity testing, hepatocytes, murine embryonic stem cell test, valpromide

Lecture in Session I: Legal, ethical and policy topics regarding alternatives

Human health risk assessment of pesticides – novel approaches explored by EFSA's PPR panel

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Both under the current Directive 91/414/EEC and the new Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market, a full risk assessment covering all possible adverse effects of active substances is required. While the Pesticide Risk Assessment Peer Review Unit (PRAPeR) is organising the peer review of these risk assessments, EFSA's Panel on Plant Protection Products and their Residues (PPR) provides scientific advice on specific questions and is in charge of drafting guidance documents and exploring new approaches in the field.

Regulation (EC) 369/2005 on maximum residue levels of pesticides in food requires the assessment of possible cumulative and synergistic effects of pesticide residues in food once appropriate methodologies are available. Currently there are no EU wide accepted methods for cumulative risk assessment. Health risks of pesticides are solely addressed on bases of individual compounds.

Therefore, in a first scientific opinion, the PPR Panel evaluated relevant existing methods based on which principles and methodologies for assessing risks of two or more pesticides in combination were established (EFSA, 2008).

In a second opinion these methodologies have been tested on a selected group of pesticides in order to find out whether or not they can also be applied in practice (EFSA, 2009).

Currently the PPR Panel is working on a third opinion identifying pesticides that can, based on their structure and effects, be grouped together for cumulative risk assessment. This opinion, completing the work on cumulative risk assessment, will be published at the end of 2011.

The OECD (2006) suggests the evaluation of the toxicity of pesticide metabolites to the best extent possible. However, within the peer review of pesticides, usually only the toxicological properties of active pesticidal substances are fully investigated in experimental studies, although often a number of plant/animal metabolites are identified in foodstuffs. Their toxicity is normally insufficiently assessed, since comprehensive experimental testing is not feasible due to their sheer number.

In order to be able to provide a guidance document on the assessment of metabolites in foodstuffs, the PPR panel is currently working on an opinion in which non-testing methods are evaluated for this purpose. Explored are the applicability of (Q)SAR models, the "threshold of toxicological concern" (TTC) concept and the identification of certain metabolic pathways leading to changes in the toxicity profile of molecules. The opinion should be published in 2011, the resulting EU guidance document in 2012.

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Keywords: pesticides, risk assessment, cumulative effects, non-testing methods

Poster

Planned future developments of lung model for use in inhalative substance testing

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Up to now there is a wide range of alternatives for animal tests, whereas there is no such alternative for research in the field of test substances applied to the respiratory system. Based upon a novel active lung model, the developments required to allow application of this model in the area of respiratory research are presented.

The currently realised lung model is able to simulate spontaneous breathing and ventilation processes. A good approximation to physiologically realistic properties is reached by using real, preserved pig lungs. Several limitations affect the application of this model as an alternative used in routine practice. By utilisation of isolated lungs, which must be kept alive artificially, the inner structure of real organs can be represented very well. On the one hand the supply of the lungs and on the other hand the size of these has to be considered when adapting the artificial nutrition of the organ. This regards not only the nutrition of the tissue with blood or blood replacement fluids, but also sufficient ventilation and controlled and regulated temperature and humidity conditions. At the moment only the ventilation is sufficiently optimised. In addition it is necessary to communicate with the provider of the pig lung, for example the slaughterer, to ensure that the lung is taken out without damaging the structure or the tissue. In order to perform concentration measurements on lung tissue that has been exposed to harmful substances, preparation and analysis of histological slices is necessary. The analysis may be realised with mass spectroscopic methods on which our cooperation partner, the "TU Vienna", is currently working.

In conclusion, analysis of the impact of the applied substances on a cellular basis has to be reviewed. For this only infrastructural requirements have been created so far, but the University of Innsbruck has experience in that area. The examination of applied particles should not be limited to chemical analysis, as the reactive properties of substances are influenced by their size and geometry. For detection of these structures the cooperation partner "University Vienna" has developed a multibeam single-particle spectrometer, which has to be further enhanced in order to integrate it into the lung model system.

For statistical, representative results several models have to be simulated in parallel. In order to achieve that a stand-alone embedded or an external master (PC) – slave solution has to be implemented. For this the LabView user interface is running as master on a laptop and signals are transmitted via Bluetooth to the slave integrated in the model. Communication between the models and the triggering has to be up to date for a parallel simulation.

In the current lung model the physiological breathing of humans is well reproduced. Despite enhancements are distinctions to real physiological breathing patterns realisable. The presented, planned developments of the current model will allow *ex vivo* tests with respiratory background, which then can actually contribute to a reduction of test animal numbers.

Keywords: artificial lung model

Lecture in Session I: Legal, ethical and policy topics regarding alternatives

A qualitative and quantitative examination of the impact of chemical regulation legislation on the field of toxicity testing

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Proposals for revising the principal United States law governing industrial chemicals, the Toxic Substances Control Act (TSCA), are currently under consideration in the US Congress, and some version of legislation is likely to be passed in the near future. At the same time a desire to move away from current testing methods for ethical, scientific, and practical reasons has led to multi-million dollar investments in *in vitro* and computational toxicology methods and programs. Such investment has been endorsed by multiple scientific bodies, most comprehensively by the US National Academies of Science in its 2007 report, *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Legislative language has the potential to endorse this transition and facilitate its fruition, or conversely enshrine *in vivo* testing methods and concepts for the foreseeable future. Additionally, legislative language and subsequent regulations have the potential to affect the numbers of animals killed in toxicity tests in the near term. There are a number of strategies and incentives that, used effectively, can reduce the overall number of animals who will be killed in tests required by new legislative mandates, while strengthening environmental and human health protections. We examine legislative and regulatory options for TSCA reform and their potential impacts on animal use and test method innovation, and the likelihood that such options will assist policymakers in successfully achieving desired legislative objectives, such as providing more information on potential chemical risks for a greater number of chemicals. Analyses like these are essential to judiciously select policies that reduce the use of animals in toxicity testing and protect human health and the environment.

Keywords: TSCA, industrial chemicals, testing strategy

Poster

Introducing a new scientific society for cellular and computational toxicology in North America: ASCCT

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Research into and development of computational and cellular toxicology testing methods is reaching unprecedented levels worldwide. In particular, scientists and regulators in the United States have begun to pursue a "paradigm shift" in the field of toxicology, spurred by ethical and legislative pressures, dissatisfaction with animal-based test methods, the need for better risk assessment tools, and advances in molecular and cellular biology. To communicate and accelerate advancements which reduce and replace the use of animals in toxicity testing, North Americans have formed a new scientific society.

The American Society for Cellular and Computational Toxicology (ASCCT) aims to provide an organized forum for discussion of *in vitro* and computational toxicology approaches, especially as replacements for animal-based toxicology methods. Through regular meetings and activities, the Society will facilitate the development, acceptance and routine use of cellular and computational methods with open dialog between industry, academic, advocacy, and regulatory scientists. The Society will strive to include the participation of young scientists to promote their contributions to the field.

An essential activity of the ASCCT will be regular meetings where scientists from a variety of fields can share developments. It is expected that interaction among scientists from the cosmetics, pharmaceutical, and chemical industries, regulatory agencies, and advocacy groups will provide diverse experiences and perspectives that are essential to a successful future for toxicology.

A key mission of the ASCCT will be to increase the routine application and use of computational and *in vitro* methods for prioritization, classification, and risk assessment purposes. North American government agencies, such as the US Environmental Protection Agency, the National Institutes of Health, and Health Canada have committed resource and operational support to promoting nothing short of a paradigm shift in toxicology. Cooperation and coordination among scientists from cellular, molecular, and computational disciplines will not only complement, but will be essential to, such a shift. Please join us in making the ASCCT the seminal scientific society for tomorrow's toxicology.

Keywords: toxicology, society, cooperation

Poster

Development of a human corneal epithelium model utilising a collagen vitrigel membrane and the changes of its barrier function induced by exposure to eye-irritant chemicals

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A collagen vitrigel membrane we previously developed is composed of high density collagen fibrils equivalent to connective tissues *in vivo* and is easily handled with tweezers. It possesses excellent transparency and permeability to protein with high molecular weight and consequently the various research applications utilising it as a cell culture substratum are advancing well (Takezawa et al., 2007a, 2007b, 2007c, 2004). We then established a reconstruction method for rabbit corneal epithelium by culturing normal rabbit corneal epithelial cells on the collagen vitrigel membrane substratum and inducing differentiation to form multilayers (Takezawa et al., 2010). However, to estimate eye irritation and permeability of chemicals in humans it is necessary to reconstruct a corneal model with barrier function utilising human cells.

In this study, as a first step, we aimed to establish a reconstruction method of human corneal epithelium with barrier function utilising both a human corneal epithelium-derived cell line (HCE-T) and the collagen vitrigel membrane substratum.

Further, to confirm the utility of the human corneal epithelium model, the changes of its barrier function induced by exposing it to eye-irritant chemicals were measured.

HCE-T cells purchased from RIKEN cell bank (Tsukuba, Japan) were seeded on the collagen vitrigel substratum and cultured for 2 days to confluence. Subsequently, the reconstruction of a corneal epithelium model was started by culturing it on air-liquid interface to induce cell differentiation for multilayer formation. The corneal model in the reconstruction process was analysed histologically by light microscopy of cross-sections stained with hematoxylin and eosin. Also, the formation of its barrier function was analysed by measuring transepithelial electrical resistance (TEER). Further, time-dependent changes of TEER after exposure to chemicals (NaOH, EtOH, Tween 20, etc.) were measured.

HCE-T cells proliferated well on the collagen vitrigel substratum and gradually differentiated into multilayers in air-liquid interface culture, resulting in a time-dependent increase of TEER. The corneal epithelium model possessing five cell layers was well reconstructed after one week of culture at the air-liquid interface. The exposure of chemicals to the model induced time-dependent changes of TEER in response to the characteristic of each chemical. These results suggest that eye irritant chemicals could be estimated by the barrier function of the reconstructed human corneal epithelium model.

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Keywords: corneal epithelium, eye irritation tests, collagen vitrigel

Poster

Secretome analysis of primary human hepatocytes in 2D- and 3D-culture for *in vitro* toxicology

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Human-related cellular systems (e.g. primary human hepatocytes (PHH) or stem-cell derived hepatocytes) are promising alternatives to today's animal testing methods for drug toxicity. The BMBF-funded HepaTox project aims at the identification of biomarkers for hepatotoxicity, characterising primary human hepatocytes in a 3D-hollow fibre bioreactor (Gerlach, 2003) by systems biology approaches, namely metabolomics and proteomics. Toxicoproteomics is a valuable and an exciting horizon to preclinical drug testing (Amacher, 2010; Bandara, 2002).

PHH were cultivated either in a 3D-hollow fibre bioreactor or in conventional cell culture flasks and secreted proteins ("secretome") were investigated by MALDI-TOF-MS/MS after trypsin digestion and nano-HPLC separation of tryptic peptides. Proteins were identified by database search (SwissProt) using the MASCOT algorithm. Albumin concentration in the culture medium was determined via ELISA specific for human albumin (Albuwell II, Exocell, Philadelphia; USA). High abundant proteins (albumin, transferrin, haptoglobin and antitrypsin) were depleted using a commercially available antibody-based spin cartridge (Hu-6 multiple affinity removal spin cartridge, Agilent, USA).

