Abstracts of the 8th World Congress, Montreal, 2011

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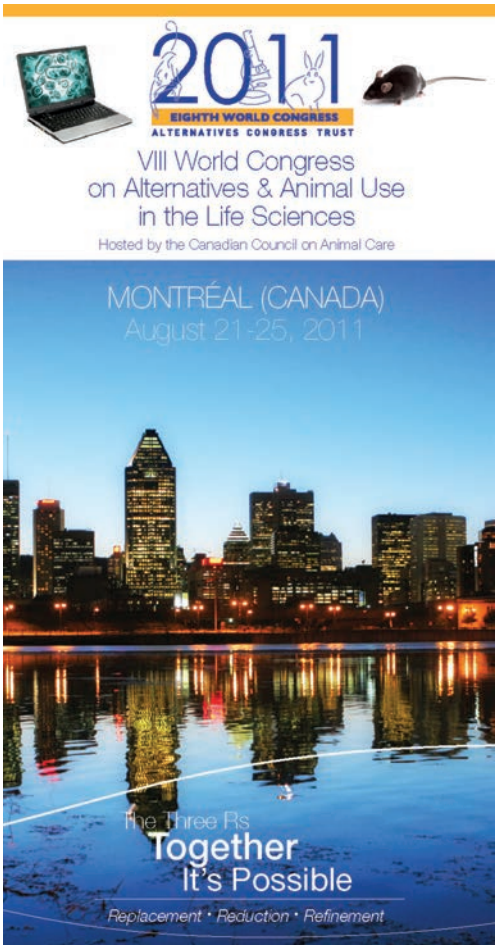
ALTEX

ALTERNATIVES TO ANIMAL EXPERIMENTATION



WELCOME

Dear WC8 participants,

The Canadian Council on Animal Care (CCAC) is proud to be the first national authority overseeing animal use in science to be given the opportunity to host a World Congress on Alternatives and Animal Use in the Life Sciences (WC). This honour recognizes Canada's, and more especially the CCAC's, contributions to the area of refinement of animal use in science. Since its establishment in 1968, the CCAC has continuously encouraged the development and adoption of best practices in animal care and use in science, a central theme at the WC3 in 1999 when the Three Rs Declaration of Bologna was adopted. So, while advances in vitro methods, particularly for regulatory testing, remain a main focus of the WCs, in keeping with the spirit of Bologna, the 8th World Congress on Alternatives and Animal Use in the Life Sciences (WC8) will also have an emphasis on refinement of laboratory animal use. In addition, through greater involvement of scientists from both the biomedical and biological sciences, the WC8 will chart new territory in examining the Three Rs as applied to basic research. Following on from the 7th World Congress Calling on Science, the WC8 will provide a forum that supports quality science and recognizes the increasing need for an integrated approach to human, animal and environmental health. In Canada, policy on the use of animals in science is developed with input from scientific researchers and teachers, veterinarians, animal care staff, regulators and the public. This tradition of consensus-building inspired the WC8 motto "The Three Rs – Together it's Possible". The goal of the WC8 is to bridge the distance between science, policy and education by identifying mechanisms to enable effective knowledge transfer and the translation of science-based evidence into good animal practices and non-animal alternatives that will result in higher quality science.

The WC8 brings together close to 900 professionals, including researchers, veterinarians, animal welfare organizations, public representatives, members of institutional animal care and use/ethics committees, and regulatory experts. These participants will join us from more than 50 countries for the unique opportunity to exchange scientific, ethical, and animal welfare knowledge, further progressing the ethical principles of the Three Rs (Refinement, Replacement and Reduction) enunciated by Russell and Burch in 1959.

We are pleased to host the WC8 in Montréal's most hospitable and gracious hotel — Fairmont The Queen Elizabeth — located at the center of Montréal's vibrant cultural and commercial district. The hotel reflects Montréal's elegance and charm. It is connected to the extensive underground city of boutiques, restaurants and cafés and is within walking distance of sports and cultural attractions.

As Co-Chairs of the WC8, we extend a warm welcome to all the participants and accompanying persons. We look forward to an opportunity to reconnect with old acquaintances and to make new friends with a view to working together to advancing Three Rs in science and education.

Sincerely,

Clément Gauthier
Executive Director, CCAC

Herman Koëter
Managing Director, Orange House Partnership

Co-Chairs, Eighth World Congress on Alternatives and Animal Use in the Life Sciences

Dear WC8 participants,

ALTEX joins the Canadian Council on Animal Care and the co-chairs Clément Gauthier and Herman Koëter in wishing you a stimulating and successful 8th World Congress on Alternatives and Animal Use in the Life Sciences.

