20th Anniversary of ZEBET at BfR and 50 Years of the 3Rs Principle

Symposium: October 26-27, 2009, Berlin

Imprint

Abstracts

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and 50 Years of the *3Rs* Principle
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2 Abstracts of Presentations

ZEBET's Role as the National Focal Point for Stakeholders: View of ZEBET Commission Members

2.1 Perspective of the German Animal Welfare Federation

Brigitte Rusche
Animal Welfare Academy, Neubiberg

In the early 1980s, in Germany, after a lot of discussions about the pros and cons of animal experimentation there was agreement between industry and the animal welfare movement that a focal point is needed, in particular on national, governmental level, to promote the development and the application of alternative methods. Together with remarkable support from the German government this agreement has been transformed into an institution that since its foundation in 1989 has received a world-wide reputation and acknowledgement from all interest groups involved: ZEBET.

From the beginning, the animal welfare community was involved in the setting-up and functioning of ZEBET, and it still is. Since 1994, ZEBET has been supported by an advisory committee. That committee is comprised of experts from various stakeholders including industry, government, and animal welfare. Its task is to support the work of ZEBET by giving recommendations concerning various issues and problems. These include the development and acceptance of alternative methods but also a critical appraisal of regulatory demanded animal tests with a view to apply the 3Rs.

In the first years, ZEBET´s role was to give alternatives an official voice and to help overcoming principle objections against alternatives. In the meantime alternatives are much better accepted. So how could ZEBET's role be expanded? At the 10th anniversary of ZEBET I claimed: "What is still needed is another type of co-operation: ZEBET's obligatory involvement in any decisions relevant to the field of animal experiments and alternatives. More than in the past ZEBET must be given the role of a co-ordinating point e.g. for other governmental institutions concerned with problems of consumer or environmental protection."Clearly, the need for such an expanded role of ZEBET is still present today.

In addition to that, from an animal welfare point of view ZEBET's tasks should therefore include particularly:

- foster cooperation regarding the acceptance of alternatives
- raise factually supported criticism of animal experiments in various scientific areas (such as abnormal toxicity testing; use of dogs as a second species)
- deliver facts and data for the discussion of animal experimentation on a societal/political level
- provide support for alternatives in the process of drafting legislation
- supply information and advice with regard to applications for animal experiments submitted to the licensing authorities. This should be obligatory, at least for contentious cases, and members of ethics committees should have the possibility to obtain expert statements from ZEBET
- act as a national partner of ECVAM

2.2 ZEBET as a National Body for the Alternative Methods – the Industry Perspective

Walter Aulmann
Director, Regulatory Affairs EMEA
Ecolab Europe GmbH

In a survey conducted in 2007 companies from different sectors collated the expectations with respect to ZEBET, the German national focal point for alternative methods. The companies voicing their standpoint in that survey are active in the cosmetics, detergents, pharmaceutical s, agro-chemicals and general industrial chemicals sector.

The role of ZEBET as a platform for information exchange and for the bundling of reseach activities in the field of alternative methods and for the dissemination of established methods is highly acknowledged. Industry indeed sees the need for ZEBET as a national focal point for alternative approaches. The scope for “alternatives” should go beyond sole experimental approaches but should also encompass assessment concepts. Especially tools such as weight-of-evidence-approaches and exposure driven testing strategies (e.g. Threshold of Toxicological concern) are promising concepts for the reduction of animal experiments in the context of the REACh and CLP regulation and last not least with respect to the testing ban for cosmetic ingredients. Our challenge is the need of over 40 million experimental animals for the execution of REACh. Only with generally accepted assessment concepts this huge figure can be substantially reduced. Industry encourages ZEBET to be a future key player in this area.

2.3 BMBF Funding Priority “Replacement Methods of Animal Experiments”

Sabine Wiek
German Federal Ministry of Education and Research (BMBF), Division of Molecular Life Sciences, Berlin

The BMBF funding measure “Replacement Methods of Animal Experiments” aims to support research projects that focus on the research and development of alternative methods to replace, reduce or refine existing animal tests according to the 3R principle. The program invites to apply for projects that range over fundamental to application-oriented research and extend to the regulatory area. Applicants are invited to submit projects outlines semi-annual (15^{th} of March and September, respectively).

Since the beginning of the funding initiative in 1984 the BMBF provided approximately 100 million Euros to support nearly 360 research projects, thereby increasing the budgets year after year. Furthermore BMBF provides an extra budget of twelve million Euros to support nine basic research projects in the field of imaging technology development to replace and refine animal tests. These and other efforts resulted in Germany to be at the forefront of providing finances for research projects following the 3R principle Europe wide.

Central aim of the BMBF initiative is to implement the results efficiently, thus, to significantly reduce animal tests and fix the alternate procedures in the relevant practices, guidelines and directives. The Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) actively contributed to the success of various projects and accompanied the initiative comprehensively. Current examples of projects sponsored by the BMBF and achieved results towards the implementation of alternative testing procedures are to be highlighted during the talk.

2.4 Perspectives of The Federal Environment Ministry and The Federal Environment Agency

Petra Greiner
Federal Environment Agency - Umweltbundesamt, Dessau, Germany

Chemicals and products belong to the daily life and needs- for consumers or for professional application. The safety of these chemicals within their lifecycle is essential with respect to human health and the environment. Therefore the marketing and use of chemicals for various purposes require hazard and risk assessment concerning human health and the environment. Depending on the respective legal context specific information including data on toxicological and ecotoxicological properties is required in order to perform hazard and risk assessment. For mutual acceptance of data reasons internationally standardised and accepted testing methods should deliver these data. The majority of the testing methods in toxicology- and to a certain extent in ecotoxicology,- involve testing with vertebrate animals. The OECD has committed to the 3 R Principle already 1982, simultaneously encouraging to discover, develop and validate alternative testing systems. The Federal Ministry of the Environment supported actively the development and validation of test methods complying with both the 3R-principle and the demands on information for hazard and risk assessment. In recent years a number of in vitro test methods have been developed, validated and published as OECD testguidelines, many of them with the engagement of ZEBET, which is not limited to toxicological working areas: ZEBET supported the development and validation work e.g. for the fish embryo toxicity test, an alternative to the widely required test on acute fish toxicity as representative organism for aquatic species. ZEBET is encouraged to further support and advice the expert group "testmethods in ecotoxicology" lead by UBA- especially with regard to increasing demand on methods for identification of endocrine disruptors and PBT-substances.

2.5 Perspective of the Competent Authorities responsible for Authorisation of Animal Experiments

Heidemarie Ratsch
Landesamt für Gesundheit und Soziales (LaGeSo)

Since 1986 it has been established in the German Animal Welfare Act that experiments on animals only shall allowed if it is ensured that no other methods or procedures are available to achieve the same purpose. A review has to be done both by the applicant and the competent authority. This legal mandate concerns not only experiments on animals which require a licence but also experiments not requiring a licence but which must be declared. Especially after the inclusion of animal welfare in the German Constitution in 2002 the competent authorities have more than ever been asked to question the statements of all applicants. Important is not only the replacement of experiments on animals but also the reduction of the amount of animals used in the procedures and the refinement of methods to minimize pain and suffering, especially in the view of the revision of EU Directive 86/609. ZEBET was established to provide help in these affairs, particularly in the form of statements. In discussions with applicants, ZEBET statements bear considerable weight. Because these competent authorities have to put their emphasis more on experiments for the purpose of the prevention, diagnosis or treatment of diseases and basic research, rather than on experiments expressly required by legal instruments, I would like ZEBET to stress the development of alternatives to animal experimentation concerning the former, as opposed to the latter.

ZEBET's Activities: From Past to Future

2.6 20 Years of ZEBET: 1989 – 2009

Horst Spielmann
ZEBET at BfR and Freie Universität Berlin

A short description is given of the history of ZEBET, the National Centre for the Documentation and Evalation of Alternatives to Animal Experiments of the Ferderal Government of Germany at the Federal Institute of Risk Assessment (BfR). ZEBET was established in 1989 as the first government centre focusing on reducing testing in animals.

To give an example of the duties assigned to validation centres, the national German centre ZEBET is serving the following mission:

- to establish a database & information service on alternatives at the national and international level
- to develop alternatives according to the 3Rs principle of Russel and Burch
- to fund research on alternatives
- to co-ordinate validation studies
- to co-operate with national & international funding agencies and validation centres
- to provide a forum for information on alternatives to animal testing.

In 1993 the EU followed the German example by establishing ECVAM (European Centre for the Validation of Alternative Methods) at the Joint Research Centre (JRC) in Ispra (Italy), in 1997 the federal government agencies of the USA formed ICCVAM (Interagency Coordinating Center for the Validation of Alternative Methods) and in 2005 the Japanese government has established JaCVAM (Japanese Center for the Validation of Alternative Methods) at the National Institute of Health in Tokyo. The decrease in experimental animal numbers during the past decade in Europe is illustrated by the situation in Germany and the contribution of international harmonisation of test guidelines on reducing animal numbers in regulatory testing is described. A review of the development of the principles of experimental validation is given and the 3T3 NRU in vitro phototoxicity test is used as an example for a successful validation study, which led to the acceptance of the first *in vitro* toxicity test for regulatory purposes by the OECD. Finally, the currently accepted alternative methods for standardization and safety testing of drugs, biologicals and medical devices will be summarised.

2.7 Animal Experiment Authorisation: Searching for Literature and Information on Alternative Methods

Barbara Grune (Presenting Author), Andreas Luch, Antje Doehrendahl, Susanne Skolik, and Daniel Butzke
ZEBET at the Federal Institute for Risk Assessment

The European Directive regulating the use of sentient animals for experimental and other scientific purposes requires that live animals may only be used if the specific scientific goals pursued cannot be achieved by any other means (i.e. non-animal procedures). In case animal research is permissible the methods applied must ensure that both numbers of animals used and suffering of individual animals are kept at the lowest level possible.

As a result there are legal requirements for identifying and harnessing all appropriate methods available to replace, reduce and refine such animal use. Scientists are obliged to undertake a valid indispensability search prior to applying for an animal experiment at authorising bodies. The aim of an indispensability search is to exclude all possibility of the presence of (i)

a suitable alternative method that can be applied instead, (ii) usable results from comparable previous animal experiments, and (iii) results from other research suited to anticipate the outcome of the planned experiment. This obligatory search has to consider the current state of scientific knowledge exhaustively. Only when the availability of a suitable alternative method or usable scientific results has been excluded in a valid search procedure, and based upon the current state of knowledge, an animal experiment may be approved indispensable to reach a vindicatory scientific objective.

The amount of accessible information grows rapidly and largely uncontrolled. Buried within this ever-growing "thicket" important innovative ideas and seminal research have become hidden over time. Scientific meta-databases like *PubMed* provide the opportunity to search almost 20 Million documents at the same time via simple and usually general keywords. After retrieving the hit list, great efforts are thus necessary to sort out irrelevant literature, to elaborate effective combination of search terms (search strategy) for subsequent queries and at the same time taking care of not losing the track. Thus, a structured search for scientific literature on a specific candidate alternative method is key in the effort to ease the handling of the ever-growing literature and to demonstrate compliancy with animal protection obligations.

2.8 The AnimALT-ZEBET Database: a Unique Resource for Comprehensive and Value-Added Information on *3R* Alternatives

Daniel Butzke (Presenting author), Antje Doehrendahl, Susanne Skolik, Andreas Luch and Barbara Grune
ZEBET at the Federal Institute for Risk Assessment

Value-added databases are the starting point for any structured search for information on suitable alternative methods. They provide short reviews on the most advanced procedures with relevance to the *3R* principle in a clear, reliable and comprehensive manner.

At the forefront of these essential resources is the AnimAlt-ZEBET database that is offered by the German Federal Institute for Risk Assessment accessible online free of charge. The documents of this database are compiled by scientific experts and provide selected high-quality information in compliance with the specific requirements of scientists, competent authorities and others who are obliged to consider the applicability of a specific alternative method. Thus, the focus is on (1) the essential technical key points, (2) the application domain, (3) advances/limitations of the most elaborate protocol, (4) the prediction model, (5) the opinion(s) of expert panels (e.g. ESAC; ICCVAM), (6) the status of validation and acceptance and, most notably, (7) the contribution of the respective method to the *3R* concept.

Because the documents of the database are written in a structured manner, they can be used as the feedstock for any up-to-date text mining application, like "semantic landscape"-producing tools. The database currently holds some 140 documents with a focus on safety testing of chemicals and drugs, but will be expanded to also cover the area of basic sciences.

2.9 Advisory Services for Public Authorities, Ministries and Scientists

Sarah Adler
ZEBET at the Federal Institute for Risk Assessment

Upon request of the competent public authorities of the *Laender*, ZEBET scientists examine whether an animal experiment for which an application has been submitted is really indispensable pursuant to the Animal Welfare Act. For this assessment the scientists examine whether the experiment reflects the latest scientific knowledge, whether the test regimen is statistically reliable and whether the number of test animals is kept at a minimum. In addition, scientists who develop alternative methods in research institutes, universities or industry frequently approach ZEBET in order to receive advice on whether the new alternative method is likely to be a suitable replacement for an internationally established animal experiment and how this goal can be achieved. In 2007 ZEBET responded to a total of 457 inquiries from inside and outside Germany.

Another main task of ZEBET is to provide scientific opinions for the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) that is responsible for consumer protection and animal welfare in Germany, e.g. in conjunction with the revision of EU Directive 86/609 which regulates the handling of laboratory animals in the European Union.