The use of foetal bovine serum (FBS) for seeding and maintenance of PHH proved to be a big obstacle, because secreted proteins were masked by their bovine equivalents. In 2D-culture, immunodepletion of four high abundant proteins was performed, leading to the identification of several low abundant proteins that were not identified reliably without immunodepletion. Furthermore, the effect of a therapeutic concentration of amiodarone on albumin produced by PHH in the bioreactor was tested in FBS free conditions. Albumin secretion was significantly reduced, but this effect was reversible upon withdrawal of the drug within a few days.

The results demonstrate that FBS should not be used when attempting identification and quantification of an extracellular

proteome. Toxicoproteomic analyses in 3D bioreactors need further optimisation, because even the use of only 2.5% FBS resulted in masking of the proteins secreted by PHH. High abundant proteins secreted by PHH, like albumin, increase the dynamic range of the secretome, hampering the identification of (probably more interesting) low abundant proteins. This difficulty could satisfactorily be overcome in 2D-culture by immunodepletion of abundant proteins. Despite these challenges, proteomics offers a potential for the dynamic study of changes at the protein level due to drug-induced liver injury (DILI), especially using (semi-) quantitative techniques.

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Keywords: alternative drug-testing, in vitro toxicology, proteomics, LC_MALDI

Poster

Animal testing issues during REACH implementation

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REACH – the EU's new legislation relating to the Registration, Evaluation and Authorisation of Chemicals entered into force in 2007 after many years of negotiations. The first deadline for the registration of chemicals produced in excess of 1,000 tonnes or already classified as very toxic is Dec 2010. The next deadline is 2013 for chemicals produced in excess of 100 tonnes. Regardless of the animal protection measures within the legislation, recent estimates of the numbers of animals that could be used under REACH range from 8 to 54 million, both truly staggering figures.

In this presentation we explore and update on the various issues that remain for the animal protection movement during the implementation of REACH. These issues include the lack of enforcement of "testing as a last resort" and inadequate mechanisms at either Member State or Commission level for the assessment of the proper use of validated alternatives. Since REACH was written a number of alternatives have been validated and it is important that industry are instructed to use these. Recent updates on the Test Methods and ECHA guidance – the agency responsible for REACH – that can provide scientifically valid ways to avoid animal use will be presented and evaluated.

Another big issue is the testing proposal facility whereby the animal-heavy test endpoints for the high tonnage chemicals are proposed rather than conducted. Interested parties have 45 days to submit evidence or existing data to help waive the testing. The European Coalition to End Animal Experiments (ECEAE), one of the key lobbying organisations in Europe on the issue of animal testing, have resourced a team of toxicologists to utilise the testing proposal system. We have developed a methodology for monitoring the proposals, prioritising them, searching for existing data using public databases and finally presenting arguments based on existing data, physico-chemical properties and exposure. Here we present our findings on how effective interested parties can be at serving the testing proposal facility from the early stages of REACH in an attempt to ensure that lessons are learned before the workload really starts to increase post the Dec 2010 deadline.

One of the problems already encountered by NGOs who are working on REACH implementation is confidentiality and transparency at ECHA. There has been a slow start to important decisions on how to evaluate confidential business claims by registrants. This has not only an effect on the ability of observers at ECHA to monitor decisions by Member States but also on those attempting to respond to testing proposals. Problems include the lack of information on substance identity and existing data. Finally, the ECEAE have joined others in asking academia to help reduce animal use by facilitating access for registrants to existing data in the form of robust study summaries.

Keywords: REACH, alternatives, regulatory testing, databases, 3Rs

Poster

Pre-validation of a two-tiered approach to determine the skin sensitizing capacity and potency of chemicals

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At present, the identification of potentially sensitizing chemicals is mostly carried out using animal models, such as the local lymph node assay (LLNA) and the Guinea pig assays (the Guinea pig maximisation test (GPMT) and Buehler's test). The EU Framework Program 6 funded the Sens-it-iv project, aiming at developing *in vitro* assays to identify and classify sensitizing agents. Results from this project have indicated the NCTC2544 activation assay and the epidermal equivalent (EE) potency assay as promising *in vitro* alternatives. This project aims to prevalidate the combination of these two assays in an *in vitro* two-tiered test strategy to identify and rank sensitizing potential of chemicals.

The first tier in the strategy determines the production of intracellular IL-18 by human-derived NCTC2544 keratinocytes and indicates the potential of a chemical to induce sensitization. The second tier in the strategy determines the viability and the production of IL-1α in a human reconstituted epidermal model as a measure of potency of a sensitizer. The project consists of three phases: Phase 1: Assessment of reproducibility and transferability (6 months); Phase 2: Pre-validation assessment and preliminary predictive capacity (15 months); Phase 3: Data evaluation; statistics and implementation (3 months).

The ring trial includes 5 different laboratories that are testing a panel of known encoded chemicals. ECVAM is involved as advisor throughout the duration of the project and TNO is commissioned to dissolve, encode and distribute the chemicals. The involved laboratories are directly responsible for setting up the assays in Sens-it-iv or have experience with setting-up and prevalidating assays.

The outcome of this project will contribute to the replacement and reduction of animals used for sensitization testing. The number of animals needed to comply with REACH legislation is estimated to be around 1 million for sensitization testing alone, whereas for testing of sensitization of cosmetics animal testing is banned completely in the EU from 2013.

Keywords: skin sensitisation, skin models

Poster

In vitro cytotoxicity and phototoxicity study of cosmetics pigments

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The aim of the work is the early identification of preventable risk factors connected with the consumer use of products of everyday use, such as cosmetic products, toys and children's products, and other materials intended for contact with human skin. The risk factor is represented by substances with irritation potential and subsequent possible sensitisation, resulting in a negative impact on human physical and psychical health with social and societal consequences. The legislation for cosmetics, chemical substances and other products requires for hazard identification the application of alternative toxicological methods *in vitro* without the use of animals.

We used a battery of *in vitro* alternative assays based on cell culture (Tomankova et al., 2009a). Progressive methods of molecular biology, based on fluorimetry and fluorescence, were

employed for identification of early morphological and functional changes on cellular level (Tomankova et al., 2009b). The four pigments frequently used in cosmetics (P-WS caramel, chlorophylin, Unicert Red K 7054-J and Unicert Red K 7008-J) were tested on the cell line NIH3T3 (mouse fibroblast cell) with and without a 5 J/cm^2 dose of UV irradiation. Fluorescence methods for cell damage study using fluorescence probes can offer results for cell viability and cytotoxicity of adherent cells and may be used for reconstruction of organ models. We detected intracellular production of ROS with the molecular probe CM-H2DCFDA, which is primarily sensitive to the increased production of hydrogen peroxide or some of its downstream products. Toxicity effects on cellular level were identified by viability tests, where the live cells reduce yellow, soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to insoluble formazan crystals. The reaction was performed on mitochondrial membranes of living cells. The type of cell death was determined by an apoptosis detection kit.

Cytotoxicity tests revealed health risks for chlorophylin and Unicert Red K 7054-J use.

This work was supported by the Ministry of Health NS9648-4/2008, Ministry of Education of the Czech Republic MSM 6198959216 and GACR 303/09 H048 and CZ.1.05/2.1.00/01.0030.

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Keywords: cosmetics pigments, phototoxicity

Poster

Animal experimentation and scientific knowledge: a Fleckian thought style?

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Animal experimentation, besides being a research method applied in scientific knowledge production, is also considered essential to science and with undeniable historical relevance in the progress of human health conditions. In this survey, a questionnaire was applied to a group of researchers with research based on non-animal models (n = 18) and another group with research based on animal models (n = 18). The data analysis used Polish microbiologist and epistemologist Ludwic Fleck's (1896-1961) epistemological assumptions.

The data suggest that there are at least two thought styles operating in consonance on the same research problem (advances in human health conditions), but with significantly different conceptions, not only about the research practices involved but also the historical conceptions related to the role of animal experimentation.

Keywords: animal experimentation, Ludwic Fleck, public understanding of science, epistemology, research methodology

Poster

Development of an *in vitro* skin sensitization test using a three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts on a collagen vitrigel membrane

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Three-dimensional (3D) human skin models have already been established to evaluate skin irritation properties of insoluble chemicals. These models did not have dendritic cells, so they could not be used for the evaluation of skin sensitization. Although some *in vitro* skin sensitization tests using human dendritic cells or the human monocytic leukemia cell line have been developed, these have difficulty in examining a wide variety of cosmetic ingredients. In this study, we established an *in vitro* skin sensitization test using a 3D human skin model containing dendritic cells.

A 3D human skin model consisting of normal human skin fibroblasts, normal human dendrite cells (NHDC) and normal human epidermal keratinocytes on a collagen vitrigel membrane (VG-KDF-Skin) was self-made. The VG-KDF-Skin was treated with test chemical for 1 h. After removal of the test chemical, the skin model was further incubated for 23 h. The supernatant was collected and IL-1α and IL-4 release were measured by ELISA. The skin model was fixed and CD86 expression on NHDC was analysed. When cytokine release or CD86 expression in the test chemical-treated model was over 150% compared to those in the control, the chemical was determined as positive.

Eight sensitizers (water-soluble: 4, insoluble: 4) and five non-sensitizers (water-soluble: 4, insoluble: 1) were examined. The chemicals tested were perfume, surfactant and solvent, etc. Among the three indicators used, IL-4 release most corresponded to the sensitization potential of the test chemicals. The accuracy, sensitivity and specificity of this method vs. LLNA were 92%, 88% and 100%, respectively.

These results suggest that the VG-KDF-Skin using an indicator of IL-4 release would be useful for evaluating skin sensitization potential of insoluble chemicals.

Keywords: skin sensitization test, 3D human skin model, collagen vitrigel membrane

Poster

In vitro prediction of teratogenic effects using pathway-specific reporters: the ReProGlo assay

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Developmental defects leading to severe malformations and dysfunctions can result from alterations in the differentiating embryo caused by exogenous embryotoxic compounds. During early embryonic development five highly conserved signalling pathways are essential for the regulation of differentiation: the Wnt-, TGF-β-, Notch-, Hedgehog- and RTK- signalling pathways. For *in vitro* assessment of teratogenic effects, alterations of these pathways could be measured using reporter constructs comprising pathway-specific DNA binding elements upstream of a luciferase reporter gene. In the context of REACH, such *in vitro* methods for the assessment of reprotoxicity are needed more than ever.

In a first approach to develop a cell-based *in vitro* test system for reprotoxic effects, D3 murine embryonic stem (MES) cells were transfected with a reporter construct specific for the Wnt signalling pathway. Stable transfectants in their undifferentiated state were treated for 24 h with potential embryotoxic chemicals. A combination of a resazurin-based cell viability assay and a luciferase activity assay was used to determine effects on the signalling pathway. For high throughput, the test, termed "ReProGlo assay", was carried out on 96-well plates. We applied the assay to several test agents with well-characterised embryotoxic potential. Besides some model compounds like retinoic acid (RA) or thalidomide, we tested valproic acid (VA) and sev-

eral of its derivatives known to possess a graduated teratogenic potential *in vivo* (Eikel et al., 2006).

In order to detect proteratogens, we tried to integrate metabolic activation in our assay using mouse primary hepatocytes. Three different approaches were used: Co-culture of MES cells and hepatocytes, use of supernatant from pre-incubation of test compound with hepatocytes and co-culture using transwell plates. We chose the well-known proteratogen cyclophosphamide as a test agent for proof-of-principle experiments.