This book contains a total of 607 abstracts, shared over the 5 Congress Themes covering safety and efficacy testing, legal and ethical issues, education and training, animal welfare and basic research. Of these, Theme I, "Safety and efficacy testing of chemicals, pharmaceuticals and biologicals" has by far the most submissions (280), followed by 131 submissions for Theme V, "Replacement and Reduction in basic research".

The abstracts were submitted from 43 countries, whereas this number does not entirely reflect the diversity of contributing authors. Like at WC7, the countries with the highest numbers of submissions at WC8 are the USA (23%), Germany (12%) and the UK (12%). These are followed by our host country Canada, as well as France, the Netherlands, Japan, Belgium, Brazil, the European Commission, India and Switzerland.

Of the countries represented by fewer abstracts, the lineup has changed substantially in comparison to WC7 in Rome. This is likely a factor of travel costs, as especially Eastern European and Middle Eastern countries are absent to whom Rome would have been better accessible. On the other hand, some countries that were not in Rome are represented here in Canada and we hope that this is a sign of growing international interest in alternative methods and concern for experimental animals.

Although the large European projects support and require the network of many different countries – we have one abstract with 27 authors representing 12 countries, it is conspicuous that countries with a substantial and growing interest in alternative methods, such as India, Brazil, and Korea, remain fairly insular and their abstracts only rarely indicate cooperation with partners from other countries. Perhaps the opportunities to meet and exchange ideas at WC8 will be a starting point for new international projects involving these countries.

The ALTEX team is looking forward to working with all presenters of talks or plenary lectures to produce the Proceedings of WC8. The Proceedings present a unique snapshot of the international state of the art in the field of alternative methods. They can be an excellent introduction to the field for interested scientists and for students and provide an opportunity for the experts to formulate and test ideas, opinions and visions more freely than in peer-reviewed scientific papers. Lastly, they provide an overview of what happened at the congress to those who were unable to attend. The Proceedings will be provided to all attendees on DVD and will be freely accessible on the ALTEX webpage (www.altex-edition.org). Please submit your manuscripts on CD to the ALTEX booth on site or send them via e-mail to editor@altex.ch by mid-September 2011.

We hope you will consider ALTEX with its highly competitive impact factor of currently 4.4 for the publication of your future manuscripts or reviews, news and letters and will continue to use the journal as an information source and forum for exchange of ideas. Please visit us at our booth.

The ALTEX team thanks the CCAC, especially Allison Guy, Nicole Fenwick and Gilly Griffin, for excellent cooperation in producing this Abstract book and wishes all participants and organizers a memorable time in Montreal. We also thank the Doerenkamp-Zbinden Foundation (Switzerland) for funding the production of this Abstract book and the Proceedings.

We are already looking forward to seeing you again at WC9 in Prague, Czech Republic, in 2014.

With best wishes

Sonja von Aulock
Editor in chief, ALTEX

Theme III
Incorporation of the Three Rs in Education and Training

Theme IV
Animal Welfare for Refinement and High Quality Science

Theme V
Replacement and Reduction in Basic Research

PROGRAM OVERVIEW

MONDAY, AUGUST 22, 2011

TIME	LE GRAND SALON MARQUETTE	JOLLIET	DULUTH	MACKENZIE
7:30 - 9:00				
9:00 - 9:15	**Opening Ceremonies**			
9:15 - 10:00	**Plenary 1** Living the good life – how far can Refinement go?			
10:00 - 10:15	**BREAK**			
10:15 - 12:15		**Scientific Session I-1** Potency and safety testing of human vaccines	**Scientific Session I-2** Addressing systems toxicology	**Scientific Session II-2** Ethics review
12:15 - 12:45	**LUNCH**			
12:45 - 14:00		**Additional Session I-18** Report on the ICCVAM International workshop on vaccines		**Additional Session I-14** Comparing the challenges of implementing new non-animal methods in the US and Europe
14:00 - 15:30		**Scientific Session I-7** Potency and safety testing of veterinary vaccines	**Scientific Session I-8** Safety testing for chemically-induced eye injuries: Recent Three Rs advances	**Scientific Session II-1** Public accountability
15:30 - 15:45	**BREAK**			
15:45 - 16:45		**Additional Session I-16** Alternatives for potency testing for rabies		**II-1 continued**
16:45 - 17:30	**Plenary 2** Engineering performance or performing engineering standards? Globalization and the application of the Three Rs			
19:00 - 22:00	**GET-TOGETHER PARTY - Bonsecours Market, Old Montréal**			