Furthermore, ZEBET scientists are sought after experts on the international level in research support programmes for the development of alternative methods and also for evaluating applications for research prizes in the field of alternatives.

2.10 The BfR-ZEBET Funding Program of 3Rs Research in Germany

Michael Oelgeschläger (Presenting Author), Daniel Butzke, Sarah Adler, Ralph Pirow, Andrea Seiler, Richard Vogel, Barbara Grune, Manfred Liebsch, Andreas Luch, and Horst Spielmann
ZEBET at Federal Institute for Risk Assessment (BfR)

Since now for some 20 years ZEBET provides funding to promote novel and innovative projects and approaches at German universities and research institutes committed to refine, reduce or replace animal experiments. Compared to the initial funding budget of around € 200,000 (400,000 Deutsche Mark) in 1990 the budget has doubled to approximately € 400,000 in 2009 . Due to this special federal financial support dedicated to the 3Rs, altogether more than 100 projects in the field could be “picked-up” and fostered from their very early beginnings, mostly at a stage where no proof of concept had been available at the time.

Still, compared to other national and international funding programs the financial volume is rather small. It is therefore the main objective of the BfR-ZEBET program to concentrate on initial and time-limited funding of highly novel and rather risky project proposals to enable innovative scientists to develop their ideas and to produce experimental data for subsequent support by larger funding programs, like the priority program "Methods to replace animal experiments" of the Federal Ministry of Education and Research (BMBF), the EU Framework Programme or direct funding by ECVAM and Industry (e.g. COLIPA).

The projects accepted encompass a wide range of experimental approaches, including cell culture techniques, organ cultures or bioreactors that mimic *in vivo* situations. In addition, the development of software programs for statistical data analysis and *in silico* tools for the prediction of potential toxicity of chemicals, were supported and some of them are now successfully applied worldwide.

Many of the researchers who enjoyed support from the ZEBET fund between 1990 and 2009 have achieved honourable distinction through prestigious national and international research awards for their contributions to the 3Rs principle. It is also this international recognition of supported projects and scientists that underpins the importance of this program for initiation and development of alternatives to animal experiments over the last two decades.

2.11 The Use of Embryonic Stem Cells for Developmental Toxicity Testing

Andrea Seiler (Presenting Author), Kristin Fischer, Uschi Gruber, Katrin Hayess, Bettina Huhse, Johanna Kaltenhäuser, Michael Oelgeschläger, Dana Sittner, Birgitta Slawik, Lena Smirnova, Julian Tharmann, Manfred Liebsch, Horst Spielmann and Andreas Luch
ZEBET at the Federal Institute for Risk Assessment

Congenital abnormalities represent a severe medical and social problem, given that approximately 3% of all human newborns are affected. About 20% of these defects are associated with gene mutations and another 5% with chromosomal aberrations. Approximately 5 to 10% of the remaining abnormalities are known to be caused by teratogenic agents. It is thus mandatory in toxicological safety assessment of chemicals and drugs to evaluate adverse effects on reproduction and embryonic development according to OECD test guidelines or so-called segment studies encompassing three crucial periods of pre- and postnatal development and fertility (ICH, 1993). All of these test methods generally comprise time-consuming and expensive *in vivo* experiments mostly performed with mammalian species such as rats or rabbits.

In recent years, embryonic stem (ES) cells became available and shifted into focus. The ability to differentiate into a wide range of different cell types has made ES cells a very popular system to study developmental processes and gene function during cellular differentiation *in vitro*. More than 10 years ago scientists at ZEBET proposed a new *in vitro* assay - the so-called Embryonic Stem Cell Test (EST) – which makes use of this capacity to detect developmental toxicants during stem cell differentiation into cardiomyocytes. The EST has been scientifically validated by the European Centre for the Validation of Alternative Methods (ECVAM).

Currently the stem cell research group at ZEBET is committed to explore and develop additional stem cell based approaches, searching for novel predictive biomarkers of developmental toxicity and to extend the experimental approach to other cellular systems, like neurons and bone, in order to establish ES cell based tests for the prediction of developmental neuro- and osteotoxicity.

2.12 International Pre-validation and Validation Studies

Horst Spielmann[1,2] and Manfred Liebsch[1]
ZEBET[1] at BfR and Freie Universität Berlin[1,2]

At a symposium that was held on the occasion of establishing ZEBET in 1989, the issue of validating alternatives to animal tests that have to be conducted for regulatory purposes was discussed. To solve this problem we agreed to hold a workshop on the validation of *in vitro* toxicity tests. In 1990 this workshop was held in Amden (Switzerland) and the basic principles of experimental validation that were defined by the participants and published as “report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures”. Later in the same year a second international workshop in Vouliagmeni (Greece) “on the promotion of the regulatory acceptance of validated non-animal toxicity test proce-

dures" was held. Thus even before ECVAM or ICCVAM existed at these two workshops some of the basic principles of experimental validation of *in vitro* toxicity tests were defined.

After the EC/HO international validation study on alternatives to the Draize eye test failed, which was conducted according to these recommendations, at a second validation workshop in Amden in 1995, biostatistically based prediction models were added as essential elements of toxicity tests and also a management structure of validation studies, to ensure that validation studies are conducted as independent and unbiased as possible. Later the same year the pre-validation concept was proposed as result of a cooperation between ECVAM, IIVS and ZEBET.

The specific modules of validation studies that were proposed until 1995 are until today the basis of international documents on experimental validation of toxicity test procedures, e.g. recommendations and guidelines accepted by ECVAM, ICCVAM and finally also by the OECD, which published in 2004 a detailed Guidance Document No. 34 "on the validation and international acceptance of new and updated test methods for hazard assessment".

To speed up the regulatory acceptance of in vitro tests methods that are meeting the requirements of an established test, e.g. to prove that a newly developed skin model meets the requirements of an EU or OECD Test Guideline, in cooperation with ECVAM ZEBET proposed the principle of " catch up validation" and proved that this concept is valid.

Finally ZEBET also contributed to the development of the "weight of evidence" validation concept which was proposed in 2004 under the guidance of Thomas Hartung at ECVAM to speed up the experimental validation process and to reduce costs.

Examples of successful pre-validation and validation studies will be presented to illustrate ZEBET's contribution to establishing *in vitro* toxicology in an "unfriendly environment".

2.13 Towards the International Acceptance of Alternative Methods!

Manfred Liebsch, Andreas Luch, and Horst Spielmann
ZEBET at the Federal Institute for Risk Assessment

The proof of validation (i.e., the process by which relevance and reliability of a method are determined for a specific purpose) of any new method to be used in a regulatory context is purely scientific exercise, until being finally approved through independent (peer) review of its overall performance. The entire process necessary to gain regulatory acceptance at the international level comprises more than just scientific evaluation: an expert judgement on the practicability, availability and costs of the new method has to be provided. Even more important, a thorough evaluation of limitations and restrictions are key to enable the specification in which particular regulatory field a new method most likely could be successfully applied in the future.

In the 1990s, the question of whether or not specialised "validation authorities" such as ECVAM, ICCVAM, ZEBET, and others should get involved in the process of international harmonisation and acceptance at the OECD level was vividly and controversially discussed. Since ZEBET itself was established within a Competent Authority (former BGA and BgVV, now BfR) scientists at ZEBET have been constantly exposed to critical responses and issues raised from colleagues involved in the regulation (i.e., notification of chemicals, the authorisation of pesticides and biocides, or the safety assessment of consumer products). The constructive and continuous input from their side has contributed to appropriately ensure full consideration of their expectations and needs in validation studies, e.g., by welcoming their suggestions during the process of selection of reference chemicals ("gold standards") to be

used in those studies. Triggered by such experiences made at their own institution (BfR), scientists at ZEBET always felt strongly obliged to the concept of attempting to fully reach general consensus on new methods at the international level. To accelerate this process ZEBET has delegated a scientist to work at the OECD office in Paris. Moreover, ZEBET staff were engaged as members of the German delegation to OECD Expert Meetings and the Centre quite often has hosted OECD expert meetings itself, such as those performed in the fields of acute inhalation toxicity, skin corrosion, skin irritation and phototoxicity.

The presentation will exemplify several specific experiences made during validation of alternative methods in the past. This figure is going to be supplemented by an interesting and actual case in which the u sage domain of a new method accepted for regulating chemicals has been gradually extended to the assessment of the biological safety of medical devices (regulated by ISO Standard 1933).

2.14 The Role of Biostatistics in the Development and Validation of Alternative Methods

Ralph Pirow
ZEBET at the Federal Institute for Risk Assessment

The technological advances to monitor the state and activity of cellular systems offer new possibilities for improving accepted alternative methods and for establishing new alternatives to animal testing. The selection from a multitude of potential toxicological endpoints, and the demand for an enhanced predictive power and high-throughput screening, represent a challenge for biostatistics. Biostatistical methods play an important role during all phases of the development and validation of alternative methods. Main targets of biostatistical support include the optimization of experimental design, the automated data processing, the data quality control (reduction of data variability), the analysis of dose-response curves, the selection of suitable endpoints for predicting toxicity, and the establishment of appropriate prediction models (Holzhütter et al., 1996, ATLA 24, 511–530). The visualization of experimental data, analytical procedures, and statistical results is an important aspect in providing a “common language” between the biostatisticians and experimental toxicologists. The present talk will give a brief overview on the application of biostatistical methods to ongoing research projects at ZEBET.

National and International Cooperations

2.15 National and International Cooperation – Cooperation with the Freie Universität Berlin

Monika Schäfer-Korting
Freie Universität Berlin, Institut für Pharmazie, Pharmakologie und Toxikologie

Since 1994 scientist from the FU Berlin cooperate with scientists from the ZEBET with respect to the use, establishment, and validation of alternative testing strategies. Funded by the German Ministry of Education and Research (BMBF) a joint research group has established and validated a protocol for percutaneous absorption testing making use of commercially available reconstructed human epidermis. Currently the biotransformation of xenobiotics by primary skin cells and reconstructed human skin as well as the associated risks are under evaluation. Scientists from the German industry and academia are partners of both projects, too. The irritant potential for eye and skin of our new investigational drug agents and innovative carrier systems is regularly estimated by HET-CAM test and the EpiSkin test which have been introduced into our lab by scientific support of the ZEBET.

Moreover, the development of a reconstructed cornea (in cooperation with the Animal Welfare Academy, Neubiberg, Germany) and an in-vitro model of angiogenesis by the Departments of Veterinary Anatomy (Berlin and München) have become possible only by funding by the European Commission (EFRE) and the ZEBET.

To increase the awareness of animal welfare in research and the impact of in vitro testing, certified training courses for PhD students and postdocs (laboratory animals, animal experimentation, alternative testing strategies) have been set-up in the 1990s by the ZEBET and the Berlin universities. More recently a training lab (INVITROTRAIN, EFRE funding) has been established at the FU Berlin (Pharmacy) which introduces scientists from all over the world to the use of in vitro test protocols. Major contribution of scientists from the ZEBET greatly improves the impact of the courses.

The cooperation with scientists from the ZEBET for more than a decade as well as funding by seed money by the ZEBET and funding by BMBF and EFRE is gratefully acknowledged.
The progress made since 1989 should allow for a further reduction in animal testing in the next decade. A high demand is due to the increasing awareness for toxicological risks of e.g. chemicals, drugs, food additives and consumer products. Besides this, in vitro disease models based on gene transfer and gene knock down in human cells have to be set up which asks not only for fundamental research but also for an extension of the fruitful co-operations.

2.16 Cooperation with the OECD and Strategies for Replacing Animal Procedures – OECD TestGuidelines

Patric Amcoff
OECD, Environment Directorate, Environment, Health and Safety Division

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries meet to coordinate and harmonise policies and work together to respond to international problems. The OECD Test Guidelines (TG), that are developed by the Test Guidelines Programme (TGP) of the Environment, Health and Safety Division, are a collection of the most relevant internationally agreed test methods used by government, industry and others to assess the safety of chemical products for the protection of man and the environment[1]. The work is overseen by the Working Group of National Co-ordinators of the TGP (WNT) and dependent on member

countries active participation in the projects and long-term commitments to the development and validation of new alternative test methods. Animal welfare, and adherence to the 3R-principles as laid down by Russell & Burch in 1959, is always considered when existing TGs are revised and when new test methods are being developed.

To facilitate alternative test method development and their regulatory acceptance, OECD has developed a Guidance Document No. 34 (GD 34) on "*the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*"[2], based on internationally acclaimed validation and regulatory acceptance principles. These principles are now requirements for all new or updated TGs. New tailored validation concepts for specific applications, such as Performance-Based TGs, are under evaluation by the Validation Management Group for Non-Animal tests (VMG NA) that oversees the development of new *in vitro* TGs for the testing of chemicals with potential endocrine disruptive properties. Following the adoption of GD 34 in 2005, Performance Standards (PS) have become commonly used for new *in vitro* TGs, *e.g,* for the TG 435 on "*In Vitro* Membrane Barrier Test Method for Skin Corrosion" and the newly adopted TG 437 "*Bovine Corneal Opacity test*" (BCOP) and TG 438 "*Isolated Chicken Eye test"* (ICE) for identification of severe eye irritants. PS are also suggested to be incorporated into the new draft TG for *in vitro* skin irritation and the existing TGs 430 and 431 on skin corrosion as well as into the revised Local Lymph Node Assay (TG 429). The PS will provide means for developing proprietary test methods into TGs and the development of similar "me-too tests". Implementation of the OECD principles of validation and regulatory acceptance enhances international harmonisation of validation of alternatives, saves resources and animals and facilitates international collaborations and adoption of OECD TGs.