Most (10 out of 14) of the known embryotoxicants tested showed an effect on the Wnt signaling pathway; all (3) compounds known to have no embryotoxic effects were also negative in our assay. Interestingly, VA and its derivatives showed a graduated response in the ReProGlo assay according to their *in vivo* teratogenic potential. Cyclophosphamide was only active after metabolic activation by primary hepatocytes; the effect was observed in pre-incubation- as well as in co-culture experiments.

The results demonstrate that the ReProGlo-assay can detect the influence of embryotoxic compounds and may help to elucidate the complexity of signalling pathways during embryonic development. The simultaneous detection of reporter activity and cell viability is crucial for the discrimination of specific and unspecific effects. Furthermore, the integration of a metabolic activation system is feasible. Overall, the initial results seem promising and we expect that the generation of reporter cell lines for more pathways and their inclusion in the test system will improve the predictive quality of the ReProGlo assay.

This study was supported by the EU (ReProTect, LSHB-CT-2004-503257) and the *Ministerium für Ernährung und Ländlichen Raum*, (Projekt-Nr. 0314 E). VA derivatives were a kind gift from Prof. Dr. Heinz Nau, Hannover.

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Keywords: murine embryonic stem cells, in vitro test system, teratogenicity, metabolic activation, wnt pathway

Lecture in Session II: Metabolism and toxicokinetics

Human hepatocyte cultures allow repeated application of drugs resulting in repeatable effects on hepatocellular metabolism

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Human hepatocytes are the *in vitro* system of choice to study drug-induced processes in man. Here, we present HEPAC2: A standardised and validated culture system in which functional human hepatocytes can be maintained in serum-free medium for several weeks. Anabolic and catabolic hepatocellular functions and cellular vitality are monitored daily. Albumin and urea are produced on a relatively constant level for up to 2-3 weeks, while the cells remain viable. Based on this, a standard protocol was established that allows repeated exposure of hepatocytes to test substances for studying drug metabolism.

Human hepatocytes were isolated from healthy tissue of liver resections due to cancer. Hepatocytes were cultured in HHMM (Human Hepatocyte Maintenance Medium) with daily medium changes. After adaptation to the *in vitro* conditions during the first 3 days, the cells were exposed to test pieces for 24 h, beginning on day 4 of culture. Subsequently, the culture medium was replaced by medium without the test substance and the same exposure scenario was repeated in intervals of 4 days.

As a first model substance we used acetaminophen (APAP) to assay the feasibility of this system. High doses of APAP (2815 mg/l) diminished urea production by 25% and albumin secretion by 70%. In addition, APAP led to a complete loss of glycogen and a switch from rough to smooth endoplasmic reticulum. These effects were reversible. After removal of APAP, secretion of urea and albumin returned to control levels, the glycogen stores were refilled and a high content of rough ER was found again. Human hepatocytes are able to compensate functional as well as ultrastructural changes due to APAP within 48-72 h after removal of the compound. Within one cell culture this exposure scenario could be repeated 4-5 times without loss of reproducibility.

As a second model substance we used Riemun, an immunomodulator derived from human blood. Riemun had no effect on albumin secretion, but led to an increase of urea release in a concentration dependent manner. After removal of Riemun, secretion of urea returned to control levels. Again, within one

cell culture this exposure scenario could be repeated 4-5 times without loss of reproducibility.

Next, APAP and Riemun were applied alternately. This rotary application of APAP and Riemun did not alter the effects that each substance had on its own on albumin and urea metabolism.

In conclusion, these data demonstrate the robustness and the suitability of our long-term culture system to serve as a tool for repetitive screening of drug-mediated changes on hepatocellular functions and it can become an alternative to animal testing. One human hepatocyte culture may be used multiple times for toxicity testing of xenobiotics. In case of nontoxic effects of the compound, the same cells can metabolise a second compound after a recovery period of 3-4 days.

Keywords: human hepatocytes, acetaminophen, drug testing

Poster

The *in vitro* BALB/c 3T3 cell transformation assay to screen the anti-transforming activity of chemical compounds

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The *in vitro* transformation test on BALB/c 3T3 cells resembles tumour onset and progression *in vivo* and represents a useful and flexible model to screen the carcinogenic potential of both genotoxic and non genotoxic chemicals. It shows good predictability, high sensitivity and specificity and is time-saving and less expensive compared to animal studies (Sakai et al., 2007).

Several improvements to the basic protocol were proposed aiming at increasing the cell transformation frequency, shortening the length of the assay and reducing the cytotoxic effects associated with chemical exposure (Matthews et al., 1993). The results obtained in our laboratory confirm the suitability of the improved protocol to discriminate carcinogenic compounds and support the use of the BALB/c 3T3 cell transformation assay as a possible alternative to predict carcinogenic risk to humans (Mascolo et al., 2010).

In order to identify compounds which could interfere with tumour development we set up an anti-transformation assay (Vaccari et al., 1999). Several molecules belonging to different chemical classes, such as estrogen antagonists, polyphenolic compounds and retinoids, were tested as promising tumour chemopreventives. The treatment was performed according to different exposure schedules to better define the potential protective effects and the steps of the multistage carcinogenesis process they affect. The chemicals were administered to BALB/c-3T3 cells: 1) for 48 hours, before cell plating (pre-treatment); 2) for 48 hours, concurrently with the carcinogen (simultaneous treatment); 3) throughout the duration of the experiment, after the exposure to the carcinogen, at each medium change (chronic treatment). A cytoxicity assay was performed to estimate the corresponding cloning efficiency.

The cell treatment with the carcinogen determines the induction of morphologically aberrant foci; the outcome of BALB/c 3T3 cell exposure to the potential anti-transforming chemicals is the reduction of the number of transformed foci/plate as well as the decrease of the transformation frequency.

Results give evidence that the proposed *in vitro* test could be useful for the preclinical screening of promising chemopreventive agents.

Keywords: transformation, in vitro test

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Poster

Towards a mechanism-based safety test for acellular pertussis vaccines

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Although pertussis vaccines are routinely administered to infants to protect them from whooping cough, a satisfactory safety test is currently not available. The histamine sensitization test (HIST) is currently the standard assay that is accepted by regulatory authorities to test for the absence of active pertussis toxin (PTx). Being a lethal animal test that is difficult to standardise, replacement of the HIST should be considered a priority. Moreover, the exact mechanism of the test is not defined, nor is it clear whether the assumed underlying mechanism, i.e. PTx-mediated ADP-ribosylation of G proteins, is the only effect that should be considered in PTx safety testing.

Thus, instead of developing an *in vitro* assay focusing solely on ADP-ribosylation to replace the HIST, we suggest to take a step back and start by outlining all physiological and clinically relevant effects of PT. To this end we will perform micro-array experiments in relevant human cell lines exposed to PTx. The human cell lines EHY926 (umbilical vein endothelial cells) and MUTZ-3 (dendritic cells) are relevant because a) these cell types are involved in the *in vivo* effects of PTx and b) PTx has been described to have *in vitro* effects on these specific cell lines. Determining gene expression patterns in these cell lines will allow us to identify the involved signaling pathways of PTx.

Ultimately, marker genes and/or proteins selected from these signaling pathways can be used to develop a novel safety test that covers the complete biological activity of PTx. Moreover, such a test does not require the use of experimental animals and may be more informative than current *in vivo* testing.

Keywords: pertussis toxin, vaccines, micro-array

Lecture in Session IV: Good cell culture practice

Improving *in vitro* methods by developing and using defined culture media

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In vitro methods are widely used to study activities at the cellular level. Furthermore, *in vitro* methods are powerful tools to replace or reduce animal experiments, either as stand-alone or as part of a testing strategy.

Cells are maintained under well-established conditions, which typically involve incubation at 37°C with a humidified gas mixture of 5% CO_2. An often used basal medium is Dulbecco's Modified Eagles Minimal Essential Medium (DMEM). Depending on the cell type this medium is supplemented with factors essential for proliferation, migration and differentiation of the cells. Usually, serum is used as supplement, often fetal bovine serum (FBS). The use of serum involves several problems: possible contamination, undefined (binding) factors, batch-to-batch variation, etc. The use of FBS also involves an ethical problem; blood collection may cause severe suffering to the animal (van der Valk et al., 2004). It was therefore concluded that the use of FBS should be strongly discouraged and chemically defined media should be preferred when using *in vitro* methods (Coecke et al., 2005; ESAC, 2008; van der Valk et al., 2004). Around 450 serum free media are now available (www.goodcellculture.com). Still, a defined medium has not yet been developed for every cell type. In addition, the formulation of most commercially available media is not released, and these media can thus not be regarded as strictly defined.

To discuss the development of culture media for specific cell types a workshop was organised by the Dutch-Belgian Society for In Vitro Methods (INVITROM), the European Society for Toxicology In Vitro (ESTIV) and the Danish In Vitro Toxicology Network in November 2009 in Copenhagen.

It was concluded that the development of serum-free media and cell adaptation processes is an ongoing process in several laboratories, often without knowledge of research processes, experiences or results of other laboratories regarding this topic. This information, particularly with regard to precise formulations, should be collected and made publicly available to facilitate the further development and use of defined cell and tissue culture media. Several databases already collect this type of information (see below).

The workshop participants supported the recent statement of the ECVAM Scientific Advisory Committee (ESAC) in which the use of serum and other animal components in cell and tissue culture was strongly discouraged and the development of defined media was required (ESAC, 2008). The use of defined media is also suggested when applying Good Cell Culture Practice (GCCP) (Coecke et al., 2005; Hartung et al., 2002). It was recommended to make GCCP part of Good Laboratory Practice and/or Good Manufacturing Practice to give it a legal basis.

During the workshop, the different components of a defined medium were discussed to facilitate the development of defined culture media. Furthermore, several approaches to adapt cells to serum-free media were discussed.

Details of the workshop and its recommendations will be discussed at the congress and are published in a workshop report in *Toxicology In Vitro* (van der Valk et al., 2010).

More information on already established media may be found on:

– http://www.focusonalternatives.org.uk/PDFs/FCS-free%20table%20May%2009.pdf
– http://www.goodcellculture.com/

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Keywords: ocular irritation, in vitro alternative, tissue model, EpiOcular

Poster

Toxicogenomics in an estrogenic screening context: added value to classical single endpoint assays?

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Due to its high throughput nature, toxicogenomics can provide a broad picture of a toxic response, offering the ideal functional platform for grouping chemicals according to mechanistic similarity. In this way, toxicogenomics has found its way into predictive toxicology. So far, however, the predictive potential of toxicogenomics in an endocrine disruptive screening context has not been extensively examined.

The aim of this study was to combine a toxicogenomics approach with an estrogen-sensitive MCF-7 cell system. In total, 18 compounds with endocrine disruptive potential were selected, of which 11 are listed as ICCVAM reference compounds for validation of estrogen receptor (ER) binding and transactivation assays. In this way, the grouping and classification potential of the gene expression profiles could be directly compared to the more classical estrogenicity screens, such as ER binding, ER transactivation and MCF-7 cell proliferation assays.