HARRICANA CHAUDIÈRE MATAPÉDIA	BERSIMIS PÉRIBONKA RICHELIEU	SAINT-LAURENT GATINEAU	SAINT-FRANÇOIS (LOBBY)	HOCHELAGA			SAINT-MAURICE
				1-3	4-6		
		Altweb Project Team Meeting (closed)		Exhibition	Theme II poster session	Theme III poster session	
BREAK							Multimedia exhibition
Scientific Session III-1 Innovative teaching in the life sciences	**Scientific Session IV-1** Indicators of animal welfare to implement refinement	**Scientific Session V-1** Novel methodologies and their potential in vitro application for drug development and safety assessment	**Additional Session II-9** The EPAA, a model for private-public partnerships supporting the advancement of Three R approaches				
	Additional Session IV-9 National cancer institute guidelines – a tool for implementing more humane endpoints?						
Scientific Session III-6 Training animal-based scientists	**Scientific Session IV-2** Farm animal research and the Three Rs	**Scientific Session V-2** Systematic reviews of animal experiments	**Additional Session II-7** The role of partnerships in advancing the Three Rs - Together it's possible!				
BREAK							
III-6 continued (panel discussion)	**IV-2 continued**	**V-2 continued**	**II-7 continued**				
GET-TOGETHER PARTY - Bonsecours Market, Old Montréal							

PROGRAM OVERVIEW

TUESDAY, AUGUST 23, 2011

TIME	LE GRAND SALON MARQUETTE	JOLLIET	DULUTH	MACKENZIE
7:30 - 8:30				
8:30 - 9:30	**Plenary 3** Implementing the Three Rs through policy – The EU Directive			
9:30 - 9:45	**BREAK**			
9:45 - 12:00		**Scientific Session I-3** Biological and biotechnology-based therapeutics	**Scientific Session I-4** Validation and Three Rs strategies for assessment of endocrine-active substances	**Scientific Session II-3** Public law – the Three Rs in regulation addressing animal use
12:00 - 12:30	**LUNCH**			
12:30 - 13:45				**Additional Session II-8** Science and politics in food safety assessment and their impact on experimental animal use
13:45 - 15:00		**Scientific Session I-9** Advances in Three Rs alternatives for reproductive and developmental toxicity	**Scientific Session I-11** Three Rs approaches to skin sensitization	**Scientific Session II-4** Implementing the Three Rs – alternatives to legislation
15:00 - 15:15	**BREAK**			
15:15 - 16:15				**Additional Session I-15** Shellfish toxin testing: how are the Three Rs being progressed in this field?
16:15 - 17:15	**Plenary 4** A challenge to the ultimate Three Rs – in silico approach to evaluate chemical safety for humans			

HARRICANA CHAUDIÈRE MATAPÉDIA	BERSIMIS PÉRIBONKA RICHELIEU	SAINT-LAURENT GATINEAU	SAINT-FRANÇOIS (LOBBY)	HOCHELAGA		SAINT-MAURICE
				1-3	4-6	
				Exhibition	Theme I poster session	Multimedia exhibition
BREAK						
Scientific Session III-3 Development of non-animal teaching/ training models	**Scientific Session IV-3** Wildlife science and the Three Rs	**Scientific Session V-3** Cell culture and tissue engineering	**Additional Session I-13** Toxicity testing in the 21st century (Tox21)			
Additional Session II-10 Role of international bodies in spreading Three Rs efforts globally		**Additonal Session V-9** Improving reporting of animal-based research				
Scientific Session III-4 Replacement alternatives and teaching objectives – determining if and when student learning objectives require the use of an animal	**Scientific Session IV-4** Multi-imaging modalities, telemetry and the Three Rs	**Scientific Session V-4** Refinement and Reduction in the use of genetically-engineered animals	**I-13 continued**			
BREAK						
Additional Session IV-7 VET2011: Advancing animal welfare training for veterinarians		**V-4 continued**	**I-13 continued**			