The German Federal Institute for Risk Assessment (BfR) and the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) is acknowledged for its long-standing commitment and deep engagement in OECD work for the development of new alternative test methods for regulatory uses. Several new TGs that are based on projects lead by Germany, or in collaboration with other member countries, and that have been developed and validated by BfR-ZEBET have been adopted in the last decade. This include alternative Test Guidelines for acute oral (TG 423) and acute inhalation (TG 436) toxicity testing, eye irritation (TG 405), *in vitro* skin corrosion (TG 430, TG 431), skin irritation (TG 404), *in vivo* and *in vitro* skin absorption (TG 427, TG 428) and *in vitro* phototoxicity (TG 432) test method, in addition to having hosted several OECD Expert Consultation Meetings, *e.g.* for the finalisation of the GD 34. The OECD TGP looks forward to future fruitful collaborations with Germany and the BfR-ZEBET on new interesting alternative test methods, and congratulates BfR-ZEBET to its 20 years of successful development of alternative Test Guidelines for the regulatory community.

2.17 Cooperation within The Framework Of International Validation Studies

Rodger Curren
IIVS, USA

The many and varied contributions of ZEBET towards the validation of non-animal methods are essentially unparalleled by any other organization. Almost since the inception of ZEBET in 1989, this group has taken a leadership role in designing, funding, conducting, analyzing and publicizing studies which show the usefulness of non-animal methods in making safety decisions. These contributions are well known to my organization – the Institute for In Vitro Sciences, Inc. (IIVS) – since over the years IIVS staff and ZEBET have been involved in many of the same validation activities. Nearly 20 years ago we first worked together on a large and costly eye irritation validation study co-sponsored by the European Commission and the British Home Office. Although the assays our laboratories personally conducted performed well, none of the methods in the study could be considered validated. In the face of this pos-

sible major set-back, ZEBET, IIVS and ECVAM worked together to propose a prevalidation approach that defined relatively inexpensive incremental steps which should be performed to make the validation process more efficient and cost-effective. Since then our approach has been adopted internationally for many successful validation efforts. Such successful ZEBET/IIVS collaborations have continued over the years, due to the fact that both organizations have strong, practical laboratory expertise which is used to ground validation programs in the realities of real life application of the assays. At the start of a study both ZEBET and IIVS share their scientific knowledge (often involving highly detailed Standard Operating Procedures) with all participating laboratories to ensure that the technical aspects are virtually guaranteed to be successful. ZEBET then follows up at the conclusion of the study by effectively communicating the results - in a scientifically credible way - to appropriate sections of the regulatory and political community. ZEBET's other strength is that they are inclusive of many different organizations, reaching out unhesitatingly to friends around the globe to forge an effective assemblage to promote the scientifically valid use of alternatives. This strategy, embraced by both ZEBET and IIVS, has resulted in numerous advances for the alternatives community, and we can only hope that ZEBET's effectiveness can last through several more 20-year periods.

50 Years of the 3Rs Principle

2.18 Bill Russell, Rex Burch and the Three Rs

Michael Balls
FRAME, UK

In *The Principles of Humane Experimental Technique*, Russell & Burch explained how the Three Rs can be used to diminish or remove the inhumanity inevitably involved in animal provide experimentation. They concluded that "*Replacement* is always a satisfactory answer, but *Reduction* and *Refinement* should, whenever possible, be used in combination". Many of the commonsense insights in *The Principles* are as relevant today as they were in 1959. However, their warnings about the limited value of models in general, and, in particular, the danger of succumbing to the high-fidelity fallacy (whereby it is assumed that the best models for humans are always placental mammals, because they are more like humans than other animals), appear to have largely gone unheeded. Of particular importance is their discussion on toxicity testing, which they saw as one use of laboratory animals "which is an urgent humanitarian problem, for it regularly involves considerable and sometimes acute distress". How, then, can it be that mammalian models are still routinely used in attempts to detect chemical carcinogens and reproductive toxins, despite the fact that the relevance to humans of the data they has not been, and perhaps could never be, satisfactorily established?

2.19 Refinement – Enrichment

Hanno Würbel
Animal Welfare and Ethology, Department of Veterinary Clinical Sciences, University of Giessen

The welfare of an animal is its state as regards its attempts to cope with the environment. Environmental enrichment is aimed to provide animals with resources and stimuli that offer them a degree of control which is fundamental for their ability to cope with the environment. Laboratory rodents housed in standard cages develop a range of abnormal behaviours and other signs of poor welfare that can be attenuated by adequate environmental enrichment. Against concerns that enrichment might disrupt standardization, we recently showed that the welfare of laboratory mice can be improved by environmental enrichment without reducing the precision and reproducibility of experimental results. In fact, these concerns were based on a flawed concept of standardization. It is based on the true finding that experimental results vary depending on environmental conditions, and on the false belief that standardization will 'spirit away' such variation. This has been referred to as the 'standardization fallacy'. Indeed, we recently showed that environmental standardization increases variation between replicate studies and the rate of false positive results, whereas systematic environmental variation (heterogenization) attenuates these deleterious effects. Taken together, these findings offer simple and effective strategies to improve both animal welfare and the validity of animal research in the best of meanings of the 3R concept.

2.20 Reduction – Animal Use in Food Safety Assessment

Herman B.W.M. Koëter
Orange House Partnership, Belgium

European food safety legislation started in 1962 with a Directive on food colours. Since these early days food safety regulations including data requirements involving animal testing, have mushroomed over the decades and, until recently, became a patchwork of some thirty-plus pieces of legislation and guidance documents. Together these regulations cover: food additives, feed additives, colours, sweeteners, enzymes, flavourings, food contact materials, food processing aids, GMO's, novel foods, food supplements and more. Only recently, the European Commission has started to harmonise food safety legislation for all food improving agents in a new single legislation.

EU has the most stringent food safety and food management policy in the world. Nonetheless, increasingly strict food safety criteria, quality controls and monitoring procedures have lead to an increasing number of food safety alerts. Current policies of openness and transparency, intended to build consumer confidence and trust, have in fact resulted in a decrease in consumer trust since at least weekly some sort of a warning appears anywhere in the newspapers. More testing has not resulted in more confidence, on the contrary, more testing reveals more 'uncertainties' and requires human and financial resources to address these which are not at hand. But there is hope: pushed by the urgency to assess the safety of large numbers of food additives, enzymes, flavourings and food contact materials, emphasis is increasingly more focussed on screening methods for priority setting. Such screening approaches, boosted by computational technologies and sophisticated in vitro approaches, have improved in power and became gradually more relevant and reliable. Added to 'older' concepts such as GRAS (generally recommended as safe) and QPS (qualified presumption of safety) and parallel initiatives such as QSARS and TTC (threshold of toxicological concern), an increasing number of food additives is currently being 'screened' only and subsequently considered as of low concern. Unfortunately, the culture of scientific food risk assessment committees and panels is still rather conservative and animal welfare is not high on the agenda. In EFSA efforts have been made to establish a general animal welfare climate among staff and external scientists but, at best, responses are lukewarm: unnecessary testing, although not based on requirements, is still frequently observed when evaluating technical dossiers but these observations remain without any follow-up such as an alert or reprimand to the notifier that such careless animal use is considered poor management.

2.21 Replacement – Replacing Animal Testing for Assessing Consumer Safety

Maxwell, G. (Presenting Author), Carmichael, P., Dent, M., Fentem, J., MacKay, C., Pease, C., Reynolds, F., Westmoreland, C.
Safety & Environmental Assurance Centre, Unilever, UK

Assuring consumer safety without the generation of new animal data is a considerable challenge. However, through the application of new technologies, the development of new experimental models and the further development of risk-based approaches for safety assessment, we remain confident it is ultimately achievable.

To tackle this challenge a substantial research programme was initiated by Unilever in 2004, to critically evaluate the feasibility of a new conceptual approach for consumer safety risk assessment [1]. Today our research efforts cover the priority areas of skin allergy, cancer and general toxicity (including inhalation toxicity). In each of these areas, a long-term investment is essential to increase the scientific understanding of the underlying biology and molecular mechanisms that we believe will ultimately form a sound basis for novel risk assess-

ment approaches. For example, within the Skin allergy programme w e are currently evaluating four non-animal predictive models and developing a prototype probabilistic model to better predict the factors that drive the prevalence of skin allergy in a consumer population [2].

Here we share our progress to date as well as highlighting where we believe the key challenges for the future exist.

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Global Developments

2.22 Revision of EU Directive 86/609

Katharina Kluge
Federal Ministry of Food, Agriculture and Consumer Protection

In November 2008 the European Commission presented a proposal for the revision of 'Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific pur poses'.

This proposal for a 'Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes' includes a comprehensive revision of the existing Directive of November 1986. The objective of the proposal is to harmonize the measures in the Member States thereby ensuring a level playing field throughout the EU, to set more stringent and transparent measures in the area of animal experimentation, to improve the welfare of animals used in scientific procedures by rising the minimum standards for their protection in line with the latest scientific developments.

The proposal includes *inter alia* requirements for the killing of experimental animals, for the re-use of animals, for the authorisation of procedures, for establishments breeding, supplying or using animals, for the accomodation and care of animals as well as for the competence of persons. A severity classification of procedures is introduced. The proposal fully takes account of the so-called 3R principle of replacing, reducing and refining the use of animals in experiments. It also promotes that more research is carried out in the field of alternative methods.

The new Directive will be adopted by codecision procedure, both the Council and the European Parliament must approve the final text. In it's first reading of the proposal in May 2009 the European Parliament proposed a series of amendments to the Commission proposal. Within the Council, the proposal was discussed in several meetings at working group level. Consultations are ongoing, probably also resulting in several suggestions for amendments. A first informal trialogue between Council, Commission and Parliament for exploring compromises took place in October. The goal is to rapidly adopt the dossier. The challenge is to bring the areas of animal welfare and freedom of research into balance.

2.23 Toxicity Testing in the 21st Century: A Vision and A Strategy

Daniel Krewski (Presenting Author) & Melvin E. Andersen
University of Ottawa (Canada) and the Hamner Institutes for Life Sciences (USA)

In 2007, the US National Academy of Sciences released a report, Toxicity Testing in the 21st Century: A Vision and a Strategy. This report envisions a not-so-distant future in which virtually all routine toxicity testing would be conducted in human cells or cell lines in vitro by evaluating cellular responses in a suite of toxicity pathway assays using high throughput tests, implemented with robotic assistance. Toxicity pathways are simply normal signaling pathways in cells that may be perturbed by test compounds. Risk assessment would shift towards the avoidance of significant perturbations of these pathways in exposed human populations. Dose response modeling of perturbations of pathway function would be organized around computational systems biology models of the circuitry underlying each toxicity pathway. In vitro to in vivo extrapolations would rely on pharmacokinetic models that would predict human blood and tissue concentrations under specific exposure conditions. This re-direction of toxicity testing will lead to use of vastly smaller numbers of animals, improve knowledge of modes of action and molecular targets for environmental agents,

enhance human relevance of test results, and provide much higher throughput, thereby permitting coverage of much larger numbers of test agents than is possible with current toxicological testing strategies. All the tools for making these changes in toxicity testing practices are either currently available or in an advanced state of development. The major prerequisites for achieving this paradigm shift are a commitment to change, and the necessary resources to enumerate the pathways by which chemicals can induce toxic responses in humans, to develop the suite of toxicity pathway assays needed to identify and prevent critical pathway perturbations, and to implement computational systems biology approaches to describe pathway function. A broad scientific discussion of this new vision for the future of toxicity testing is needed to motivate a departure from the current reliance on traditional animal-based toxicological tests towards a new approach more firmly grounded in human biology. This paper provides and overview of the original NRC report, and an update on subsequent events that have served to move the NRC vision forward.

2.24 Applications of High Throughput Screening to Identify Profiles of Biological Activity

Robert J Kavlock
National Center for Computational Toxicology, Office of Research and Development, US Environmental Protection Agency

ToxCast, the United States Environmental Protection Agency's chemical prioritization research program, is developing methods for utilizing computational chemistry and bioactivity profiling to predict potential for toxicity and prioritize limited testing resources (www.epa.gov/tocast). This presentation will provide an overview of the rationale, design and status of ToxCast. In Phase I, our proof-of-concept component, we have focused upon evaluating chemicals with an existing, rich toxicological database in order to provide an interpretive context for the high through put screening data. This set of 320 reference chemicals, largely food use pesticides, and represents numerous structural classes and phenotypic outcomes. The in vivo datasets include chronic cancer bioassays in the rat and mouse, multigenerational studies in the rat and developmental toxicity studies for the rat and the rabbit. Bioactivity data is derived from a broad spectrum of nearly 500 readouts from biochemical assays, cell-based phenotypic assays, and model organisms. A variety of supervised and unsupervised computational tools are being used to derive signatures of boactivity in the in vitro data that are predictive of phenotypic outcomes in the whole animal bioassays. Examples of resulting models will be presented. ToxCast is part of a larger government effort (Tox21) being conducted jointly by EPA, the National Toxicology Program of NIEHS, and the NCGC that is obtaining high throughput screening data on more than 2000 chemicals, with plans to expand to nearly 10000 chemicals in 2009. *This is an abstract of a proposed presentation.*

2.25 The European Partnership on Alternative Approaches to Animal Testing (EPAA)

Odile de SILVA
L'Oréal

The EPAA is an innovative and dynamic approach, as it brings together for the first time in a collaborative manner the European Commission and major companies from seven industry sectors. The partners are committed to accelerate the development, validation and acceptance of alternative approaches over an initial five-year period.