Results indicated that this toxicogenomics approach was clearly capable of grouping compounds into strong, weak and non estrogenic groups. The chemical groups were represented by interesting marker genes, such as PGR, ERBB2, CXCL12, AREG and EGR3, including some early-responsive (i.e. 4 h) potential biomarkers. Moreover, compound classification reflected largely the classical *in vitro* estrogenicity screens. The present study however also demonstrates the value of toxicogenomics for evaluating gradual differences in related modes of action, in addition to the more common search for opposite modes of action. This could indicate that omics data can also provide information on chemical potency, so far considered an important bottleneck in using toxicogenomics

data in a toxicity screening context. However, statistical tools to implement this 'potency' information in the classification process of omics data need to be further evaluated to fully comprehend the true value of these gradual expression profiles. Nevertheless, these results open the discussion on the added value of a broader endpoint evaluation in estrogenicity screens provided by omics-techniques compared to the classical single endpoint based assays.

Keywords: toxicogenomics, estrogenicity, compound classification

Poster

PreadyPort™, a novel ready-to-use system for *in vitro* drug transporter evaluation

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Membrane transporters can be major determinants of the pharmacokinetic, safety and efficacy profiles of drugs. Recent progress has been focused on the interaction of drugs and their metabolites with mammalian transporters present in epithelial and endothelial barriers. Many questions for drug development, including which transporters are clinically important in drug absorption and disposition or which methods are suitable for studying *in vitro* drug interactions with important transporters, are now being raised.

Solvo Biotechnology and Advancell, in close collaboration, have developed a PreadyPortTM platform for *in vitro* drug transporter evaluations. PreadyPortTM consists of three ready-to-use reagents based on differentiated MDCKII cells overexpressing MDR1, BCRP, and OATP2B1/BCRP transporters in a 24-transwell plate format. PreadyPortTM -MDR1 and PreadyPortTM -BCRP model the net transport event of barriers like the human blood brain barrier (BBB) and the intestinal barrier and can be used for regulatory submission studies. The kits allow the performance of transporter experiments without in-house cell propagation, cell line development or acquisition. The innovative shipping medium, developed and patented by Advancell, preserves the properties of the barrier throughout transportation and storage. Results of different substrates and inhibitors obtained with PreadyPortTM -MDR1 and PreadyPortTM -BCRP will be presented and discussed.

Keywords: transporters, MDR1, cell-based, ready-to-use

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Poster

ISOCYP-TOX: A new ready-to-use concept for *in vitro* evaluation of biotransformation-mediated toxicity

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The toxicity of a compound may be caused not only by the parent molecule but also by its metabolites. Metabolism can result in a bioactivation phenomenon rather than in a detoxification process, leading to metabolism-dependent toxicity that cannot be assessed by cell lines currently used for basal cytotoxicity screening as they lack biotransformation enzymes (Gómez-Lechón et al., 2001). Toxicity is responsible of 30% of the attrition rate in drug development. Thus there is an ur-

gent need to develop methods to evaluate compound toxicity at early stages.

To this aim, Advancell has developed a new technology platform based on adenoviral transduction for transient expression of biotransformation enzymes (Jover et al., 2001) that allows: a) to modulate the CYP450 enzymatic activity and reach comparable levels to those obtained in subcellular fractions or fresh hepatocytes, b) to perform studies either with each of the CYP isoforms individually or customised combinations and c) to reproduce human metabolic idiosyncrasy.

Based on this concept, Advancell has generated a new cell-based and ready-to-use reagent, ISOCYP-TOX, for *in vitro* screening of CYP450 biotransformation-mediated toxicity in 96-well plate format amenable to HTS platforms. The reagent consists of a HepG2 cell line transiently transduced with individual CYPs, making these cells metabolically competent. ISOCYP-TOX represents a cost-effective method for the early detection and screening of bioactivation-dependent acute toxicity of compounds through rapid analysis for curve fitting and IC_{50} generation, as well as identification of CYP isoforms involved in production of toxic metabolites. We have studied CYP450 biotransformation-mediated toxicity of a total of eleven reference compounds in HepG2 control cells and HepG2 cells, transiently transduced with single CYP3A4, CYP1A2, CYP2E1 and CYP2A6. The results showed that ISOCYP-TOX is a good cellular model to evaluate biotransformation-mediated toxicity.

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Keywords: CYP450, isoforms, screening, ready-to-use, cell-based

Poster

Connexin32 hemichannels facilitate apoptotic-to-necrotic transition during Fas-mediated hepatocyte cell death

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Although it is widely accepted that the establishment of gap junctional intercellular communication (GJIC) is a prerequisite for maintaining liver homeostasis, its role in the occurrence of apoptotic hepatocyte cell death still remains elusive. Most hepatic gap junctions are formed by two hemichannels of neighbouring hepatocytes, which are composed of connexin32 (Cx32). The present study was set up to investigate the fate of Cx32 and its channels in Fas-mediated hepatocellular apoptosis.

Primary hepatocytes were exposed to Fas ligand and Cx32 expression was studied by means of immunoblotting and qRT-PCR analysis. GJIC was monitored by fluorescence recovery after photobleaching analysis. Cx32 hemichannels were approached through Cx32 siRNA-mediated gene silencing, cell surface biotinylation, application of a Cx32 mimetic peptide and measurement of ATP release.

GJIC rapidly declined upon progression of the cell death response, which was associated with a decay of the gap junctional Cx32 protein pool. Simultaneously, levels of newly synthesised Cx32 protein increased and gathered in a hemichannel configuration. This particularly became evident towards the final stages of the cell death process and was not reflected at the transcriptional level. Both the silencing of Cx32 expression and the inhibition of Cx32 hemichannel activity prior to cell death induction resulted in a delayed termination of the cell death response.

In conclusion, (i) Cx32 hemichannels facilitate the apoptotic-to-necrotic transition, which typically occurs in the final stage of Fas-mediated hepatocyte cell death and (ii) primary hepatocyte cultures are suitable *in vitro* tools to elucidate the mechanisms that underlie hepatocyte cell death.

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Keywords: connexin, gap junction, hemichannel, cell death, primary hepatocyte culture

Poster

Multi-walled carbon nanotubes – toxicological effects on human cells

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Nanoparticles have recently received much interest because of increasing epidemiological and experimental evidence for their effects on human health. Human exposition to nanoparticles occurs either during their manufacture at the workplace or during application of nanoparticle-containing products. The aim of the present study was the establishment of an *in vitro* test system to reveal the potential risk on human health of multi-walled carbon nanotubes (MWNTs) at the workplace. The essential advantage of *in vitro* investigations is that they are non-invasive, the employees are not bothered and the work routine does not need to be interrupted.

Transwell® inserts with co-cultures consisting of differentiated macrophages (PMA pretreated U-937 cells) and human lung epithelial cells (A-549 cells) were exposed to aerosols containing MWNTs using a CULTEX® system. To measure the amount and size of the particles in the aerosols a Scanning Mobility Particle Sizer (SMPS) was connected parallel to the feed line of the CULTEX® system. In a first step co-cultures were exposed to well-defined aerosols produced by an aerosol- or particle generator. Two different types of MWNTs (type 1 and type 2) were investigated in these experiments. In a second step co-cultures were exposed to workplace atmospheres. Atmospheres emerging during different production steps with open and closed nanoparticle handling were investigated. To reveal the toxic potential of MWNTs containing aerosols the following endpoints were examined: cytotoxicity (WST1 assay), oxidative stress (DHR-assay), secretion of pro-inflammatory cytokines (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL 10, IL-12p70, TNF-α und TNF-β) and genotoxicity (COMET-Assay).

MWNTs of type 1 were cytotoxic and induced oxidative stress, whereas these effects could not be observed with MWNTs of type 2, which can be explained by the about two-fold higher amount of carbon in the MWNTs type 1 compared to MWNTs type 2. Workplace atmospheres did not induce cytotoxic effects, independent of the production step investigated.

Increased oxidative stress was observed in one of the experiments during the open handling production step. In the other experiments (one with open and two with closed handling), no increase could be observed. SMPS measurement showed that the induced oxidative burst was related to a higher particle exposure. No increase of cytokine release could be measured, either with generator produced aerosols or with workplace atmospheres. Investigation of DNA damage revealed a very slight increase after exposition to MWNTs type 1, whereas MWNTs type 2 showed no genotoxic effects.

Our results indicate that high MWNT particle exposition induces reduction of cell viability as well as induction of oxidative stress and DNA damage. At the working place the MWNT exposition was very low owing to safety precautions and was about 1000 fold lower than in the experiments with the well defined aerosols. Thus, effects of the working place atmospheres on cells could be observed only in one case. Consequently, high demands on employee protection as well as further development of safety standards are necessary to maintain low exposure at the working place.

Keywords: multi-walled carbon nanotubes, in vitro inhalation exposure, A-549 cells, U-937 cells, cytotoxicity, immunotoxic effects, genotoxic effects

Lecture in Session III: Skin sensitisation and eye irritation

Models of localised *Candida* infections based on *in vitro* reconstituted human epithelia

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Basic research on the biology and immunology of microbial infection requires appropriate model systems. Due to the complexity of the processes, most studies involve animal testing. Besides ethical concerns, these models are not always representative of infections in humans, which holds true particularly for the human pathogenic fungus *Candida albicans*. *In*

vitro models that closely parallel the *in vivo* situation and allow studies of relevant physiologic functions are thus highly desirable.

Possible alternatives, especially for localised infections, are provided by models using *in vitro* reconstituted human epithelium or epidermis. In recent years, these model systems have been successfully established to evaluate the effectiveness of topical anti-infectives, to characterise the role of fungal virulence factors and to study immune responses during localised *C. albicans* infections (Schaller and Weindl, 2009; Schaller et al., 2006). Early studies focused on the consequence of gene disruption in *C. albicans* on pathogenicity and the epithelial cytokine pattern. Most recently, these models have been supplemented with immune cells such as lymphocytes and polymorphonuclear leukocytes to study their role during the course of infection and to characterise the interaction between the skin barrier and accessory immune cells. Using the *in vitro* model, it has been demonstrated that an immunological crosstalk between *C. albicans*-infected oral epithelium and polymorphonuclear leukocytes induced an immune cell-mediated upregulation of epithelial Toll-like receptor 4, a member of an important receptor family which plays a critical role in innate immune recognition of pathogens (Weindl et al., 2007). The increased receptor expression was directly responsible for protecting the mucosal surface from fungal invasion and cell injury. These studies will help us to gain insight into the complex mechanisms by which appropriate innate and acquired immune responses are initiated and to identify factors that contribute to an increased susceptibility to *Candida* infection in patients.

Although any conclusion from these models for an *in vivo* infection has to be made with caution, the available systems reflect more and more closely the physiological situation found *in vivo*, thereby providing a valid matrix to model the events under controlled experimental conditions. In addition, such model systems can also be used to study infections with other fungi or bacteria.

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Keywords: host pathogen interaction, Candida albicans, oral epithelium, innate immunity

Poster

Evaluation of anti-inflammatory and atrophogenic effects of glucocorticoids on reconstructed human skin models

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Topical glucocorticoids (GCs) are extensively used in the therapy of inflammatory skin diseases. One of the most important side effects of long-term GC treatment is skin atrophy. Currently, preclinical studies involve animal testing, which is not always representative of the situation in humans.

The aim of this project was to establish a 3D *in vitro* model for the evaluation of the anti-inflammatory and atrophic potential of topically applied GCs. Initial studies in fibroblasts and keratinocytes confirmed the known effects of GCs as decreased cytokine production and collagen mRNA expression. In reconstructed human skin (RHS) models, topical application of GCs for 7 days strongly reduced IL-6 secretion. GC-induced skin atrophy, known to appear only after prolonged treatment, was not detected by analysis of epidermal thickness and collagen mRNA expression.