PROGRAM OVERVIEW

WEDNESDAY, AUGUST 24, 2011

TIME	LE GRAND SALON MARQUETTE	JOLLIET	DULUTH	MACKENZIE	HARRICANA CHAUDIÈRE MATAPÉDIA
7:30 - 8:30					
8:30 - 9:30	**Plenary 5** Alternative training methods for clinical education – considerations				
9:30 - 9:45	**BREAK**				
9:45 - 12:00		**Scientific Session I-5** Nanotoxicology and the Three Rs	**Scientific Session I-6** Advances in alternative methods for ecotoxicology	**Scientific Session II-5** Validation of Three Rs alternative methods	**Scientific Session III-5** Introducing multi-media to the curriculum
12:00 - 12:30	**LUNCH**				
12:30 - 13:45			**Additional Session 1-17** Update on new in vitro models for detection and potency assessment of botulinum neurotoxin		
13:45 - 15:00		**Scientific Session I-10** Safety testing for carcinogenicity and genetic toxicity: recent Three Rs advances	**Scientific Session I-12** Epigenetics and its increasing relevance in toxicology and risk assessment	**Scientific Session II-6** Setting limits and resolving conflicts between the Rs	**Scientific Session III-2** Innovative training in human and veterinary medicine
15:00 - 15:15	**BREAK**				
15:15 - 16:15			**Additional Session II-11** The International Cooperation on Alternatives Test Method	**II-6 continued**	
16:15 - 17:15	**Plenary 6** Pursuing Medawar's challenge for full Replacement				
19:30 - 23:00	**Gala Dinner - Windsor Station**				

BERSIMIS PÉRIBONKA RICHELIEU	SAINT-LAURENT GATINEAU	SAINT-FRANÇOIS (LOBBY)	HOCHELAGA			SAINT-MAURICE	KAMOURASKA 1-2 (3RD FLOOR)
			1-3	4-6			
			Exhibition	Theme IV poster session	Theme V poster session	Multimedia exhibition	
BREAK							**BREAK**
Scientific Session IV-5 Can pain research benefit research animals?	**Scientific Session V-5** Developments in stem cell research as the basis for sustainable availability of differentiated human cells and tissues	**Additional Session V-7** Relevance, reproducibility and robustness – the other Three Rs important to science and animal welfare (SCAW)					
Additional Session IV-8 Ending severe pain and distress in animal experiments by 2025?							**Additional Session V-8(a)** Tutorial for Go3Rs search engine
Scientific Session IV-6 Broadening the application of Refinement	**Scientific Session V-6** Animal reduction through the better use of mechanistically-	**Additional Session I-19** Toxicity testing strategies – progress in skin sensitization testing (COLIPA)					
BREAK							**BREAK**
	based translational animal disease models						**Additional Session V-8(b)** Tutorial for Go3Rs search engine
Gala Dinner - Windsor Station							

JOHNSON & JOHNSON'S COMMITMENT TO A 3Rs CORPORATE CULTURE

Worldwide, we make every effort to maximize the use of *in vitro* alternative methods in our companies' laboratories

Johnson & Johnson has engaged in the 3Rs since the 1980's

3Rs Award

Established in 2005 by Johnson & Johnson Corporate Office of Science and Technology (COSAT) and the Bioresearch Subcommittee to recognize employees who make a major contribution in advancing the 3Rs' mission.

3Rs Post Doc

Established in 2006 by COSAT recognizing the importance of focused research in the area of 3Rs.
To nourish a creative corporate culture where top scientists and engineers serve as role models for others.

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Established in 1985 to recognize outstanding performance by animal technicians responsible for the care of laboratory animals in Johnson & Johnson facilities.

Community of Practice

Animal Testing Alternatives internal "wiki" to share best practices and information as well as Animal Testing Alternatives discussion forum to promote collaboration.

Johnson & Johnson

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Plenary Lectures

Living the good life – how far can Refinement go

D. M. Weary

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Refinement is typically viewed as a means of reducing the harms to animals used in laboratory research, a key example being improved use of analgesics following experimental surgery. I review other similar examples of recent research on refinements, including improved methods of handling and euthanasia, to show how science can be used to assess the effects of these refinements on the welfare of animals. Focus in the animal welfare literature is now shifting from simply reducing harms we cause to animals to promoting positive experiences. The question has now become: Do the animals under our care experience "a good life"? Achieving a good life might require that we provide environments that allow animals to express natural behaviours that they are motivated to perform and provide opportunities for positive emotional experiences. The goal is that, on balance, positive experiences far outweigh any negative experiences. Our challenge now is in developing scientific methods that can address the question of a good life. Thus I also review recent research in animal welfare science that has begun to develop methods of identifying and assessing positive emotional states and assessing how the animal views its own condition. I conclude that refinement research should increasingly focus on providing laboratory animals a good life, and that research focussed only on reducing harms should be viewed as insufficient.