Now in its fourth year, the Partnership currently focuses on 3 areas of delivery where EPAA offers distinctive competence: implementing effective sharing across sectors, reinvigorating 3Rs research and removing barriers to the implementation of 3 Rs.
This is illustrated by several ongoing projects, such as

- reviewing the role of acute toxicity testing across several industry sectors and identifying opportunities for test waiver or improved study design , building on the successful achievement of the pharmaceutical industry,
- testing the feasibility of a huge reduction in animal numbers in reproduction studies based on the experience of the agrochemical sector and applying it to the chemicals sector,
- exploring cutting edge research for new non animal alternative approaches and strategies for safety , more specifically computational chemistry, mathematical modelling, stem cell biology and systems biology.
- Discussing and developing guidance for the validation of complex testing strategies , taking into account the multiplicity and specificity of existing Integrated Testing Strategies in the different industry sectors
- Exploring the dissemination of targeted information on 3Rs activities to young scientists, regulators and 3 Rs methodologies users.

These projects will be outlined as well as the planned future activities.

2.26 The concept of Evidence-Based Toxicology

Thomas Hartung
Johns Hopkins University, Center for Alternatives to Animal Testing (CAAT), USA

Evidence-based-medicine (EBM) has been a revolution in clinical medicine over the last three decades, showing the advantage of objective, critical and systematic reviews of current practices as well as formal meta-analysis of data and central deposits of current best evidence for a given medical problem. Toxicology might benefit from a similar rigorous review of traditional approaches and the development of meta-analysis tools as well a central quality-controlled information portal. Already in 1993 Neugebauer and Holaday in their book “Handbook of mediators of septic shock” showed that EBM methods can be applied to animal studies and in vitro work. With Sebastian Hoffmann and his thesis “Evidence-based *in vitro* toxicology” in 2005 we developed initial concepts of an evidence-based toxicology (EBT).

EBM was born from the need to somehow handle the flood of information in medicine and to sort the available evidence in an objective manner, which includes traditional approaches and new scientific developments of variable quality. More than half a million papers included in MedLine per year of an estimated more than 2 million in medicine every year address questions relevant to the life sciences and therapy. For example, entering the search term “toxicology” for the time period since 2003 results in 28,500 article hits in PubMed, a database not even covering all relevant publications in the biomedical field. Instead of expecting individuals to determine what is the best evidence for a specific question or approach at a given time-point, high-quality reviews available at a central deposit should represent a primary resource of information. This requires agreed quality standards, so that the individual physician can rely on the information received. This is the key difference between evidence-based and traditional (“narrative”) reviews: most reviews represent a story told by (knowledgeable) authors who present their personal views on their topic of interest. They tend to select their own papers and those that fit the story line of their review. The systematic review proceeds differently: The sources and a search strategy, i.e. which decides which papers shall be considered and which not, are defined upfront. Before collecting the actual articles, the procedure for information analysis is defined. Ideally, these search and analysis strate-

gies are peer-reviewed to safeguard objective and efficient processes. The analysis of the collected evidence requires weighing the quality of individual pieces of evidence and summarising these as objectively as possible. The latter often involves meta-analysis, i.e. statistical approaches to combine results from different studies.

Obviously, toxicology has a similar problem of information flooding and coexistence of traditional and modern methodologies, as well as various biases. It is most difficult to find and summarise the relevant information for any given major question. This has been nicely illustrated by Christina Ruden (2001): She showed the divergence in judgment and limitations of analysis for 29 cancer risk assessments carried out for trichloroethylene – 4 assessments concluded that the substance is carcinogenic, 6 said it is not and 19 were equivocal. The main reason for this divergence was a selection bias in the materials considered, i.e. an average reference coverage of only 18%, an average citation coverage of most relevant studies of 80%, as well as an interpretation difference of most relevant studies in 27%, and the lack of study/data quality assessment not documented in 65% of the assessments.

The similarities between the problems of toxicology and clinical medicine, and especially the similarities between making a diagnosis in medicine and deciding on whether a substance is hazardous (Hoffmann and Hartung, 2005), prompted us to think about whether EBM tools could be suitable for toxicology (Hoffmann and Hartung, 2005).

A major step toward the formation of an EBT movement was the first International Forum toward an Evidence-based Toxicology in 2007 (www.ebtox.org). The Forum formulated a declaration and ten defining characteristics of EBT, but not a consensus definition yet. Proceedings are now available. The first major development of EBT was the ToxR-Tool to systematically assign quality scores to existing in vivo and in vitro studies (Schneider et al. 2009), which can be downloaded from the ECVAM website. Such evaluation is critical for any meta-analysis but also for programs like REACH using existing information for notifications.

With the creation of the first chair for EBT at Johns Hopkins in 2009, the EBT idea has been institutionalized for the first time in a major academic institution. It is hoped to become a starting point for further developments of an EBT movement.

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2.27 Expectations of Animal Welfare Organisations in Europe

Roman Kolar
German Animal Welfare Federation, Eurogroup for Animals

Animal experimentation has always been an issue of particular concern to the animal welfare community. By definition, in this field of animal use, in contrast to others, pain, suffering or distress is deliberately and systematically inflicted on sentient animals. The history of opposi-

tion to animal experimentation is as old as animal experiments themselves, but scientific approaches to reduce, refine or replace such experiments have only emerged 50 years ago. Particularly after intense societal debate, making animal experiments a truly political issue, and resulting legislation on national and international level in the 1980s, some success could be observed and particularly became evident in the decreasing numbers of animals recorded in the regular statistics on animal experiments. However, this progress has stopped around the year 2000, and the numbers have begun to rise again. What are the reasons for this development that seems to counteract all the efforts animal welfare organisations, scientists, and decision-makers have put into this issue? Apart from the dramatic numbers of animals that are used in gene technology related fields, one can be seen in the slow development of alternative methods in contrast to the fast growing number of applications of animal experiments, especially in the light of increasing interest in better protection of humans and the environment from unwanted effects of new substances and products. The EU chemical legislation (REACH), is a perfect example of this dilemma.

While science and its methods have enormously advanced in both aspects, technically and conceptually, it seems that many animal experiments still follow scientific principles that stem from the beginning of the last century. The mere fact that these methods have been used for decades for certain purposes, such as risk assessment, seems to be a sufficient justification for regulators and politicians to cling to these. The tradition of *in vivo* techniques seems to be a stronger driving factor than the exploration of new scientific pathways and concepts which could replace such use. This is also true for basic research. While an abundance of new scientific questions, new substances, new products, new technologies and new regulations has emerged over the time, existing animal experiments have persisted as a method, independent of whether they can be regarded appropriate to respond to these new challenges.

In addition to this it has become obvious that existing legal demands on animal experiments are not enforced to a satisfactory extent. The provision that an animal experiment must be indispensible and ethically justified, which is in the core of most legal requirements in animal research, is obviously not met in a significant proportion of animal use in science.

To overcome these problems, several measures are needed, such as:

- Legislation regulating animal experiments has to be revised in a way that allows for realisation of the fundamental ethical principles underlying that legislation.
- More efforts must be put into the development and application of alternative methods. This concerns resources, acceptance processes, and particularly coordination of the various activities on national and international level.
- On national and international level, a coherent strategy is needed to approach the issue. Among other things, testing requirements in different fields need to be harmonized – e.g. alternatives that have been validated for a particular use have to be made available for other types of uses.
- Research funding bodies should not give new incentives to animal use by asking for new animal "models" to investigate particular questions, and instead promote the use of non-animal methods.

The revision of the EU animal experimentation Directive 86/609 is a historical opportunity to respond to the societal concern of animal protection in research and testing and implement at least some of the above mentioned approaches.

2.28 What Animal Welfare Organisations in the U.S. Expect in the Future

Martin Stephens
The Humane Society of the United States

The Humane Society of the United States (HSUS) is the nation's largest animal protection organization, with 11 million supporters. Although we speak only for ourselves, we suspect that our expectations for the future are similar to those of other animal protection organizations in the United States and perhaps even in Europe. The HSUS would like to see all stakeholders (Three Rs centres, regulatory agencies, industry, academia, nongovernmental organizations, etc.) embrace "21st Century Toxicology" as exemplified in the 2007 National Research Council report on "Toxicity Testing in the United States." To bring this vision to fruition, we need international cooperation, strategic focus, and adequate funding. The HSUS is spearheading the Human Toxicology Project Consortium to help facilitate this process in the US. Our sister organization, Humane Society International, is working with European Union partners on a 7th Framework Programme project to coordinate existing large-scale EU alternative efforts and create a research and development roadmap to spur 21st Century Toxicology in the EU. At the same time, stakeholders should support more immediate Three Rs goals, including animal-friendly implementation of the Cosmetics Directive, REACH, 86-609 revision, reform of US chemicals legislation, and similar programs, as well as effective execution of individual alternatives programs.

Round Table: Views of International Experts on the 3Rs Principle in the 21st Century

2.29 FRAME – Fund for the Replacement of Animals in Medical Experiments

Michael Balls
FRAME, UK

FRAME was established in 1969, to promote the development, acceptance and use of new, advanced and valid methods that will *replace* the need for laboratory animals in medical and scientific research, education, and testing. Where the use of animals is currently necessary, FRAME supports the *reduction* of numbers involved to an unavoidable minimum and *refinement* of the experimental procedures to minimise any suffering caused.

FRAME believes that the current scale of animal experimentation is unacceptable, but recognises that the immediate abolition of all laboratory animal use is not possible. Essential medical research must continue, so that effective treatments for diseases that lessen the length and quality of human and animal life can be found. New products, including medicines and vaccines, and industrial and agricultural chemicals, must be adequately tested, in order to identify potential hazards to human and animal health, and to the environment.

FRAME's ultimate aim is the elimination of the need to use laboratory animals in any kind of medical or scientific procedures.

2.30 IIVS – The Institute for In Vitro Sciences, Inc. – A Non-Profit Organization Dedicated to the Advancement of Alternative Testing Methods

Dr. Rodger Curren, Institute for In Vitro Sciences, Inc.

The Institute for In Vitro Sciences was founded in 1997 as a non-profit, technology driven, organization dedicated to the advancement of alternative test methods. The Institute functions as an independent technical and educational resource which strives to coordinate various efforts taking place in the field nationally and harmonize them with international activities.

To meet these goals the Institute:
1. Provides high quality non-animal research and testing services
2. Sponsors workshops and training courses in in vitro methods
3. Actively participates in outreach programs that provide education, and scientific and technical counsel on emerging issues including alternative methods validation and implementation in the US and Europe.
4. Provides a forum where Industry, Government and Animal Welfare proponents can meet to determine constructive programs which effectively reduce animal use.

By doing the above IIVS can:
• Protect public health and safety through the use, refinement and development of in vitro tests
• Facilitate prompt reduction, refinement and replacement of animals used in testing through in vitro technology

Since its inception, IIVS has taken a global view of the non-animal testing field and has effectively co-operated with numerous international organizations. Collaborations with ZEBET have provided some of the most effective activities, and we expect that continuation of such joint programs will provide momentum to

the alternatives field for the foreseeable future.

2.31 U.S. EPA – U. S. Environmental Protection Agency
Meeting the Needs of a Paradigm Shift: A Regulatory Perspective

John R. Fowle
Health Effects Division, Office of Pesticide Programs, U.S. Environmental Protection Agency

EPA has identified the need to move to a new risk assessment paradigm to increase the speed with which toxicity assessments can be performed while reducing both the cost of testing, the use of animals, and also efficiently identifying and generating focused data for risk assessment. The challenge is to develop a scientific basis to determine what specific data, for which chemicals and which exposures, are essential for the appropriate assessment and management of risks in order to build an efficient and economical test regimen. This requires a combination of scientific/technology activities as well as political and program management activities to obtain regulatory acceptance. Activating a paradigm shift will require sustained effort over many years. Some efforts will require many years, such as research to enhance the interpretation of data, and others can be done in the near- and mid-term, such as guideline revision. To ensure regulatory acceptance of alternatives to current approaches we must clearly articulate their utility and develop public confidence that the approaches are protective of public and environmental health. Successful implementation will require partnerships between scientists and program managers across the international community to coordinate the scientific advances with the evolution of regulatory programs from animal intensive to alternative testing paradigms.

2.32 FICAM – The Finnish Centre for Alternative Methods
Non-animal alternative methods in the 21st century

Tuula Heinonen
FICAM, University of Tampere

The common goal in all research is to replace animal experiments with non-animal alternatives as soon as possible and preferably within the time frames set by EU-regulations, without jeopardising safety and efficacy in testing of chemical and biological substances concerning both human health and safety of environment. We regard the following points crucial to obtain alternatives soonest possible into the routine testing and basic and applied research:

1. Systematic and coordinated development of alternative methods.
In this context, maybe the most important role is played by Directive 86/609/EEC, which is now being updated. Obligation to the member countries to nominate a body of expertise (a centre or reference laboratory) that focus on development of alternative non-animal methods, validate those and give education and training would be an important instrument together with ECVAM to promote systematic and coordinated development.