Noteworthy, reproducible inflammatory conditions in the epidermis were established for the first time in RHS models. Topical treatment with TNF increased IL-6 release and strongly reduced epidermal thickness accompanied by severe parakeratosis. Subsequent GC treatment reduced IL-6 levels and completely resolved parakeratosis, leading to increased epidermal thickness.

Inflammatory conditions mimic more closely the *in vivo* situation in which GCs are used and therefore appear to be more suitable for future investigations for the establishment of human-based *in vitro* models to estimate wanted and unwanted GC effects.

Keywords: glucocorticoids, inflammation, skin atrophy, reconstructed human skin model

Poster

Proposal of a chemical dataset for the development and evaluation of *in vitro* methods as alternatives to *in vivo* eye irritation testing

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The Colipa (European Cosmetics Association) Task Force Eye Irritation (TFEI) is actively involved in the development of *in vitro* methods to replace the Draize rabbit eye test, now banned for evaluating cosmetic ingredients. One of its key projects is focused on method development/optimisation using Reconstructed Human Tissue (RHT) assays for both MatTek EpiOcular® and SkinEthic® HCE human corneal models. The completion of pre-validation datasets was performed after evaluation of numerous chemicals, enabling these two methods to enter a prospective ECVAM validation study now in progress to reliably discriminate non-classified (non-irritant) chemicals from all classes of eye irritants, as defined according to the United Nations Globally Harmonized System (GHS). In parallel, additional efforts are still required and so a key research project of the COLIPA TFEI programme is development of new *in vitro* test systems/endpoints that are based on an understanding of mechanisms of action of eye injury/recovery to predict human ocular responses to chemical exposure. In order to develop *in vitro* assays that accurately identify eye irritation potential, selection of appropriate test chemicals is of critical importance. Here, we provide a set of around 50 chemicals which are: 1) single chemical entities; 2) supported by relevant and reliable high quality *in vivo* data; 3) cover the whole range of irritant effects/potencies; 4) include relevant classes of chemicals; 5) include relevant physical states and 6) are readily available. All the chemicals listed have been tested in at least one RHT assay and were tested in the Bovine Corneal Opacity and Permeability (BCOP) test method and referenced in the ICCVAM BCOP Mild/Moderate Ocular Irritants Background Review Documents.

Use in the method development phase of selected chemicals from such a reference list would facilitate early assessment of the performance of a method with respect to existing tests, and of its possible contribution to a tiered testing strategy, for example according to that published from the ECVAM 2005 expert meeting. This poster provides a detailed analysis of a chemical dataset proposed by Colipa for use in development and evaluation of *in vitro* methods for eye irritation testing.

Keywords: eye irritation, in vitro methods, human corneal models, chemical dataset, Bovine Corneal Opacity and Permeability test

Poster

IMOLA-IVD: a cytosensor microphysiometer of the 2nd generation

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Cell based assays for live cell monitoring are useful tools in the field of toxicology. For example, live cell monitoring was shown in 1989 with the cytosensor microphysiometer (Parce et al., 1989). This device was designed to analyse the acidification rate of living cells with a silicon based pH sensor. Recently, the cytosensor microphysiometer was evaluated in a Bottom-Up/Top-Down approach as an alternative testing strategy for eye irritation (Scott et al., 2010) and a catch-up validation of similar instruments was discussed (Hartung et al., 2010).

In the group of Prof. B. Wolf at the Technische Universität München (www.lme.ei.tum.de) a cell based assay was developed using a BioChip platform manufactured in thin film technology. Parameters were – beside pH – extended to include dissolved oxygen, impedance and temperature. Thus it is possible to address extracellular acidification, cellular respiration and changes in cellular

morphology. A closed fluid system running in a stop-and-go mode allows supply of the cells with fresh cell culture medium or the addition or removal of drugs or toxins. State of the art electronic circuitry and software for control and analysis of the data yielded the IMOLA-IVD (Intelligent mobile lab for in-vitro diagnostics) system, which was spun-off to cellasys GmbH (www.cellasys.com) and extended to a six channel version. This system allows multi-parametric, long term, marker-free, online analysis of living cells and their response to compound addition or removal.

The IMOLA-IVD system (Wiest et al., 2006) has applications in various fields including toxicokinetics. As an alternative method for animal experiments there is a high potential for full replacement. Proof of principle experiments using mouse fibroblasts have shown a high sensitivity of the different microsensors toward known toxic profiles. To fully evaluate cell based assays for live cell monitoring as alternative methods, non-endpoint based standard operating procedures have to be developed and validated. Ongoing work includes the development and validation of alternative methods and development of technology for investigation of metabolic chains and non-water-soluble compounds.

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Keywords: living cell, acidification, respiration, morphology

Poster

Fulfilling data requirements for the U.S. EPA Endocrine Disruptor Screening Program using existing and non-traditional data

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New methods of generating and evaluating toxicity data for chemicals are needed to cope with the increasing demands of new testing programs. One such approach involves the use of existing data combined with non-testing strategies to create a custom, integrated testing strategy (ITS) based on the specific properties of a chemical. ITS can be applied to new or existing programs to increase efficiency and save animals and resources. We recently proposed an ITS approach to the U.S. EPA Endocrine Disruptor Screening Program (EDSP), which the EPA launched in October 2009 by issuing orders for the first 67 chemicals to be tested in the EDSP Tier 1 battery of assays. As a condition of approving EPA's plan to collect data for the program, the Obama Administration instructed the EPA to promote and encourage the use of Other Scientifically Relevant Information (OSRI) in lieu of performing some or all of the Tier 1 assays. The Phase I chemicals consist of 58 pesticide active ingredients and nine High Production Volume (HPV) pesticide inert chemicals, all of which have been subjected to dozens of toxicity tests, often including reproductive and chronic/lifecycle studies in rodents, fish and birds. Many of these chemicals also have information available from mechanistic *in vitro* assays that supplement *in vivo* information already available. Building on our previous work using ITS principles, we show that OSRI-based arguments are sufficient to satisfy Tier 1 data requirements for a number of Phase 1 chemicals. This approach can be used to address the endocrine disrupting potential of chemicals in any regulatory context. In fact, development of a Guidance Document for the use of Test Guidelines for assessing endocrine disruptors within the context of existing information is a current high priority project at the Organization for Economic Cooperation and Development.

Keywords: alternative approaches, endocrine, integrated strategies

Lecture in Session VI: Acute and long term toxicity B

Transcriptomic, proteomic and metabolomic alterations in RPTEC/TERT1 cells in response to Cyclosporine A

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Cyclosporine A (CsA) remains one of the most widely used immunosuppressive drugs for use in organ transplantation; however, it is also associated with the development of chronic renal disease. While there is thought to be a vascular and glomerular component to CsA nephropathy, evidence shows that proximal tubular cells are also directly affected by this compound.

In the present study we investigated the effect of long term repeat exposure of CsA on the human renal proximal tubule cell line RPTEC/TERT1. Cells were cultured to confluence on aluminium oxide, microporous supports and allowed to differentiate for two weeks prior to exposure. Cells were exposed to a fresh daily bolus of CsA at 5 or 15 μM for 1, 3 and 14 days. Transepithelial electrical resistance (TEER) and lactate production were monitored on a daily basis. At each time point cells were harvested for transcriptomics, mass spectrometry based peptide profiling and NMR based metabolomics analysis.

CsA exposure caused an increase in TEER of RPTEC/TERT1 cells over the duration of the experiment, at both 5 and 15 μM. CsA also caused a sustained increase in glycolysis, as previously reported at 15 μM. Transcriptomic analysis revealed the prominence of genes involved in cell cycle and p53 pathways. The effect of CsA on the RPTEC/TERT1 proteome and metabolome was also demonstrated. We show here for the first time a full omics analysis of the effects of CsA in cultured renal epithelial cells.

Keywords: Cyclosporine A, transcriptomics, proteomics, metabolomics

Lecture in Session III: Skin sensitisation and eye irritation

Human liver cell cultivation in a miniaturised 3D bioreactor system for *in vitro* studies on hepatic drug toxicity

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Hepatic drug toxicity is one of the leading causes of drug withdrawal from the market. While conventional 2D hepatocyte culture systems or suspension cultures are suitable for detection of acute toxicity, the assessment of subchronic to chronic hepatic toxicity requires models that allow prolonged maintenance of hepatocyte functionality.

Based on a multi-compartment bioreactor technology for clinical bioartificial liver support (Schmelzer et al., 2009) we developed a miniaturised bioreactor prototype that allows 3D perfusion culture of liver cells in a capillary scaffold providing decentralised mass exchange and integral oxygenation. The device is based on the use of interwoven hollow fibre capillary

membranes for independent medium and gas perfusion of the cells located in the extra-capillary space (cell compartment). The maintenance of physiological functions of hepatocytes over several weeks was shown in human hepatocytes co-cultured with non-parenchymal liver cells in the bioreactor.

To reduce the required cell number and medium volume for screening studies on drug metabolism and toxicity, miniaturised bioreactor prototypes with a reduced cell compartment volume of 2.0 ml or 0.5 ml were constructed based on the existent multi-compartment culture technology. The use of transparent material in the bioreactor housing facilitates microscopic access to the cells. In addition, optic sensors can be integrated into the 0.5 ml-prototype for monitoring of metabolic parameters, e.g. oxygen concentrations, in the cell compartment. The bioreactor is integrated into a perfusion device that allows electronic control of major system functions (temperature, gas supply, medium flow and pressures).

Primary human liver cells cultured in the miniaturised bioreactor showed stable metabolic activities in terms of urea and albumin production, glucose metabolism and cytochrome P450 dependent enzyme activity. The time profile of metabolic parameters was comparable in the 2.0 ml- and the 0.5 ml-prototype in relation to the cell number and medium volume. The presence of markers for hepatocytes, biliary cells and non-parenchymal cells as well as hepatic transporter proteins in the cell aggregates was demonstrated by immunohistochemical staining.

The results indicate that the miniaturised multi-compartment bioreactor provides an innovative tool for *in vitro* investigations on human hepatic drug metabolism and toxicity. The option or simultaneous operation of up to four independently perfused bioreactors in the same perfusion device facilitates the performance of parallel experiments with cells from the same donor, e.g. for dose-finding studies. Moreover, the co-cultivation of hepatocytes with other cell types from the liver (e.g. endothelial cells, biliary cells, Kupffer cells) in the bioreactor could be valuable for studies of toxic effects of drugs in the liver.

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Keywords: human liver cells, 3D culture, bioreactor, drug toxicity, metabolism, cytochrome P450

Poster

A test system with high sensitivity for developmental neurotoxicants based on differentiation of murine embryonic stem cells to neurons

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The understanding of mechanisms and the screening of a larger panel of environmentally-present compounds has been hampered by the lack of suitable and cost effective experimental systems. *In vitro* neural differentiation of embryonic stem cells (ESC) has been shown to approximately recapitulate *in vivo* differentiation. It may thus form the basis for a suitable *in vitro* system to assess DNT.