Engineering performance or performing engineering standards? Globalization and the application of the Three Rs

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This paper reflects on the insights a geographer might bring to understanding the international application of the 3Rs. The landscapes of scientific research are increasingly extensive, with established research centres in Europe and North America now supplemented by the emergence of new scientific initiatives in China, India and elsewhere. Scientific research is also becoming more interconnected, with an intensification of patterns of international collaboration. The use of laboratory animals is following similar patterns, perhaps most notably through a series of initiatives for generating, characterizing and archiving mutant mice across the international scientific community. Whilst publication metrics and other quantitative data give a sense of the patterns of these emerging collaborations, qualitative research is essential for understanding the processes through which such collaborations are forged and the challenges they present to established research practices and the governance of science in different localities. This paper outlines these processes, drawing on in-depth interviews and participant observation with researchers and stakeholders involved in the changing practices of laboratory animal research in North America, Europe and South-East Asia. In particular, it explores the often-entrenched debates between the use of performance and engineering standards to argue for animal welfare in the USA and Europe. It suggests both are linked to these specific landscapes of laboratory research, and the debate between them may need revision to further both animal welfare and meaningful research within the increasingly global landscapes of laboratory animal research.

A challenge to the ultimate 3Rs – *In silico* approach to evaluate chemical safety for humans

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The safety assessment of chemicals is one of the most important issues in regulatory science. Until recently, at least in Japan for industrial chemicals, the safety assessment process has been hazard-based. The toxicity of chemicals has been evaluated mostly using rodents and extrapolated to humans. Because of animal welfare and the requirement of high-throughput, *in vitro*, omics, and also *in silico* systems have been developed and introduced. Our final goal is the assessment of safety for humans, and of the many assay systems only *in silico* systems can focus directly on human beings. Any one (Q)SAR alone cannot do this task, but evaluating mutagenicity of chemicals by a combination of models revealed an acceptable performance, because of the relative simplicity of correlating chemical structure with mutagenicity. However, general toxicity is not easy to evaluate because of the many factors that can affect the results. Any single SAR or (Q)SAR cannot evaluate general toxicity of chemicals. Our project sponsored by NEDO/METI aims at the safety assessment of chemicals evaluated *in silico* and the final goal is to assist toxicology experts in making safety assessments of chemicals for humans accurately and efficiently. We constructed databases containing not only the results of animal repeat dose toxicity tests but also metabolism of chemicals, and of mechanisms of toxicity. We are constructing a platform to integrate these databases and are also including categories of chemicals to assess, based not only on structure but also on activities. We are also building a Bayesian network to incorporate the knowledge of experts.

Alternative training methods for clinical education – considerations

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The use of animate models in medical education and clinical training continues to be a controversial subject often clouded with emotion and experiential bias. On one side, there is clear feeling that it is ethically wrong to sacrifice animals for educational purposes. This position argues that medical and surgical skills needed to perform clinical procedures can be effectively acquired by alternative methods and will provide equivalent or superior results to training with animate models. The opposing viewpoint believes that appropriate exposure to concepts within an animate laboratory setting results in a level of understanding that is different and superior to other learning methods. The belief here is that hands-on animate experience allows mastery of interrelated complex concepts, where other methods cannot. This presentation will explore information available in the open literature comparing animate vs. inanimate training models, limitations of the available data, and considerations of necessary models based on approaches to learning.

Pursuing Medawar's challenge for full Replacement

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William Russell and Rex Burch, who pioneered the Three Rs approach in the 1950s, considered refinement and reduction of animal use in biomedical procedures as interim steps on the path towards replacement. In the 1960s, Peter Medawar, a Nobel Prize winning scientist who helped guide Russell and Burch's work, predicted that scientific innovation would someday completely replace animal use in science. Medawar correctly forecast the leveling off and subsequent decline in animal use in the last quarter of the 20th century. That decline has been checked by a rise in the breeding and use of genetically engineered animals in recent years, but this probably will be a temporary pause in a long-term trend of declining animal use. A 2007 report by the U.S. National Academy of Sciences, *Toxicity Testing in the 21st Century*, proposed a strategy that is likely to replace routine animal testing in toxicology with innovative methods within one to two decades. While replacing animals in biomedical research is more challenging given its diverse nature and larger scale, full replacement is a goal worth pursuing for a host of reasons. This presentation will call for coordinated, targeted, and sustained efforts to fully replace animals in research and testing, review current replacement initiatives, and offer policy and programmatic proposals for moving forward. Such replacement efforts will advance scientific progress and animal protection, and help steer the animal research controversy into a more productive phase.