Naturally, sufficient funding of large multinational projects is critical. The new funding in FP-7 will be very welcome.

2. Human cell and tissue based methods
The generally accepted principle is that a new method (alternative method) must be at least as relevant and reproducible as the existing methods if it is to replace them. In evaluation of the safety of chemical substances in man, human-based cell and tissue models will be needed to replace animal experiments.

3. Automation

It is expected that a large number of in vitro assays are needed to cover an animal test. Therefore, automation possibility is an important part that should be included into model/test development. Already now techniques enable automation of cell culture tests and even tissue models.

4. Validation and GLP
The test performed must give reliable and reproducible results. The test conditions must be traceable. Therefore, validation of test methods and performance in compliance with GLP are the crucial elements.

5. Knowledge and information sharing, education and training
Risk assessment is today mainly based on animal experiments. Thus knowledge and information sharing, education and training are essential in promoting alternatives. It is important that all key players i.e. students, researchers and decisions makers in industry and regulatory bodies are involved. In this the roles of centers that focus on alternatives, the network of the centers, university education and regularly organised national and international meetings are of utmost importance.

2.33 JaCVAM – Japanese Center for the Validation of Alternative Methods Japanese views on the 3Rs in the 21st century

Hajime Kojima
JaCVAM, NIHS, Japan

The Ministry of Health, Labour and Welfare (MHLW) created the Japanese Center for the Validation of Alternative Methods (JaCVAM) at the National Institute of Health Sciences (NIHS) in Japan in 2005. JaCVAM has promoted the 3Rs in animal experiments for the evaluation of chemical substance safety in Japan and established guidelines for new alternative experimental methods through international collaboration for 4 years. Many Japanese colleagues have supported JaCVAM activities by performing validation studies, peer reviews and regulatory acceptance for new alternative experimental methods. Furthermore, we plan to push forward with international harmonization efforts by both OECD activities and the International Cooperation on Alternative Test Methods (ICATM) framework, which was organized this April.

Japan Health Sciences Foundation established the Center for Accreditation of Laboratory Animal Care and Use in 2007. The center aims to promote the optimum enforcement of scientific animal testing. The center assesses and verifies institutes of animal experiments for their compliance with the “Basic Guidelines for Proper Conduct of Animal Testing and Related Activities in the Research Institutions under the Jurisdiction of the MHLW”. The other ministries control animal experiments in universities and other institutes.

We think developments and continued activities of these Japanese centers will contribute much to achieve the 3Rs. We also expect these Japanese activities to contribute to International harmonization in the 3Rs in the 21st century.

2.34 ECVAM – The European Centre for the Validation of Alternative Methods

Joachim Kreysa
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1. Introduction of ECVAM, incl. aims and goals (mission) and sponsor

- ECVAM was created in 1991 in order to support the implementation of the 3Rs in particular through validating alternative toxicological test methods.
- Since then it developed generally accepted principles for the efficient validation of alternative tests, such as pre-validation/validation and the modular approach.
- Validation is only useful if it is deemed trustworthy. Therefore the independent peer review of the validation, for which ESAC is responsible, is an essential element of the process.
- So far ECVAM validated more than 30 test methods, mostly in vitro, and produced 37 ESAC statements.
- Of these ECVAM-validated tests a number is already accepted for regulatory purposes as full replacements, other are in the accep tance process
- Since the 7th amendment to Cosmetics the full replacement of animal tests for the cosmetic endpoints is determining the agenda and progress is made – most topical toxicology endpoints can be tested without using animals.

- The more complex endpoints (in particular reproductive tox, Toxicokinetics, and repeated dose tox) are still far off and require new testing approaches, also because the available animal tests are neither perfect nor cost efficient nor fast.
- REACH is another driver that becomes more and more evident, clearly stating the intention to return to animal testing only as last resort while creating a strong demand for more and reliable data.
- Today ECVAM is a Policy Support Action of the IHCP/JRC, supported primarily by two of its scientific units, the in vitro methods unit and the system toxicology unit. This provides ECVAM with access to stable validation capacities and to much increased laboratory capacities.
- ECVAM is primarily funded by Community funds, mostly directly, e.g. for staff and infrastructure cost, and sometime indirectly, e.g. via co-funded R&D or other projects.

Today ECVAM's mission is:

- We support the EU policies in the field of Consumer protection, Environmental protection and Animal protection
  - by validating alternative methods for safety testing that implement the 3Rs and provide the same or a better basis for risk assessment and risk management as current methods,
  - and by promoting their development, their application in industry and their acceptance by regulators.

2. A short statement on the future of the 3 R's in the next decade, e.g. chronic systemic toxicity testing and toxicology in the 21rst century.

- Sorry, no crystal-ball!
- With growing/continuing economic crisis we might expect a decreasing general public attention for the issue, but
- With increasing allergies, resistances, etc. the public might get more concerned about safety of chemicals and related products, also because with increasing understanding of the complex physiological pathways in the human body and how chemicals might disturb them, science might ring more alarm bells
- Therefore I expect an increasing demand for better, faster, reliable, and affordable safety testing, also stimulated by REACH, generating a need to screen very fast many chemicals with a high likelihood to catch potential risks.
- If alternatives can successfully respond to this demand, and I believe this is not excluded, the future for the 3Rs will be good, but has to be driven by scientific and technical progress.
- The challenge is to integrate modern science into toxicology and into toxicology testing.
- We will be able to measure more and better at the cell and sub-cellular level – the challenge will be to understand the information. Integrating measurement technology (including those based on biological in vitro test systems) with computational toxicology, in particular modelling will become critical.
- For ECVAM the challenge will be to validate complex testing systems, consisting of building blocks that influence each other (or not). We have to convincingly establish that the generated data are reliable and relevant.

3. Assuming that national 3R centres will be created in the EU Member States. Will ECVAM support a network of European centres on alternatives?

- YES, ECVAM would support these centres by running and managing an effective network.
- For example,
- incoming test methods could be tried out, as part of its potential-assessment, by the centre that has the capacity and expertise. Based on this and other information the validation priorities will be fixed.
- Validation studies could be carried out in the best suited and available laboratories.
- Dissemination activities could be coordinated,…
- As to budgetary support - this depends what the legislator decides. Experience warns to put the expectations too high.

2.35 NICEATM and ICCVAM - NTP Interagency Center for the Evaluation of Alternative Toxicological Methods and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
Advancing Public Health and Animal Welfare in the 21st Century with Sound Science and Collaborations

William S. Stokes
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The U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) work collaboratively to promote the validation and regulatory acceptance of new, revised, and alternative test methods that are based on sound science and that will provide continued or improved protection of people, animals, and the environment while reducing, refining, and replacing the use of animals where scientifically feasible. ICCVAM was organized in 1997 and U.S. law established it as a permanent interagency committee in 2000. ICCVAM consists of representatives from the15 Federal regulatory and research agencies that generate, use, require, or provide toxicological testing data to safeguard the health of people, animals, and the environment. NICEATM administers ICCVAM and provides scientific and technical support, including the conduct of high priority validation studies. Since its establishment, ICCVAM has contributed to the evaluation of 27 alternative test methods that have now been accepted or endorsed by national and international authorities. NICEATM and ICCVAM developed a Five-Year Plan in 2008 in conjunction with member agencies to advance alternative methods. The plan identifies priority areas for research, development, translation, and validation activities necessary to support the regulatory acceptance of alternative methods, and increased collaborations with stakeholders. The plan emphasizes the application of advances in science and technology to the development of new more predictive alternative test methods. In 2009, the U.S., Japan, Europe, and Canada signed a Memorandum of Cooperation on Alternative Test Methods (ICATM). The participating national validation organizations will work to strengthen and expand cooperation, collaboration, and communications on the scientific validation and evaluation of new alternative methods in order to achieve more rapid international adoption of alternative methods. Implementation of the NICEATM-ICCVAM five-year plan and expanded international collaborations are expected to result in significant progress on alternative methods that will support improved safety assessments while further reducing, refining, and replacing animal use.

3 Abstracts of Poster

P1: In Vitro Evaluation of 3D Skin Models for congenital Ichthyosis

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For the testing of skin absorption, the OECD approved guideline 428 for in vitro testing legitimating the replacement of animal experiments by alternative tests. A German research consortium has previously validated an in vitro test protocol using reconstructed human epidermis to quantify skin absorption. In vitro models for skin diseases, however, are in their infancies only.

Thus, the aim of the present study was to appraise a recently established 3D model for congenital ichthyosis, a hereditary severe epidermal barrier function defect. Cultivating normal keratinocytes at the air-medium interface, control models and models mimicking congenital ichthyosis through RNAi-mediated gene knock down were generated. Moreover, keratinocytes from skin biopsies obtained from two ichthyosis patients were used to build up the disease models. The tissues were evaluated for permeation of OECD standard substances. Major and disease typical differences in permeability and barrier function were observed, as the permeability coefficients were markedly increased in patient and gene knock down models when compared to control samples. Moreover, the models were evaluated for the penetration enhancement by innovative nanocarrier systems, solid lipid nanoparticles and dendritic core-multishell nanotransporters. Loaded with nile red as a model dye, both nanoparticles demonstrated strongly enhanced penetration into control models and healthy human skin compared to conventional cream. Penetration was even more pronounced in models created from patient samples thus once more reflecting the defective epidermal barrier. In conclusion, standardized procedures were successfully applied to characterize in vitro disease skin models rendering future investigations on potential new drug targets possible.

P2: Quantification of Esterase Activity in Human Keratinocytes and Fibroblasts

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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. 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 confluency. 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 determination of the enzyme kinetics. Initial reaction rates were used for the calculation of maximum transforma-

tion velocity (V_{max}) and Michaelis-Menten constant (K_m). As expected, a higher esterase activity was observed in keratinocytes which are superior in the cleavage of e.g. glucocorticoid esters. In conclusion, the assay for FDA hydrolysis provides an accurate, reliable, and reproducible *in vitro* test to determine esterase activity in human keratinocytes and fibroblasts. Future investigations will address the esterase activity in human skin *ex vivo* and commercially available 3D skin models.

P3: 3R Projects funded by ZEBET: Angiogenesis in vitro: All-in-one-assay

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Angiogenesis, the growth of endothelial sprouts from pre-existing blood vessels, is required for tumour growth. Until now most of the studies on antiangiogenic substances are done in particularly painful animal experiments (e.g. cornea angiogenesis assay) since existing in vitro models of angiogenesis prove a disappointing choice.

In order to replace these animal experiments the focus of our projects is to establish a realistic in vitro model of angiogenesis which allows investigation and quantitation of all steps of the angiogenic cascade in only one assay.

An in vitro system based on bovine endothelial cells was developed and validated providing the opportunity of both, stimulation and inhibition of angiogenesis within each step. However, adaptation to human endothelial cultures showed that not all of them could be stimulated to angiogenesis comparably. Therefore, the current project deals with comparison of the protein expression profile of different angiogenic and non-angiogenic endothelial cultures.

P4: Mucilair™: An In Vitro 3D Human Cell Model for Repeated Dose Inhalation Toxicity Assessment

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Most of the in vitro cell models for long term testing of chemicals suffer of at least two shortcomings: 1.The failure of reproducing the in vivo physiological characteristics of the corresponding tissues, such is the case for the immortalized cell lines. 2. A limited shelf-life, for example, the freshly established primary cell cultures. Our company, Epithelix, has developed and is commercializing a novel in vitro cell model of the human airway epithelium (MucilAir™) made of primary cells which is free of these limitations.

MucilAir™ is morphologically and functionally differentiated and it can also 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 W.cm2 ± 5 (N=1470)). 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. Remarkably, the epithelium has a strong capacity of regeneration after mechanical or chemical injuries. Epithelia from several different pathologies can be reconstructed (e.g. Asthma, COPD, CF, smoker, etc.).

Due to its unique long shelf-life of one year, this model is used for studying the human respiratory diseases, and for testing the long-term/repeated dose effects of drugs/chemicals on the respiratory tract. Several applications of MucilAir™ relevant to inhalation toxicity assessment will be presented:

_ Acute, Long-Term and Repeated Dose Toxicity testing
_ Inflammatory effect assessment
_ Wound healing experiments

P5: Evaluation of Glucocorticoid-induced Skin Atrophy on Full-thickness Skin Models

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Glucocorticoids (GCs) are the most widely used class of anti-inflammatory drugs, but their therapeutic use is limited by serious side effects, with skin atrophy being the most prominent limitation. Thus, determining the atrophogenic potential of novel compounds is of great importance for drug development. Currently, there are no predictive *in vitro* models available. The aim of the present study was to establish an *in vitro* model for the evaluation of GC-induced skin atrophy by quantification of collagen synthesis and degradation upon topical treatment. In initial experiments primary human fibroblasts and keratinocytes were pretreated with TNF-ÿ to induce an inflammatory response followed by incubation with GCs for 72 h. Prednicarbate (PC) and clobetasol propionate (CP) were selected as a mid- and high-potency GC, respectively. Quantification of gene expression by real-time RT-PCR showed a strong downregulation of COL1A1 and COL3A1 mRNA expression by CP in fibroblasts, whereas PC only minor affected COL3A1. No effect was observed in keratinocytes. Analysis of IL-6 release by ELISA confirmed the anti-inflammatory effects. After the successful establishment of monitoring collagen expression in monolayer cultures, GCs were applied topically to full-thickness skin models (EpidermFT, Mattek™) for 7 days. Under inflammatory conditions, CP again downregulated collagen expression, whereas PC even slightly increased collagen expression. Accordingly, histological analysis revealed a reduction of the epidermal thickness in the CP-treated samples, thus correctly reflecting the atrophogenic potential observed *in vivo.* In summary, the results indicate that skin models appear to be a promising alternative method to determine and predict the atrophogenicity of GCs.