Here, murine ESCs were differentiated to neurons under feeder-free conditions. The mature postmitotic neuronal phenotype was corroborated on day 20 by staining for general neuronal markers and several markers for neuronal subtypes or synaptic proteins. Differentiation kinetics were assessed by q-PCR and chip analysis. DNT testing was performed by exposure to compounds during differentiation. DNT was evaluated by e.g. Western blot or q-PCR, and the acute neurotoxicity was assessed by the release of lactate dehydrogenase (LDH) or resazurin reduction. Test compounds included known neurodevelopmental toxicants (CH_3HgCl (methylmercury)). The concentrations for the DNT assay were chosen so that they did not affect the overall cell viability (as assessed by e.g. LDH assays). This upper toxicity threshold was determined in acute neurotoxicity experiments.

When ESC-derived, terminally differentiated neurons were exposed for three days to known neurotoxicants, we detected EC_{50} values that were similar to those found in murine primary neuronal cultures, but about one log lower than in non-neuronal cells. All DNT tests were performed with concentrations lower than those triggering acute neurotoxicity. Under these conditions, DNT compounds showed a pronounced adverse effect on differentiation. The mRNA levels of neural markers were significantly downregulated compared to control cultures not treated during differentiation. Notably, DNT, e.g. of CH_3HgCl, was detected at more than 10,000 times lower concentrations than the acute neurotoxicity. This reflects the *in vivo* situation where

mercury is neurotoxic only at high concentrations while it is toxic for the developing fetal brain at very low concentrations. Significant effects of CH_3HgCl were robustly and reproducibly detected at concentrations as low as 50 pM.

To our knowledge this test system has an unprecedented sensitivity and will be used both for mechanistic studies on the background of DNT of known compounds and for the pre-screening of unknown compounds.

Keywords: embryonic stem cells, mercury, neuronal differentiation, developmental neurotoxicity

Poster

Protective effect of curcumin and its soluble form on benzoquinone treated human erythrocytes *in vitro*

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Benzene, one of the known environmental pollutants, is mainly released to the environment from fossil fuels, oil and its derivatives, contaminating air, water and soil. It is readily absorbed by all routes of exposure, rapidly distributed throughout the body and metabolised to a variety of intermediate carcinogenic compounds. This can lead to occurrence of leukaemia especially Acute Myelogenous Leukaemia (AML). It is suggested that benzene's adverse effects are mainly due to oxidative stress induction especially by its metabolites. Extensive research during the last 50 years has proved curcumin (the yellow pigment in curry powder, a widely used food spice) as a potent antioxidant chemopreventive agent with anti-tumoral activities. In this presented work we have examined the ability of curcumin to counteract oxidative stress induced by one of benzene's metabolites, benzoquinone, on *in vitro* treated human erythrocytes.

Erythrocytes were divided into four portions. The first portion was pre-incubated for 4 h at 37ºC with different concentrations (0, 10, 20, 50, 250 μM) of benzoquinone. The second portion was pre-incubated with curcumin for 30 min followed by incubation with benzoquinone for 4 h. The third portion was incubated with the same concentration of curcumin solubilised with dendrosomes for 30 min and was subsequently treated with benzoquinone. The last portion was used as the control with no treatment. Malondialdehyde (MDA) concentrations, CAT and SOD activities, as well as haemolysis percentage were measured in all samples of erythrocytes.

Benzoquinone induced oxidative stress in erythrocytes leading to elevated activities of CAT and SOD. These were significantly decreased by treatment with 50 μm dendro-curcumin. The presence of dendro-curcumin in the benzoquinone treated sample also reduced haemolysis by up to 15%. MDA levels declined but were not completely eliminated using either curcumin or dendro-curcumin.

Altogether, dendro-curcumin seems to have considerable antioxidant effects and can decrease benzoquinone induced oxidative stress in erythrocytes.

Keywords: curcumin, nanocurcumin, benzoquinone, oxidative stress, erythrocyte

Poster

Corporate social responsibility and laboratory animals

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Protection of laboratory animals is not an exclusive government task. Businesses and organisations too should take responsibility in this matter. Corporate social responsibility (CSR) offers a sound framework within which the particular responsibility towards laboratory animals can be modelled. Corporate ethics with respect to people, planet and profit are at stake. Animal testing is not covered however, whereas surely it can be considered a social issue.

For this reason, the Dutch Society for the Replacement of Animal Testing has initiated a round table discussion with key

players from corporations, academia and not-for-profit organisations to ensure that a policy on laboratory animals is encompassed in CSR.

An initial result of the talks is the Transparency Code, whereby organisations undertake to report on their animal testing policy according to set guidelines. Organisations adhering to the code agree to report on numbers of animals and species used, research goals, as well as their policy regarding alternatives to animal testing. The next step would be to report on their policy and to evaluate its implementation.

The round table is presently elaborating on a dialogue between stakeholders, another essential element in advancing CSR. Supply chain responsibility will also be addressed. An organisation that outsources its animal tests or buys products having entailed animal testing, must likewise assume responsibility.

The new approach in the debate on animal testing opens up innovative ways to achieve a reduction of animal testing. Cooperation between companies, academia and animal welfare organisations yields new insights, to the benefit of laboratory animals.

Keywords: animal use policy, stakeholder dialogue, corporate social responsibility, transparency

Late Abstracts

Poster

The ReProTect project: development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies

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Integrated testing strategies using in vitro tests for the detection of reproductive toxicity are urgently required since reproductive toxicity safety evaluations demand the highest number of animals per tested substance, e.g. a two generation study, which is requested in several legislative frameworks, requires up to 2,600 animals per substance. An integrated project called ReProTect (www.reprotect.eu) started in 2004 within the 6th EU Framework Programme, assembling 33 European partners from academia, SMEs, governmental institutions and industry. It aimed to develop and/or optimise *in vitro* assays that provide mechanistic information relevant for assessing the hazard of compounds to the mammalian reproductive cycle.

Several *in vitro* systems were developed that are able to assess a chemical's hazard in the areas of male/female fertility, implantation and prenatal development. A statistical analysis to evaluate the intra/inter-laboratory variability of these assays has been conducted. A huge amount of data has been produced comprising information on the experimental results of all assays along with information on the evaluation of test results (e.g. EC_{50} values and other relevant parameters). In addition, information on the toxicological *in vivo* profiles of ~150 test chemicals has been recorded. Successfully developed tests have been combined into a testing battery that was challenged with ten blinded compounds.

This scientific approach has for the first time demonstrated that complementary *in vitro* tests performed in parallel can deliver predictive toxicological profiles of substances for very complex human health endpoints. The ReProTect battery identified toxicologically relevant hazardous properties of ten test compounds with relatively high accuracy in the majority of cases. The poster will present major achievements of the project.

Keywords: ReProTect, reproductive toxicity, in vitro tests, FP6 integrated project

Poster

Meiotic recombination is increased in *in vitro* human fetal oocytes by Bisphenol A

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Bisphenol A (BPA) is a chemical substance with a wide distribution around the world. It is the major component of polycarbonate plastics, an additive used to line food and beverage cans and containers and is contained in many dentistry products. BPA works as a hormonal disruptor with weak estrogenic effect. It can cross the placenta and recent studies have reported high levels in placental tissues and amniotic liquid. In some cases, the concentration in the fetuses was higher than in the maternal serum. Studies in rat and mouse fetuses have reported a wide and differing spectrum of effects like morphological alterations, premature puberty or aneuploidy. In that sense we could say that the fetus is very sensitive to BPA. Like in other species, the reproductive cycle starts at the fetal stage with meiosis. During this stage pairing, synapsis and recombination of chromosomes takes place, and only concludes with fertilisation.

The aim of this study was to evaluate the effect of BPA in human fetal oocytes in culture. To accomplish the objective, samples of 4 euploid fetuses were used. The ovarian fetal tissue was cultivated following the technique described by Brieño et al., (2010). The tissue was cultivated with D-MEM medium (supplemented with SCF 90 ng/ml and ITC) and divided into three groups: control group, estradiol group (1, 5, 10, 20 and 30 nM) and BPA group (1, 5, 10, 20 and 30 μM). The cultures were analysed at T7, T14 and T21 (after 7, 14 and 21 days in culture). Pairing and synapsis were followed by immunofluorescence with antibodies for SYCP1 and REC8 and recombination with MLH1. Pairing and synapsis did not show any difference between fresh tissue and the different groups. For recombination, first results indicated that the number of recombination points of MLH1 was in direct relation with the concentration of BPA.

The increase of the mean foci number indicates that the compound increases the recombination of human fetal oocytes in *in vitro* culture.

Keywords: oocytes, human, meiosis, bisphenol A, recombination

Poster

ZEBET's activities to promote alternative methods for the ID_{50} potency test in botulinum neurotoxin product testing

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Botulinum neurotoxin (BoNT) products are used for treatment of many medical disorders such as cervical dystonia or blepharospasm. Prior to release, every production lot of BoNT requires characterisation in a mouse LD_{50} potency test according to the monograph "Botulinum Toxin Type A for Injection" issued in the European Pharmacopoeia (EP) 6.0. Due to the paralysing toxic syndrome of BoNT, the treatment of animals is associated with severe suffering.

As part of the German Federal Institute for Risk Assessment (BfR) in Berlin, the Center for Alternative Methods to Animal Experiments (ZEBET) has recently increased its efforts to promote the implementation of the 3Rs in BoNT potency testing. In April 2009, ZEBET organised an international expert meeting on the issue of alternative methods to replace the LD_{50} Potency Test for BoNT Testing. As a main result of this meeting a "BoNT Expert Working Group- BoNT EWG" was established dedicated to convene several times per year at BfR to further discuss all aspects of BoNT potency testing. It aims to clarify the regulatory requirements regarding BoNT product testing on an international level, to select the most promising alternative test methods and to pinpoint further steps necessary to succeed with their validation and regulatory acceptance. ZEBET in collaboration with the BfArM (Federal Institute for Drugs and Medical Devices) has taken the chair of the BoNT EWG. Members are representatives of national and international competent authorities, validation organisations and manufacturers of BoNT products as well as academia.

Keywords: botulinum neurotoxin, potency testing, LD_{50} test

Poster

Chromium exposure induced cell cycle arrest in human osteoblasts

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Chromium (Cr) is a naturally occurring heavy metal usually found in the environment in the trivalent Cr (III) or hexavalent Cr (VI) valence state. Chromium is used in numerous industrial processes and as a consequence it is a contaminant in many environmental systems. Besides, modern prostheses are made of metal articulating on metal (cobalt and chromium alloys). As metals in contact with biological systems undergo corrosion, these implants are associated with high levels of metals in the serum and blood of patients.

Using *in vitro* systems we evaluated the effects of the most toxic valence of Cr, i.e. Cr(VI), on human osteoblast cell lines (MG-63) exposed for different periods to this metal. The doses selected were in the range of the concentrations previously detected in patients with metal implants (0.1 and 0.5 μM) and 10 times greater [1 and 5 μM of Cr(VI)]. Putative induced genotoxicity (clastogenicity and ploidy level) was analysed using flow cytometry as described by e.g. Calejo et al. (2010), Oliveira et al. (2010). Also, cytostatic effects or changes in cell cycle dynamics were evaluated by flow cytometry. At physiological doses, Cr(VI) did not affect cell proliferation or cell cycle progression. However, the concentration of 5 μM reduced cell division/confluence and viability to about 70% of the control. Cell cycle progression was severely affected with significant decreases of cells in G0/G1 phase of the cell cycle and an increase of cells in S-phase and in G2. The cell proliferation activity index was assessed by the SPF (S-phase fraction) and PI (proliferation index) that may evaluate putative changes in the period necessary to complete a cell cycle (Sun et al., 2007), but may also demonstrate the occurrence of cytostatic effects.