P6: Biological Effects in Human Lung Cells exposed to Platinum Nanoparticle Aerosol

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Platinum nanoparticles (Pt NPs) are under intense study because of their unique catalytic properties, however, Pt and other platinum group elements are released by cars at an increasing rate. Pt concentrations of 135-303 ng/g have been determined in street dust along highways in Germany (Djingova et al., 2003). Potential health effects of inhaled Pt NPs are rarely studied so far. In order to evaluate the potential risk for human health *in vitro* cell-based assays have been performed under submerged conditions and at the air-liquid interface modelling the situation during inhalation.

P7: Prevalidation of a Hemi-corneal model for Corneal Safety and Permeability Testing

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Made possible by financial support granted by the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET), we have developed a hemi-cornea model to provide a tool for corneal safety- and transcorneal drug permeability testing, in order to replace animal experimentation. The hemi-corneal model is exclusively based on human corneal cell lines cultured under serum-free conditions, which have been optimized for the maintenance of functional and structural characteristics of the tissue.

The use of this hemi-cornea for corneal safety testing involves topical application of test materials to the surface of the epithelium and the subsequent assessment of their effects on cell viability using the MTT assay. For determination of the transcorneal drug permeation the permeation coefficients of aqueous solutions of test compounds are calculated.

Recently, the hemi-cornea project entered the prevalidation phase to prove collaboratively with industry partners the applicability of hemi-cornea models in eye irritation testing and transcorneal drug permeation studies.

This includes:
• inter-laboratory method transfer for the hemi-cornea construction,
• assessment of the reproducibility of the hemi-cornea construction (intra- and inter laboratory variability) in all participating laboratories,
• definition of a preliminary prediction model for eye irritation and transcorneal permeation,
• transfer for the test protocols.
Keywords: hemi-cornea, corneal irritation, transcorneal permeation, prevalidation

P8: Immunological Substance Testing on Human Lymphatic Microorganoids in vitro

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Pharmaceutical drugs and compounds used for consumer products may bear the risk of unexpected immunotoxicological effects such as sensitization, allergy, anaphylaxis or immunogenicity. Modern biopharmaceuticals of a high potency and target specificity like antibodies and cytokines need to be tested for their immune functionality before first-in-man application, with respect to therapeutical dose and exposition regiment.

Existing *in-vitro* tests and commonly used animal models do not reflect the complexity and specificity of the human immune system. However, novel humanized animal models have limitations in systemic reactions. Monolayer or suspended cell culture don't have tissue functionality and organ physiology and could not be used for long-term culture. In contrast, solid tissue biopsies, e.g. tonsil preparations of tonsillitis patients typically show inflammatory artefacts and degrade in long term culture due to damages induced by the preparation procedure.

The construction of tissue-like structures *in vitro*, so called "organoids" can overcome these limitations. Key structures of secondary lymphatic organs, e.g. lymph nodes or spleen are the primary lymphatic follicles and germinal centres, in particular in the "activated-state" of inflammation or infection. For the remodelling of lymphatic follicles, functional and structural cells, e.g. lymphoid cells (PBMC) and stromal cells need to be combined with biogenic or artificial matrices and scaffolds in a suitable 3D-environment. Tissue formation can be induced under controlled conditions.

A human lymph node model should be designed for the induction and monitoring of both, cellula r and humoral immune responses under operation of several weeks, long-term drug exposition and repeated doses. Cellular immunity is monitored e.g. by cytokine release patterns, and humoral immunity e.g. by analysis of B cell activation, plasma cell formation and antibody secretion profiles (IgM, IgG). Cellular composition and micro-organoid formation is analysed by flowcytometry, histology and *in-situ* imaging.

P9: Metabolic Capacities of in vitro Alternatives for Chemical Testing in Skin: Insights from the COLIPA Skin Metabolism Project

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This work was funded by The European Cosmetics Association (COLIPA).

Human skin represents a large contact site for all kinds of potentially harmful substances. The relevance of testing chemicals for skin irritation, sensitization and genotoxicity in cosmetics is unquestioned, but using animals for that purpose will be prohibited in the EU from 2009 on by the 7th Amendment to the EU Cosmetics Directive. Skin models as alternative testing methods exist, but little is known about the differences in their xenobiotic metabolism capacities compared to human skin.

Therefore, the aim of this study is to characterize enzymatic activity of human skin compared to the following skin cell-derived *in vitro* models: keratinocyte-based cell lines, primary keratinocytes and a three-dimensional epidermal model. Skin was processed to yield cytosolic and microsomal extracts, while the epidermal model was examined in intact form as well as in cytosolic and microsomal preparations.

Phase I detoxification enzymes assayed in our project include Cytochrome P450 (CYP) and Cyclooxygenases, while phase II activity tests are carried out for GST, NAT and UGT. Obtained results of undetectable basal CYP activity were consistent in skin and all models. Production of PGE2 metabolites was measurable in cell lines and epidermal model and will be checked for skin extracts. Preliminary results of phase II detoxification enzymes indicate a good correlation of human skin and skin models in the detoxification of chemicals.

Our findings will help evaluating the potential of these *in vitro* models to serve as alternative toxicological screening methods for human skin.

P10: In silico Modelling of Skin Absorption – a further Step towards Realizing 3R

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To assess potential risks for human health and environment, animal experiments have been a valuable resource. With the REACH legislation, the number of animal experiments may be expected to rise even further.

We present an alternative in silico method, which allows for assessing skin absorption of arbitrary substances and which may help in reducing the number of animal experiments. For this purpose, we have successfully developed a computational model that solves the diffusion equation for discrete points in space and time. Our model allows for computing steady-state permeability coefficients as well as drug concentration-depth profiles after arbitrary times of incubation. It may not only use arbitrary two-dimensional structures, but also more realistic three-dimensional geometries.

Although our work does not replace animal experiments, it may help to reduce the number of animal experiments considerably.

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P11: A new in vitro Model for Testing Drug Permeation of New Aerosol Formulations: the Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC)

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New aerosol medicines have to be tested for safety and efficacy by animal experiments. Based on the 3R principle of animal welfare there is a demand in in vitro test systems to determine the efficacy of these aerosols, but they are rarely developed. Due to the complexity of the processes which are involved in the inhalation process, animal models are used by default. Besides ethical concerns these models often lack of inter-species differences. Therefore in vitro test systems should be based on human cells to ensure species-specific results.

We developed a new **P**harmaceutical **A**erosol **D**eposition **D**evice **O**n **C**ell **C**ultures (PADDOCC) to mimic the inhalation of a single metered aerosol dose and its subsequent deposition on filter-grown pulmonary epithelial cell monolayers exposed to an air interface. In first experiments the reproducibility of deposition with commercially available dry powder inhalers could be demonstrated as well as subsequent transport studies across human bronchial epithelial monolayers.

PADDOCC appears as an attractive alternative to animal testing in developing aerosol medicines, allowing the investigation on drug permeation, but at least it will be possible to reduce the number of animals in such studies.

P12: Reference Laboratories Can Make Validation More Efficient

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The validation of *in vitro* methods is a lengthy process encompassing multiple phases. It progresses from initial test development, through test optimization and prevalidation, to a formal validation assessment, and eventually to regulatory acceptance. Each of these phases relies heavily on the outcome of laboratory activities - even the regulatory acceptance step involves careful inspection of the data to determine their applicability to the regulatory need under

consideration. The competence and experience of laboratories participating in each phase have a significant effect on the efficiency of the entire process. History has shown that the process is never as fast as we would like; however, it can be even slower if technical errors are made along the way. High-quality laboratory work is required to maximize the opportunity for success at each stage. This emphasizes the need for a group of experienced, competent laboratories (reference laboratories) capable of readily participating in any of the phases. Such laboratories should be able to conduct assays under GLP-compliant conditions, and should optimally be independent from the developers. Reference laboratories experienced in each of the phases are particularly valuable to the process since they will be able to help test developers at an early stage to design robust protocols that can withstand the rigors of validation and subsequent routine usage. They will also be able to support the successful implementation of assays to naive laboratories post-validation, and assist the regulatory agencies in training reviewers to correctly interpret data from newly approved in *vitro* assays.

P13: Comparison of Human Patch Test and 3D Human Skin Model Results with Classification of Chemicals based on Rabbit Draize Test

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Efforts to replace the *in vivo* rabbit Draize test for skin irritation have been underway for many years and various *in vitro* protocols have been assessed. However, one key difficulty in determining the validity of any particular protocol/prediction model is that the *in vivo* rabbit data is both scarce and often of limited utility for the prediction of the biological effect in man.

In the current study, using the 4h human skin irritation patch test, we examined 15 irritants and 10 non irritants. The outcome of human patch test was compared with results obtained with reconstructed epidermis model EpiDerm using two *in vitro* skin irritation protocols. Of the 15 tested chemicals reported to be irritating in the rabbit, only 5 substances were found to be significantly irritating in human skin to merit the R38 classification. Using the Epiderm test protocol evaluated in the ECVAM skin irritation validation study (15 min exposure), 7 out of 15 rabbit irritants were identified as R38 with one false negative prediction compared to human data. With the modified protocol (60 min exposure, 10 of 15 rabbit irritants were classified as R38, without the false negative outcome compared to 4h human patch test results.

Consequently, when approaching validation of alternative methods, existing human data should be taken into consideration, as only those may provide a final judgment about the predictive ability of a new alternative method. The in vitro models derived from human skin cells, if used in appropriate test designs and optimized by reference to human hazard data, may prove to be more useful than the animal tests for the prediction of human hazard from previously untested substances.

The study was supported by ZEBET at the BfR, Germany, and a grant project of the Ministry of Health of the Czech Republic (No. NS9648-4/2008). The commercially non-available samples from the ECVAM Skin Irritation Validation Study were provided by CORRELATE at JRC, Italy.

P14: Adenoviral Gene-transfer in Cultured Adult Cardiomyocytes as an Alternative Approach to Transgenic Animals

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The enzymatic isolation of cardiac myocytes is known for more than 30 years. Once isolated, the cells dedifferentiate and lose the cardiac specific morphology and gene expression pattern.

We developed a cardiac myocyte culture with minimised dedifferentiation by (i) cultering the cells in serum free medium supplemented with insulin, transferrin and selenite, (ii) plating them on elastic surfaces coated with a mixture of extracellular matrix proteins and (iii) applying a continuos electrical stimulation to keep the isolated cells contracting. Based on this treatment we can conserve rod shaped cells with a distinct cross striation in culture for one week. This provides a time slot to allow an adenoviral mediated gene expression, which typically takes place within 20 to 40 hours. We constructed adenoviruses for (i) a number of fluorescent fusion proteins to visualise subcellular structures in living cells, (ii) genetically encoded biosensors, like the calcium sensors YC3.6 or TN-XL and (iii) signalling molecules such as several isoforms of protein kinase C.

For optical investigations of the living cardiomyocytes we developed 24-well plates and the adjacent hard- and software to allow automated high resolution imaging while cells are electrically stimulated.

In summary we set the scene to go for optical high content screening of genetically modified primary adult cardiomyocytes without the necessity to breed transgenic animals. This allows high impact research and developments with a minimum of sacrificed animals and without animal experiments.

P15: ECVAM Feasibility Study: Can the Pre-validated EpiDerm In Vitro Phototoxicity Test be Upgraded to Quantify Phototoxic Potency of Topical Phototoxins?

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The determination of phototoxicity of a chemical in the 3T3 NRU-PT according to OECD Test Guideline 432 is often the first step in the sequential phototoxicity testing strategy. If the chemical provides a negative result in the 3T3NRU-PT, in most instances no further testing is required. However, if the result is positive, the chemical may be still applied topically at safe concentrations, depending on the absorption and accumulation of the chemical in the skin.

Thus, in addition to the information on inherent phototoxicity potential assessed by the 3T3NRU-PT, additional testing may be required to obtain combined information on the *photo-toxicity* and *bioavailability* of the chemical in the skin.

Ideally, confirmatory tests should be performed *in vivo* on human volunteers, but for ethical reasons, this is not acceptable if the 3T3NRU-PT has provided a positive result. Thus, to avoid confirmatory testing *in vivo* in animals, reconstituted human 3-D skin models are offer-

ing an attractive *in vitro* alternative for testing, since such models are characterized by both skin barrier function and viable primary skin cells.

In the current study, several substances (mostly cosmetic ingredients) which are known to be safely used in humans, and which provided positive results in the 3T3 NRU PT were evaluated on the reconstructed human skin model EpiDerm and if the result was negative, tested in a limited group of human volunteers. First results we obtained show that the human skin model phototoxicity test represents a useful step in the sequential strategy for phototoxicity testing.