These *in vitro* studies show that although chromium does not induce toxicity at physiological concentrations, it is able to induce severe toxicity at slightly higher levels.

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Keywords: flow cytometry, human osteoblasts, metal toxicity, cell cycle

Poster

Identification of antiangiogenic effects of antitumour agents based on screening for HIF-1 pathway inhibitors: characterisation in comparison with *in vivo* tumour angiogenesis models on examples from studies on imidazoacridinones

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Hypoxia-inducible factor-1α (HIF-1α) is a pro-survival transcription factor that particularly promotes tumour angiogenesis by increasing the production of angiogenic factors, such as vascular endothelial growth factor (VEGF). Here we present how a simple, cell-based approach for the identification of HIF-1 pathway inhibitors was useful in the prediction of antiangiogenic effects of imidazoacridinones, a group of DNA-damaging antitumour alkylators. The most promising imidazoacridinone,

C-1311, is undergoing phase II clinical trails for breast cancer (Kuśnierczyk et al., 1994; Skladanowski et al., 1996).

Hif-1α DNA-binding activity was determined using an ELISA with a double-stranded DNA probe encompassing the canonical HIF-1α binding site, hypoxia-responsive element (HRE sequence). *In vitro* studies were conducted on human colon adenocarcinoma HCT116 and mouse melanoma B16/F10 cells. Real-time PCR analysis was applied to determine HIF-1α and VEGF mRNA expression. Secretion of VEGF into conditioned media was examined using ELISA. The mouse Matrigel assay and the alginate beads model with angiogenesis stimulated by encapsulated B16/F10 melanoma cells were used to test the effect of imidazoacridinones on angiogenesis *in vivo* (Hoffman et al., 1997; Passaniti et al., 1992).

We found that in a cell-free system C-1311 interfered with HIF-1α binding to an oligonucleotide containing the HRE sequence present in the VEGF promoter. In hypoxic human colon adenocarcinoma HCT116 and mouse melanoma B16/F10 cells, C-1311 significantly decreased HIF-1α mRNA and protein expression. Consistent with HIF-1α inhibition/depletion, C-1311 (1-10 μM) markedly reduced the level of hypoxia-induced VEGF mRNA expression and protein secretion into the extracellular medium. Based on these promising *in vitro* results, C-1311 was subjected to further analysis in animals. At a single dose (40 mg/kg), C-1311 inhibited angiogenesis *in vivo* by 70%, monitored as bFGF-stimulated vascularisation of Matrigel plugs injected into mice. Of note, the observed decrease in haemoglobin content within Matrigel plugs was similar to that found for 170 mg/kg cyclophosphamide used as a positive control for antiangiogenic effects. Antiangiogenic activity of C-1311 was further confirmed in experiments where angiogenesis was stimulated by proangiogenic B16/F10 melanoma cells encapsulated in alginate beads and implanted into mice. Importantly, imidazoacridinone C-1310, the second top putative HIF-1α DNA binding inhibitor, as revealed in experiments with the HRE probe, also reduced neovascularisation of alginate implants in mice; however this effect was less profound than that of the parental C-1311.

To conclude, using both cell-free and cell-based approaches, we identified C-1311 as a novel compound, that targets the hypoxia pathway by inhibiting HIF-1α. Importantly, C-1311-mediated inhibition of HIF-1α and VEGF *in vitro* correlated well with the suppression of angiogenesis *in vivo*, which emphasises the fact that simple *in vitro* screening for HIF-1-targeting drugs with concurrent evaluation of their inhibitory potential may be useful in predicting antiangiogenic properties of antitumour compounds.

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Keywords: angiogenesis, imidazoacridinone C-1311, hypoxia, HIF-1, VEGF

Poster

The AltTox website – devoted to non-animal methods for toxicity testing

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AltTox.org is a website dedicated to advancing non-animal methods of toxicity testing, both to better protect the health of humans, animals and the environment, and to reduce the numbers and suffering of animals used in current toxicology assessments. The website is designed to encourage the exchange of technical and policy information on *in vitro* and *in silico* methods for all types of toxicity tests. The target audience includes stakeholders in industry, government, academia and nongovernmental organisations.

AltTox consists of three interconnected components:

- Online forum/message board – *The AltTox Forum* (moderators Martin Stephens, Grace Patlewicz, Horst Spielmann, Hajime Kojima)
- An informational section on toxicity testing – *Toxicity Testing Resource Center (TTRC)*
- Invited commentaries – *The Way Forward*

The AltTox Forum is a message board for the AltTox community to use for posting news, information and perspectives as

well as encouraging feedback and commentary. This online community is intended to foster progress internationally in the development, validation and acceptance of *in vitro* methods, with the goal of decreasing our reliance on animal-based safety testing. The Forum is moderated by a group of internationally-recognised subject matter experts.

The website's informational section – the TTRC – provides a comprehensive source of information on non-animal methods of toxicity testing not easily found anywhere else on the Web. It features concisely summarised information for anyone interested in toxicity testing and alternative (non-animal) test methods.

The Way Forward invited commentaries, which are posted in the TTRC, are opinion pieces written by experts in each relevant subfield. These essays are meant to help chart the course for future developments by advancing opportunities to overcome challenges and barriers to progress. Stakeholders are invited to comment on these essays in the AltTox Forum.

AltTox users are encouraged to contribute to the website and interact with other users in several ways, including:

- Participating in the online forum
- Providing invited expert commentaries
- Suggesting or submitting content, events, monthly features, data and graphics
- Providing feedback through the "Website Feedback" survey

Future opportunities for participation may include online workshops and virtual meetings. AltTox is supported through a collaboration of the Procter & Gamble Company (P&G) and The Humane Society of the United States (HSUS). To encourage objectivity, the website content is overseen by an editorial board of distinguished subject matter experts.

AltTox is sponsored by the Alternatives Research & Development Foundation (ARDF), the American Chemical Council (ACC), Procter & Gamble (P&G) and the Humane Society of the United States (HSUS).

Keywords: AltTox, alternative methods, website, in vitro toxicology, 3Rs

Lecture in Session VIII: Reproductive toxicology and stem cells

Predicting developmental toxicity with human embryonic stem cells and metabolomics

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Birth defects are a prominent problem, occurring in 6% of births, nationally. Teratogens, or substances that cause one or more fetal abnormalities in development, cause 5-10% of these defects. While animal models are currently used to predict developmental toxicity of drugs, they are costly, time-consuming and, most importantly, do not adequately correlate to human response. The overall ability of animal models to predict developmental toxicity in humans can be as low as 62%.

Stemina Biomarker Discovery, Inc. (Stemina) addresses these issues by using an *in vitro* method that utilises both human embryonic stem (hES) cells and metabolomics to discover metabolite biomarkers of developmental toxicity. As a proof-of-principle experiment, hES cells in a 6-well format were exposed to five non-teratogens and seven teratogens. The spent media from the "dosed" cells was collected, filtered to exclude high molecular weight moieties, then analysed using liquid chromatography electrospray ionisation quadrupole time of flight mass spectrometry (LC-ESI-QTOF-MS). Using statistical analysis, a Random Forest predictive model was constructed using the small molecules analysed and this model was tested by performing a blinded study. Seven of the eight blinded drugs were predicted correctly, making the overall accuracy of the model 88%.

Recent efforts have been made in the transfer of this low throughput format of this developmental toxicity model to a high throughput format. Instead of culturing and dosing hES cells in 6-well plates, 96-well plates were used. Six non-teratogens and eight teratogens were dosed on hES cells in order to form a new statistical model to predict drug teratogenicity. An initial blinded study was performed using the newly formed statistical model from the high throughput model by dosing hES cells with three non-teratogens and two strong teratogens. All five blinded drugs were predicted correctly, giving a preliminary accuracy figure of 100%. Future efforts are being made to perform another blinded study and to analyse the small molecules which are driving the model in order to arrive at potential biomarkers of developmental toxicity.

Keywords: developmental toxicity, human embryonic stem cells, metabolomics, teratogenicity, biomarkers

Poster

Quality QSAR as an alternative assessment tool: cross-cutting issues

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The roots of QSAR (Quantitative Structure-Activity Relationships) can be found in late 19th century papers. However, only recently has QSAR gained enough reliability and trust to be used as a dependable tool for assessment of toxicology endpoints for regulative and a variety of other purposes. Based on the assumptions that all chemical activity is related to the structure (structural similarity gives rise to similar properties), QSAR relies on descriptors for the quantitative representation of chemical structures. QSAR models for toxicity predictions are constructed from data obtained from past animal experiments, no further animal tests are required for toxicity assessment using QSAR.

At present, QSAR models have great importance in completing the data requirements under different EU Regulations and Directives (Chemicals, Cosmetics, Biocides, etc.). EC Regulation 1907/2006 (REACH Regulation), which affects all of the chemical industry over the whole supply chain, states in its Article 1: The purpose of this Regulation is to ensure a high level of protection of human health and the environment, including the promotion of alternative test methods for assessment of hazards of substances. QSAR, as one of the most potent alternative methods, may be used instead of testing when results are derived from a QSAR model whose scientific validity has been established when the substance falls within the applicability domain of the QSAR model and results are adequate for the purpose of classification and labeling and/or risk assessment (Annex XI). During the period 2010-2018 more than 150,000 chemicals should be registered under the REACH Regulation. This will require a number of studies and a lot of resources – time, finance, personal and laboratory animals (for example for OECD TG 453, 480 laboratory rats are used, duration of study is 2 years and cost € 1 million). The use of QSAR models will considerably reduce the time and cost and save large numbers of animal lives.

The validation of the models has been the crucial issue throughout the history of QSAR. Validation – both statistical and by scientific peer-review – can be the only guarantee for meaningful predictions. Recently, the Joint Research Center (JRC) has been trying to standardise and validate QSAR Models. Molcode – an international comp-chem and biotech company has pioneered in the attempt to have their QSAR models peer reviewed and validated with the JRC. Currently Molcode has over 25 models (by far the largest number), both linear and nonlinear, published with the JRC and validated for REACH application and other purposes.

In the presentation, the quality control in the process of collecting data, building statistical models and the different steps for validation, as well as the quality of predictions based on the unique Molcode QSAR technology and years of experience will be discussed.

Keywords: QSAR, validation

Lecture in Session III: Skin sensitisation and eye irritation

Development, refinement and combination of *in vitro* assays to hurdle skin sensitization complexity

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Contact sensitizers are reactive molecules (haptens) that have the ability to modify skin proteins to form an antigen which will be recognised by specific T cells activated during the sensitization process. In addition to the haptenation mechanism, contact sensitizers induce several phenotypic and functional changes of dendritic cells (DC) either directly or indirectly through intercellular signaling pathways implicating keratinocytes (KC), fibroblasts and other skin cells. This rather complex and still unraveled maturation process of DC induced by contact sensitizers allows them to migrate to the lymph node, present antigen and efficiently prime hapten-specific T cells. A range of *in vitro* methods have been developed, each of which is based on distinct events of the immunobiology of skin sensitization, like chemical reactivity assays (DPRA, GSH assay, others…),

which reflect the haptenation mechanism, and DC-based assays (MUSST, hCLAT), which measure DC maturation markers (CD86, CD54).

Due to the complexity of the sensitization process it is now commonly agreed that hazard identification and risk assessment could only be addressed by combining a battery of assays. Such a combination should cater for the physicochemical diversity of ingredients. Therefore it is crucial to (i) characterise the applicability domains of each assay, (ii) better understand the mechanistic links between the different endpoints evaluated by those assays and (iii) identify the gaps in terms of events of skin sensitization that need to be evaluated through further assay development. All these aspects will be illustrated in the presentation.

Keywords: in vitro, skin sensitization, haptens

Lecture in Session III: Skin sensitisation and eye irritation

In vitro safety assessment of cosmetic products: investigation of new endpoints for eye irritation

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Cosmetic industry is working to assure a more accurate, sensitive and predictive safety assessment of finished products in order to comply with the new incoming European cosmetic regulation asking for deeper and robust testing and reporting on finished products.

Linked to the complexity of both the eye irritation mechanism (involving the corneal epithelium, the conjunctiva and iris) and the cosmetic formulations and also for ethical reasons, the safety assessment of cosmetic products for their eye irritation potential is a complicated issue.

The role of corneal epithelium is crucial because it represents the first line of defence against many types of injury, trauma and infection and contributes to the maintenance of corneal transparency and rigidity. *In vitro* Reconstituted Human Corneal Epithelial models are under a validation process in ECVAM for discriminating non classified from classified chemicals for their eye irritation potential.

We previously showed (Pauly et al., 2009; Meloni et al., 2010) the occludin gene expression to be an early and relevant biomarker for eye irritation potential. This membrane protein associated to tight junctions appears to play a regulatory rather than a structural function in tight junctions and could be an early marker of physical disorder and damage, suggesting that occludin transcriptional activity could be a valuable tool for *in vitro* eye irritation assessment.

By performing dynamic exposures (6 h, 24 h, 72 h and post incubation) and testing complementary end-points (cell viability, TEER measurement, histology, IL-8 release) on defined concentrations of BAK and several tear substitutes, the toxicogenomic approach has been confirmed to be a valuable and promising tool for *in vitro* eye irritation assessment with the power to detect mild irritants and subclinical eye irritant potential from non classified chemicals.

Application to the assessment of cosmetic products (mascara and surfactants) will be presented.

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Keywords: eye irritation, Human Corneal Epithelium, toxicogenomic, cosmetic products, tears substitutes

Poster

Risk assessment for nanotoxicology: animal welfare aspects

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Due to their special properties like altered physical properties or new behaviour in the interaction with biomaterials, manufactured nanomaterials (MN) are becoming more and more important in science, technology and commerce. Funding for new projects already is in the range of billions of € per year. An ever increasing number of products is available on the market and consumers may already be directly exposed to MN in their everyday life.

But what makes MN special may also pose serious risks for human health and for the environment. These risks are likely to depend on many more factors than those normally considered in safety assessments for bulk scale materials. For example, MN may possess the ability to penetrate cell membranes and/or interact with DNA molecules. Furthermore, MN of the same substance but produced by different manufacturers show substantially different properties.

The current methods employed to observe the toxicity of nanoparticles mainly focus on the use of animals. Results point to serious adverse effects on the health of the species tested in some of these studies, but animal tests have limited value in predicting effects on humans. There are numerous basic nanomaterial-specific scientific problems related to *in vivo* experimentation. Practical problems include challenges related to tracking nanoparticles *in vivo* and delivering a relevant dose to animals. In addition, differences in anatomy, physiology, biochemistry, metabolism and pharmacokinetics make the extrapolation of test data between species, gender and breed of animals (including humans) very difficult.

It would be a grave mistake and a waste of time and resources to rely on adapting common *in vivo* methods that have been developed decades ago for risk assessment to the use on nanomaterials. Such methods were developed decades ago for bulk scale materials, and numerous scientific problems are already known. Furthermore, their suitability for toxicity testing of MN has never been scientifically approved, and no historical data exist. Instead, efforts should be undertaken to develop and validate animal-free test strategies.

To gain human-relevant results and to circumvent suffering of animals, non-animal tests offer several advantages. They represent the most recent advances in biotechnology compared to outdated *in vivo* methods. *In vitro* methods are already developed in related modern fields, such as particulate matter toxicology, that appear to be applicable to nanotechnology with some adjustment. They avoid species differences by using human cells or sub-cellular components. High-throughput systems allow the rapid and cost-effective testing of multiple nanomaterials at the same time, allowing the assessment of differences in mode of action and toxicity of nanomaterials of the same substance but produced by different manufacturers.

The German Animal Welfare Federation and its 800,000 members demand that current and future research and innovation efforts be focused on the promotion, development and validation of non-animal test methods and testing strategies for the safety testing of nanomaterials. Consistent with related European legislation like REACH, the replacement and reduction of animal experimentation should be the main objective.

Keywords: manufactured nanomaterials, nanotoxicology, animal experimentation, alternative methods

Lecture in Session AXLR8 '21st Century Toxicology' Info Forum

VITROCELLOMICS – Reducing animal experimentation in preclinical predictive drug testing by human hepatic *in vitro* models derived from embryonic stem cells

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The objective of the VITROCELLOMICS project has been to establish stable cell lines that reliably reflect human hepatic properties by the development of *in vitro* models derived from human embryonic stem cells (hESC). The aim has been to de-

liver *in vitro* models that are so reliable that they can be used by the pharmaceutical industry in order to replace experimental animals in investigations on liver toxicity, drug metabolism, uptake and efflux properties of compounds in the drug discovery and development processes. In the pharmaceutical industry reliable *in vitro* cell models have potential to replace current techniques and animal experimentation in the selection and optimisation of lead compounds and in documentation of a selected drug candidate before it enters clinical phases.

The experimental design of the project involved improvement of culturing procedures to direct hESC differentiation towards a mature hepatocyte phenotype and expansion and handling of the derived hepatocytes. This work also included identification of hepatocyte markers and a thorough characterization of the hepatocyte phenotype of cells after undergoing the new differentiation protocols (Synnergren et al., 2009). 3D bioreactor culture procedures were developed and adapted in order to further improve hepatic functionality over longer time spans, and miniature bioreactors were developed to increase capacity for high-throughput screening (Gerlach et al., 2010; Miranda et al., 2009). Methods to assess metabolism of drugs, clearance and induction of liver enzymes (CYPs) were evaluated and adapted (Niklas et al., 2009). Established assays for toxicity were evaluated with reference hepatocytes for comparisons with the hESC-derived hepatocytes, and new biosensor technologies were evaluated for use in toxicity screening. The comparative studies of hepatocytes derived from hESC with established *in vitro* models and hepatocyte reference cell types were carried out in order to validate the new models and methods. The biosensor technologies include the optical sensing of oxygen for the measurement of respiration. Respiration can be correlated to viability of the cells (Noor et al., 2009). This method is used for non-invasive dynamic monitoring of effects of tested drugs. The method is correlated with commonly used toxicity assays. An example of another optical biosensing method developed in the project with potential for pre-validation was multi-wavelength fluorescence spectroscopy (Fritzsche et al., 2009).

The presentation will give a brief overview of the accomplished results of the project and provide some recommendation for further actions.

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Keywords: hESC-derived hepatocytes, 3D bioreactors, metabolomics, CYP activity, cell culture respiration

Poster

Analysis of use and reliability of statistics on the use of laboratory animals in the EU

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One major document concerning animal welfare in the European Union is the statistical report entitled "Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union". Despite or because of its great complexity it is generally only used to quote large numbers of "used" animals and standard statistics. But these statistics contain much more information that can be used to enhance the understanding of current animal use with regard to the 3Rs. However this is not presented in an adequate and accessible way.

To evaluate this information according to its importance we have performed an analysis of different aspects of these statistics. Use of specific animals, areas of use and changes over time on different calculation bases (intra-, inter- or me-

tanational) were performed and are presented graphically. Furthermore some unique derivates, e.g. the numbers of animals used per capita, illustrate the differences between the different Member States.

Despite its status as an official document, the report contains some minor and major errors, like differences in calculation from 10 to 100.000. The statistical data is corrected as far as possible and errors are highlighted. For the future a complete compendium of corrected statistical data, with former miscalculations highlighted, and an adequate graphic interactive presentation to facilitate accurate interpretation and use is envisaged.

Keywords: analysis, statistical data, European animal use

Poster

Scaffold-free microscale tissues for *in vitro* compound testing

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Growing cells *in vitro* has become a key technology to assess biological responses of substances regarding their efficacy and toxicology. In recent years protocols to culture most primary cells and cell lines have been established. However the current standard culture format, growing cells in monolayer, does not reflect the 3-dimensional structure and cell composition in a tissue and leads to an altered phenotype and thereby altered functionality.

Microscale tissue engineering provides new strategies to reflect cellular structure and function more closely in a format which enables high throughput analysis. We developed a platform technology based on the hanging drop method, facilitating the introduction of organotypic 3D cell culture devoid of any foreign material straight into established processes. The 96-well GravityPlus plates can be processed similar to normal monolayer cultures in a high throughput process. Applying our technology, microtissues from primary cell sources, such as hepatic, neuronal, myocardial tissues or tumor tissues, from cell lines as well as co-culture models can be produced. For example, dissociated primary rat and human hepatocytes reform within 3-5 days to liver-like microtissues. Over time albumin secretion increases, indicating maturation of hepatocytes within the microtissue format. Cytochrome P450 activity (Cyp3A) remains high over 14 days and induction of activity by dexamethasone results in over 20x increased CYP3A4 activity.

The GravityPlus technology enables the production of highly functional microtissues from a wide range of cell types. Together with its high-throughput capabilities based on the standard 96-well format, the GravityPlus system represents a highly interesting alternative to *in vivo* testing for compound efficacy and toxicity.

Keywords: scaffold free 3D-culture, organotypic microtissues, hepatoxicity testing, high-throughput drug screening

Authors

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Aims and Scope – Protecting More Than Animals

ALTEX, edited by the Swiss Society ALTEX Edition, is the official journal of CAAT, the Center for Alternatives to Animal Testing at the Johns Hopkins University in Baltimore, USA, EUSAAT, the European Society for Alternatives to Animal Testing (former MEGAT), t4, the transatlantic think tank for toxicology (Baltimore, Utrecht, Konstanz) and the Doerenkamp chairs in Germany, India, The Netherlands, Switzerland and USA. ALTEX is devoted to the publication of research on the development and promotion of alternatives to animal experiments according to the 3R concept of Russell and Burch: Replace, Reduce and Refine.

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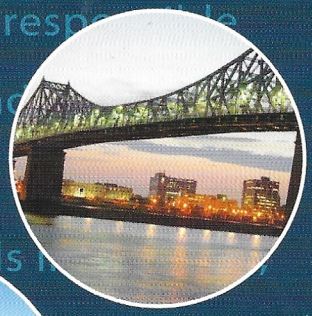

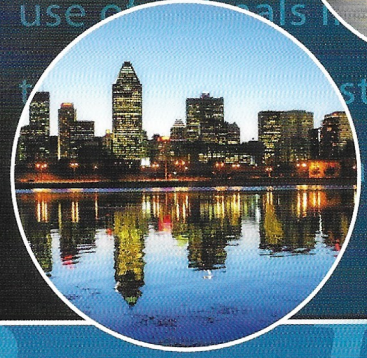

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