P16: Primary Hepatocytes and hepatocyte-like cells in Research and as a possible Tool for Drug Development

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The knowledge of degradation of new pharmacological drug candidates in the human body is essential , because of their possible toxic metabolic products and the subsequent side effects. The liver is the main organ to metabolise these drugs by various enzyme systems. Theoretical models of catabolic decomposition are far from answering all questions needed. Up to now, most drugs in pharmaceutical research are tested by the use of animal models with very limited success. In order to replace these 'insufficient' models, human hepatocytes isolated from patient with primary tumours are a valuable alternative to animal study. However, the limited availability of human hepatocytes leads to the investigation for alternative cell types which possess metabolic capacity. Recently, we and others have developed with monocyte-derived hepatocyte-like cell, which contains a variety of metabolic systems found in primary liver cells, an alternative to primary human hepatocytes. Throughout differentiation, NeoHepatocytes showed a continuously increasing expression of drug-metabolizing enzymes (*e.g.;* CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, and 3A4), resulting in stable basal activity [Ehnert et al. 2008]. These results showed a possible application of NeoHepatocytes for the pharmaceutical industry. However, because of their relatively low phase I basal activity further studies are needed to reach the same basal phase I level like in primary human hepatocytes. An important step towards this aim was done when we modified our culture conditions.

Literature:
Ruhnke M, Nussler AK, Ungefroren H, Hengstler JG, Kremer B, Hoeckh W, Gottwald T, Heeckt P, Fandrich F. Human monocyte-derived neohepatocytes: a promising alternative to primary human hepatocytes for autologous cell therapy. Transplantation. 2005 May 15;79(9):1097-103.
Ehnert S, Nussler AK, Lehmann A, Dooley S. Blood monocyte-derived neohepatocytes as in vitro test sys tem for drug metabolism. Drug Metab Dispos. 2008 Sep;36(9):1922-9.

P17: Fundamental Experiments for Risk Assessment of Nanoparticulate Carrier Systems Using Validated In-vitro Test Procedures

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Nanoparticulate carrier systems are of increasing interest as drug delivery systems for topical application. Thereby, the assessment of the tolerability is of crucial importance. Solid lipid nanoparticles (SLN) and new-typed dendritic core-multishell nanotransporters (CMS NT) can increase the skin penetration of various substances significantly and have the potential to reduce side effects. Nevertheless, the local tolerability of the promising carrier candidates has to be assessed.

Thus, to test the dermal safety of SLN and CMS nanotransporters the EPISKIN® skin irritation test was performed. Prior to the main test, the MTT reduction potential of SLN and CMS nanotransporters was tested to avoid a misinterpretation of the data. The results predict no irritant potential according EU classification R38. Interestingly, the acute irritant potencial of the positive control sodium dodecylsulphate was reduced when loaded onto CMS nanotransporters.

As the nanocarrier systems can accidentally or intended come into contact with the eyes the eye irritation potential of both nanosystems was tested, too, using the HET-CAM test. The evaluation was carried out concerning the endpoints haemorrhage, coagulation and vessel lysis. SLN as well as CMS showed no eye irritating potential.

In conclusion SLN and dendritic core-multishell carriers are promising systems to increase effectiveness and tolerability of the local treatment of skin diseases. Furthermore, the *in-vitro* approaches Episkin® skin irritation test and the HET-CAM test are suitable test procedures for the risk assessment of nanoparticulate carrier systems according the principle of the 3Rs. With regard to the application as drug delivery system or cosmetic product the exposure time should be adapted and, thus, prolonged according the regular use in humans.

P18: International Validation of In Vitro Test Protocol (EpiDerm- SIT) to Replace the In Vivo Rabbit Test for Hazard Identification of Chemicals

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In April 2007, ECVAM endorsed 2 alternative test methods (EPISKIN and EpiDerm Skin Irritation Tests (SIT)) as replacements of the in vivo rabbit skin irritation test. While EPISKIN was recognized as a stand alone method, EpiDerm SIT was endorsed for use in a tiered testing strategy (OECD TG 404), where irritating results are accepted and non-irritating results may require further testing by another method.

Based on published data and analysis of results of the ECVAM validation study, there was evidence that differences in the barrier properties between the 2 models were responsible for the lower sensitivity of EpiDerm SIT when using an identical protocol as used for EPISKIN. Therefore, modifications of the exposure conditions were introduced to the EpiDerm SIT protocol: a)exposure time was increased from 15 min to 60 min; b)the temperature during the exposure was increased to 37 °C. With these modifications, significant increase in sensitivity was obtained, while maintaining an acceptable specificity of the method.

In autumn 2007, an international validation study was performed to evaluate reproducibility and confirm the predictive ability of the modified EpiDerm SIT method. Results of the study are presented here. Overall, sensitivity and specificity of 80 % were obtained, which is comparable to results for the EPISKIN SIT for the same set of chemicals (sensitivity of 70 %, specificity 80 %). The inter-laboratory reproducibility of the modified EpiDerm SIT and its concordance with the in vivo rabbit data was also very good. The method was endorsed by ECVAM in November 2008 as full replacement method and has gained regulatory acceptance in the EU as one of three methods described as B.46 of Annex III of the REACH regulation.

P19: Effects of Collection, Transportation and BCOP Methodology on bovine corneal Histology Evaluation

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The bovine corneal opacity and permeability (BCOP) assay (Gautheron, 1992 & Sina, 1995), is used as an *in vitro* eye irritation screen for industrial hygiene, product development, and safety testing by measuring changes in corneal opacity, and permeability to fluorescein after chemical exposure. Histopathology has been used in BCOP studies to detect potential corneal injury, where the mode of chemical action might not induce opacity and permeability changes (Curren and Evans 2000). Artifactual changes in the cornea associated with the collection, transportation, or BCOP methodology of the enucleated eyes have not been evaluated; therefore, corneas were excised and fixed in 10% buffered formalin at various steps in the assay process, paraffin embedded, H&E stained and evaluated using light microscopy. Stromal thickness and Descemet's Membrane (DM) thickness was measured along the entire length of the cornea. The epithelium, endothelium, and stroma were similar histologically among all groups. The normalized stromal thickness of the whole globe corneas (903.8 µm ± 122.9 µm), immediately after enucleation (876.7 µm ± 84.2 µm), after the refrigerated transport (829.8 µm ± 63.4 µm), and at the end of the BCOP assay (721.2 µm ± 17.2 µm) suggest corneas undergo minimal artifactual changes as a result of refrigerated transport and the BCOP assay procedures.

P20: PEEK-WC-PU Membranes for Expansion of Rat Embryonic Liver Cells

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Biomaterials play an important role in directing tissue growth and may provide another tool to manipulate and control stem cell behaviour, having a significant impact on the fields of regenerative medicine and tissue engineering. Herein, we designed and developed new bioactive membranes to be used for the expansion of rat embryonic liver cells.

New modified polyetheretherketone PEEK-WC membranes were prepared in hollow fibre configurations, by phase inversion technique. Their surface was modified by means of different plasma processes, introducing amino group. The performance of the developed biomaterials was evaluated by analysis of the expression of the liver specific functions of cells cultured in the 6-well bioreactor. Liver progenitors on the membranes exhibited higher functional activities compared to those cultured on conventional plates as demonstrated by higher albumin and urea production. They showed gene expression of AFP and albumin in a time-dependent manner of the hepatic differentiation process. LDH assay revealed that a high number of viable liver stem cells attached to the membranes. Unexpectedly, liver progenitors cultured on membrane bioreactors had higher telomerase activity than ones in the plates. Further, FACS analyses showed that cells grown on membranes had longer G1 phase while S phase was shortened. Thus, membrane bioreactors are able to sustain the same *in vivo* liver functions *in vitro* and to allow the expansion of stem cells.

P21: Fluorescence Based Spatial Biomarker Profiling of HSP27 for Quantification and Classification of Mild Skin Irritation

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Background: Mild skin irritation is characterized by subtle protein expression changes despite unchanged histological appearance of the skin. At the example of HSP27 we studied in how far image processing of histological sections could be used for an automatic classification of mildly SDS treated organotypic skin cultures.

Methods: 48 Mattek EFT-400™ full thickness tissue cultures were treated with 0.4 % SDS or PBS or none at time points 1h, 6h, 16h, 24h. Histological sections were stained for heat shock protein HSP27 with Laminin-5 as a spatial reference. Multicolor-fluorescence slides were scanned using the full slide scanner Hamamatsu Nanozoomer HT®. We developed image processing software for automatically segmenting, profiling and classifying histological sections of the tissue cultures

Results: Application of 0.4% SDS leads to an altered premature and plateau-like HSP27 expression profile depending on treatment duration . At medium tissue differentiation (40-70 %), cultures treated 24h revealed a 2.6 fold HSP27 expression compared to none (1.7 to PBS). Different measures for quantifying the expression changes and thus quantification of

mild skin irritation were developed. A first potential classifier rated as positive: 0/16 none treated, 0/4 PBS 1h, 0/4 PBS 6h, 1/4 PBS 16h, 2/4 PBS 24h, 1/4 of 1h SDS, 3/4 of 6h SDS and 4/4 of 16h SDS and 4/4 of 24h SDS.

Conclusion: We have shown a first example for quantifying mild skin irritation on the basis of changes in spatial expression patterns in histological sections using the Mattek EFT-400™ skin model.

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P22: Critical Factors Impacting Interlaboratory Transferrability of the Mouse Embryonic Stem Cell Test

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The mouse Embryonic Stem Cell Test (EST) assesses a compound's ability to inhibit differentiation of embryonic stem cells into myocardiocytes in parallel with cytoxocity endpoints in adult and embryonic stem cells. Though intralaboratory transferability was found to be acceptable among European labs during the validation process, we had difficulty consistently running the EST in the United States. The problems encountered most often were: 1) a low number of contracting myocardiocytes in the control cultures; and 2) attachment and loss of embryoid bodies that require suspension culture. We initiated a program to identify critical factors impacting the outcome of the assay, and have identified and resolved three technical issues. First, the plasticware specified in the ECVAM protocol has a different catalog number when ordering from the United States. Switching plasticware eliminated attachment of the embryoid bodies. Next, using FCS at a concentration of 15% rather than at 20% improved the reliability of differentiation. Finally, using different lot numbers of serum, even from the same supplier, significantly impacted the consistency of the differentiation assay, indicating that prequalifying lots of serum is a necessary step to successfully running the EST. We have developed a serum qualification procedure and have significantly improved the performance of the assay in our hands . We recommend establishing a public forum for researchers who are working with the EST to facilitate communicating serum lot qualification efforts, and to address additional technical difficulties laboratories may encounter while setting up the EST.

P23: Human Neurospheres Can Identify Neurotoxicants In vitro

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

We have established a 3D test system for DNT screening based on primary human fetal neural progenitor cells which is now embedded in the BMBF joint project "Development of predictive in vitro test for developmental neurotoxicity testing". Within this project, different cell models are compared with regard to their DNT predictability employing a battery of test compounds.

In our system first results indicate that the well known developmental neurotoxicant methylmercury effects proliferation, migration and differentiation of neurospheres in a nanomolar range, while a negative test substance, the liver toxicant paracetamol, showed interference with these processes in millimolar concentrations. Furthermore, the DNT compounds MAM, valproic acid and lead also affect these endpoints, while glutamate, which is not developmentally neurotoxic, is well distinguishable. At the shorter timepoints, specific effects on those DNT endpoints are observed at concentrations which do not cause cytotoxicity.

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

P24: HEPAC2: Serum-free, Standardized and Validated (Re-usable) Primary Human Hepatocytes for the Analysis of Xenobiotics

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Hepatocytes are used as biosensors for pharmacological-toxicological assays to monitor the metabolism and the influence of xenobiotics on hepatocellular functions. We established HEPAC2, a long-term culture system in which hepatocytes are plated and cultured under serum-free conditions for up to 3-4 weeks. The hepatocytes retain their specific functions determined by the quantification of urea and albumin release and stay vital.

Due to the maintenance of functionality, the same hepatocytes can be used more than once for assaying the effects of xenobiotics on hepatocellular functions, viability and metabolism studies, since the readout is done non-invasively and the hepatocytes remain intact.

We used Acetaminophen to demonstrate the feasibility of our culture system. The drug-mediated effects did not even change between the first cycle of application on day 4 and the fourth cycle started on day 20 (Ullrich et al. 2007).
In addition, this model was also used successfully to screen the toxic potential of the anticancer drug cis-4-hydroxy-proline. The safety of the drug was documented and in line with the clinical data from a human study, while the data generated in animals were not consistent with the clinical data and let to false conclusions (Dickens et al. 2008).

The robustness of HEPAC2 is also shown by repetitive inductions of CYP450 activities. Prototypical inducers increased the enzyme activities to identical levels no matter if induction is started on day 3, day 7 or day 11 of culture.

However, at present, there is no regulatory acceptance for this model. The method has to be tested with further model substances and in external laboratories in order to become an accepted method.

P25: A Crossed Hollow Fiber Membrane Bioreactor for Liver Tissue Engineering as a Tool for Drug Testing and Toxicology

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Tissue or organ physiological model can be a reproducible and economical tool for the in vitro study alternatively to animal models and for the human enzyme expression prediction. A crossed hollow fiber membrane bioreactor was developed to support the long-term maintenance and differentiation of human hepatocytes. The bioreactor consists of two types of hollow fiber (HF) membranes with different molecular weight (MW) cut-off and physico-chemical properties cross assembled in alternating manner: modified polyetheretherketone (PEEK-WC) and polyethersulfone (PES), used for the medium inflow and outflow, respectively. The combination of these two fiber set produces an extracapillary network for the adhesion of cells and a high mass exchange through the cross flow of culture medium.

Primary human hepatocytes were cultured in the extraluminal compartment among the PEEK-WC HFs, devoted to provide the cells oxygenated medium containing nutrients and metabolites, and the PES HFs devoted to remove from cell compartment catabolites and cell specific products. In this way the two HF membrane systems mimic the in vivo arterious and venous blood vessels. In the bioreactor human hepatocyte maintened their differentiated specific functions at high levels up to 18 days of culture and re-established their polygonal morphological behaviour with an high level of cell adhesion on the fibers surrounded by an extracellular matrix-like structure. The individual cytochrome P450 isoenzymes involved in the diazepam metabolism were expressed at high levels and the same metabolites found in humans were produced in the bioreactor.

The good performance of the bioreactor demonstrated its workability as device with a controlled environment that may provide an inexpensive and reliable in vitro physiological model for in vitro study on engineered human liver tissue constructs.

P26: Factors Influencing the Yield of (Transgenic) Mouse Embryos

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Generation and cryopreservation of transgenic mice are depending on a reliable and continuous production of pre-implantation embryos. However, the outcome of embryos following superovulation and mating varies and is a limiting factor. To suppress circannual and circadian rhythms, laboratory animals are housed under standardized conditions. It remains to be elucidated if the artificial climate can cover all environmental effects. Using the data received together with the cryopreservation of 140.000 mouse embryos, the role of embryo yield limiting and raising factors was analyzed: The humidity in an animal facility is affecting the embryo production. The weather at the location of the facility, especially the temperature, influences the climate within a facility. Weather peaks are not fully covered, even if the air-conditioning supply is powerful. Subsequently, external weather changes interact with the environment within the facility affecting the outcome of mating and resulting in circannual oscillation.

A virus infection was paralleled with dropped yields and environmental noise and/or vibrations as generated by construction works, negatively affect the embryo yield. The mating frequency of males individually interferes with the outcome.

To increase embryo yields pheromonal effects were investigated: The housing of female donors in small groups (Lee-Boot effect) and the exposition to the future males' bedding before mating (Whitten effect) resulted in remarkably higher embryo yields.

Schwab A, Schenkel J (2008) Scand J Lab Anim Sci. 35(4), 283-296
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P27: Differentiated Human Neuroprogenitor Cells for Neurotoxicity Testing

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The current protocol for neurotoxicity testing is based on animal experiments. Furthermore, mechanistic insights of neurotoxicants are assessed mainly *in vitro* employing tumor cell lines. Because there are species differences between rodents and man and cell biological differences between tumor and normal cells, this study aims to investigate if neurotoxicity can also be studied in primary human cells which have differentiated from human neural progenitor cells (hNPCs). hNPCs form the three neural celltypes of the brain: neurons, astrocytes and oligodendrocytes and are thus a promising model to assess toxicity in this co-culture system.

Differentiated hNPCs express marker genes for gabaergic, cholinergic, dopaminergic and serotoninergic neurons as well as NMDA receptor subtypes. Moreover, they react to glutamate and acetylcholine with an intercellular Ca2+ increase. First results with model compounds show that treatment of differentiated cells with subcytotoxic methylmercury concentrations reduces beta(III)tubulin expression in Western blot analyses. Furthermore, MPP+ (1-Methyl-4-phenyl-pyridin) which is selectively toxic to dopaminergic neurons reduces protein levels of dopamin decarboxylase.

More compounds are needed to evaluate if these cells are capable of predicting neurotoxicity and thus serve as an alternative to animal models in these human-derived cells

P28: Prevalidation study for testing the toxic effects of inhalable substances (gases) on human lung cells using an air/liquid culture technique

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The increasing demand for assessing inhalation toxicity hazards calls for new testing strategies comprising both in vitro and in vivo assays. For this purpose, we are currently evaluating a direct exposure strategy, in which cells are exposed to toxic gases at the air/liquid interface. The human carcinoma alveolar epithelial cell line A549, grown on microporous membranes, is exposed to test atmospheres in a system enabling at the same time steady state nutrification, humidification and direct gas exposure. Under coordination of the Fraunhofer Institute, we are assessing the intra- and interlaboratory reproducibility and pre¬dictive capacity of the method by characterizing the toxicity of four gases, i.e. NO2, SO2, formaldehyde, and ozone. The aims of this study are: optimisation and refinement of experimental protocols; generation of standard operating procedures; assess

ment of reproducibility within and between laboratories; establishment of test acceptance criteria; determination of the in vitro vs. in vivo dose-response relationships. After transfer of the method, optimization of protocols and experimental procedures the four partners started definite testing of the gases. Each gas, together with an online analytical monitoring system, is passed from one lab to the next after six weeks of experimentation. The test design comprised one hour gas exposure followed by direct determination of cytotoxicity (electrical current exclusion method, CASY®, Innovatis) and genotoxicity (COMET assay). So far, the project has proven satisfying transferability of the test system, depending on the laboratory being practiced in this complex methodology.

P29: Assessment of the Sensitising Potential of Textile Disperse Dyes and some of their Metabolites by Loose-fit Coculture-based Sensitisation Assay (LCSA)

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Introduction: Certain textile disperse dyes are known to cause allergic reactions of the human skin, such as allergic contact dermatitis and contact urticaria. However, by now only few quantitative data on the sensitising potential of these dyes exist. We have tested 3 disperse azo dyes (Disperse blue 124 - DB124, disperse red 1 – DR1, Disperse yellow 3 – DY3), 3 products of azo-cleavage of these dyes (ANT - 2-Amino-5-nitrothiazole (DB124), AAA - p-Amino-acetanilide and ApC - 2-Amino-p-Cresol (both DY3)) and one disperse antraquinone dye (Disperse blue 1 – DB1) to achieve data on their sensitising and irritative potential.

Therefore we used a loose-fit coculture-based sensitisation assay (LCSA) of primary human keratinocytes and of allogenic DC related cells to emulate the *in vivo* situation of the human skin. Sensitisation was determined by analysing the expression of the DC maturation marker CD86 by flow cytometry. Estimation of the concentration required to cause a half-maximal increase in CD86-expression allowed quantitative risk assessment. Furthermore we used 7-AAD (7-amino-actinomycin D)-staining to achieve data on cell viability and thus the irritative

potential of the tested substances. The dyes were categorised as weak or strong irritating substances by estimation of the concentration required to devitalize 50 % of the examined cells compared to a zero control.

Results: DB1, ANT and AAA were tested up to concentrations of 100, 200 and 300 ÿmol/l , respectively, and showed no sensitising potential. All other substances were categorised as extreme sensitisers. DB124 showed the strongest sensitising potential, followed by DY3, DR1 and ApC. The irritative potential correlated with the sensitising potential. We observed most pronounced cytotoxic effects for DB124. DY3, DR1 and ApC also turned out to be highly cytotoxic substances, whereas ANT and DB1 showed only weak irritative potential. AAA did not show any cytotoxic effect at the concentration range tested.

Conclusion: The LSCA proved to give adequate results for the sensitising potential assessment of coloured substances. In addition we were also able to achieve data on the irritative potential in the same series of tests. Hence the LCSA provides a stable test system to simultaneously analyse two crucial properties of substances relevant for allergy induction.

P30: Murine Embryonic Stem Cells as a Model for *in vitro* Developmental Neurotoxicity

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Mouse embryonic stem (ES) cells are derived from the inner cell mass of growing blastocysts. These cells are considered pluripotent and capable of differentiating into a variety of endodermal, mesodermal and ectodermal cell types including neurons. This unique feature makes ES cells a favorable tool for studying developmental processes and the pathways affected upon exposure to toxic compounds.

In the present study, we have developed a new and efficient protocol for differentiating murine ES cells into neural cells. This method is based on the formation of neuronal spheres and takes only about 12 to 14 days to produce mature neurons. The differentiation process has been well characterized my means of immunofluorescence staining of selected marker proteins specific for neuronal precursor cells as well as mature neurons and glial cells. In comparison to previous protocols our method significantly shortens the differentiation time needed for the development of mature neurons from ES cells. At the same time, the number of maturing cells is sufficiently high to be applicable in developmental neurotoxicity testing. To assess adverse effects on neural cell differentiation and proliferation predictive toxicological endpoints have been established using flow cytometry of neuron-specific as well as glia-specific marker proteins and proliferation assays. Preliminary chemical testing revealed differences in the sensitivity of stem cells, 3T3 fibroblasts, and stem cells differentiating into neurons when compared to each other. Furthermore, we could show that the mouse embryonic stem cell model provides trustful and reliable results in the detection of developmental neurotoxicants.

P31: Green Fluorescent Protein Monitoring of P-glycoprotein Mediated Chemoresistance and its Regulation by Glycolysis in Multicellular Tumour Spheroids

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ABC transporters like P-glycoprotein (P-gp/ABCB1) are membrane proteins responsible for the transport of toxic compounds out of non-malignant cells and tumour tissue. Most pharmacokinetic studies on P-gp function are currently performed in laboratory animals.

Aims:
1) to establish a green fluorescent protein (EGFP) reporter gene based in vitro model to study expression and function of P-gp in living three-dimensional tumour tissues.

2) to investigate the effect of glycolysis and the tissue redox state on P-gp expression in multicellular tumour spheroids derived from prostate adenocarcinoma cells (DU-145), glioma cells (Gli36), and the human cervix carcinoma cell line KB-3-1.

Results: During cell culture of DU-145, Gli36 and KB-3-1 tumour spheroids P-gp expression was observed as well as increased lactate and decreased pyruvate levels and expression of glycolytic enzymes. Inhibition of glycolysis for 24 h by either iodoacetate (IA) or 2-desoxy-D-glucose (2-DDG) downregulated P-gp expression which was reversed upon coincubation with the radical scavenger ebselen as shown by semi-quantitative immunohistochemisty in DU-145 and Gli36 tumour spheroids, and by EGFP fluorescence in KB-3-1 tumour spheroids. Consequently endogenous ROS generation in DU-145 tumour spheroids was increased in the presence of either IA or 2-DGG, which was abolished upon coincubation with ebselen. Exogenous addition of pyruvate significantly reduced ROS generation, increased P-gp expression as well as efflux of the P-gp substrate doxorubicin. In summary our data demonstrate that P-gp expression in tumour spheroids is closely related to the glycolytic metabolism of tumour cells and can be monitored in living multicellular tumour spheroids transfected with a EGFP-Pgp reporter gene construct.

P32: An Ontology to represent Knowledge on Animal Testing Alternatives

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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 (3Rs principle). To meet this regulatory obligation, scientists must consult the relevant scientific literature prior to any experimental study using laboratory animals. More than 50 million potentially 3Rs relevant documents are spread over the World Wide Web, biomedical literature and patent databases. In April 2008, the beta version of Go3R (www.Go3R.org), the first knowledge-

based semantic search engine for alternative methods to animal experiments, was released. Go3R is free of charge and enables scientists and regulatory authorities involved in the planning, authorisation and performance of animal experiments to determine the availability of alternative methods in a fast and comprehensive manner.

The technical basis of this search engine is specific 3Rs expert knowledge captured within the Go3R Ontology containing 87,218 labels and synonyms. A total of 16,620 concepts were structured in 28 branches, where 1,227 concepts were newly defined to specifically describe directly 3Rs relevant knowledge. Additionally relevant headings from MeSH where referenced to reflect the topics associated with the definition of Animal Testing Alternatives. Therefore it is distinguished between thematic-defining and directly 3Rs relevant branches. In addition to the assignment of direct parent-child relationships, further relationship types were introduced to allow to model 3Rs relevant domain knowledge. Examples for such knowledge are e.g. (1) the characteristics of cell culture tests methods, which usually utilize “specific cell types” or “cell lines” and are associated with a specific “endpoint” and “endpoint detection method” or (2) named test methods like “PREDISAFE TM”, which replaces an animal test namely the “eye irritation test” in rabbits and uses specific cells namely “SIRC Cells” or (3) the “Haemagglutinin-Neuraminidase Protein Assay”, which detects a protein of the “Newcastle disease virus”.

Thereby, an article in which e.g. a specific 3Rs method is not explicitly mentioned could still be recognized as relevant for the specific topic searched for in an indirect manner, for example if it mentions specific cells, endpoints or endpoint detection methods, which are relevant for the respective application.

The search engine Go3R with its novel ontology is already well recognized by the 3Rs community and will be further maintained and developed.

Methods: A platinum nanoparticle aerosol was generated using a spark discharge generator in nitrogen as the carrier gas. The Pt NP aerosol was diluted by the factor of 10 with synthetic air directly after the generation process. The aerosol was directed to the Karlsruhe Exposure system (Paur et al., 2008; Diabaté et al., 2008) to analyze the toxicological potential of the freshly generated Pt NP aerosol.

For the bioassay we employed the human alveolar epithelial cells A549 and the bronchial epithelial cells BEAS-2B, which was co-cultured with diffe rentiated THP-1 macrophages, growing on Transwell inserts. The responses of the cells were analyzed by measuring viability (AlamarBlue assay), release of lactate dehydrogenase (LDH) as an indicator of membrane integrity, induction of heme oxygenase-1 (HO-1) as an indicator of an anti-oxidative response and release of Interleukin-8 (IL-8) as an indicator of a pro-inflammatory response. Additionally, Pt NPs collected on polycarbonate filters (pore diameter 0.4 µm) were used.

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