Theme I
Safety and Efficacy Testing of Chemicals, Pharmaceuticals and Biologicals

Session I-1: Potency and safety testing of human vaccines

Session I-1: Oral presentations

I-1-107

EDQM's Three Rs activities in the field of quality control of vaccines

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The EDQM (European Directorate for the Quality of Medicines & Health Care, Council of Europe) is a standard setting body involved in ensuring the quality of medicines, including vaccines for human and veterinary use. This is done through the elaboration and publication of the European Pharmacopoeia (Ph. Eur.). In addition, the EDQM runs the Biological Standardisation Programme (BSP) which elaborates reference standards and validates methods, including Three Rs approaches for the quality control of biologicals. Furthermore the EDQM is the secretariat of the European network of public control laboratories that are involved in the official control authority batch release of human and veterinary vaccines. It is through this process that the public control laboratories use animals for the quality control of vaccines.

The Three Rs activities of EDQM are based on the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes", which was published by the Council of Europe in 1986 and which represents the first international legal text in this field.

A highlight of EDQM activities in the application of Three Rs principles will be presented including insight on challenges for development and implementation of alternative methods. Examples will include projects with successful introduction of alternative assays for the assessment of the potency of human and veterinary vaccines. An overview of future plans in this field will also be presented.

I-1-048

Development of 3Rs alternatives for determining potency and toxicity of vaccines in Cuba: Current challenges and research projects in progress

M. L. Chovel Cuervo, A. Mandiarote Llanes, I. Ontivero, L. Herrera and J. F. Núñez

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Vaccines have been traditionally released for immunization purposes once a set of relevant quality control parameters have been controlled. To do so, classical animal potency and toxicity tests have played a determinant role. Over the last decade, efforts have been made in Cuba to introduce and develop Three Rs alternatives for potency and toxicity testing of vaccines. Significant progress has been made for some vaccines such as the potency of Hepatitis B (neutralization ELISA), the toxicity/potency of Diphtheria (guinea-pig serology-Vero cell) and the potency of Tetanus (mouse serology-ELISA). The remarkable reduction in the number of animals used, the refinement of the procedures and the potential for the full replacement of the challenge potency tests and some other animal assays have been considered as ways of implementing Three Rs alternatives for vaccines. The Finlay Institute is the major manufacturer of vaccines in Cuba and it is undoubtedly interested in this field. We aim to provide an overview of the state of the science and to show the progress we have made in the introduction/development of Three Rs alternatives for the evaluation of vaccines, as well as the upcoming research projects and those in progress. The Finlay Institute, along with some other Cuban institutions, are planning to introduce some of the most updated Three Rs approaches for classical animal tests for Potency and Toxicity tests of vaccines, including *in vitro* methods (ELISA, cell culture assays, biochemical and immunological functional tests), serology and consistency approach.

I-1-167

Three Rs acceptance and implementation: obstacles and opportunities for new technology

M. Long[1], M-J. Schiffelers[2], G. Griffin[1] and C. Hendriksen[2]

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The development, production and quality control of vaccines is characterized by extensive laboratory animal use and a high frequency of procedures inflicting severe pain and distress to the animals involved. Alternatives to traditional animal methods do exist which follow the Three Rs principles of reduction, replacement and refinement of animal use. However, despite progress made over the last decades to adopt Three Rs methods into vaccine testing and the existence of legislation such as European Directive 2010/63/EU, which requires the adoption of Three Rs alternatives when and where possible, the acceptance and use of Three Rs methods moves forward at a slow pace.

The authors have identified the factors which support or challenge the acceptance and use of Three Rs methods, as well as the motivations behind these factors. First, we describe the factors involved in the acceptance of Three Rs methods in vaccine quality control as identified through a Canadian case study. These factors are compared with factors identified through a second study, in the broader context of regulatory testing in Europe. We use the concept of technology transitions and technology paradigms to explain the mechanisms of acceptance and use of Three Rs models in regulating the risks of products such as vaccines. Recognition and understanding of these mechanisms will provide regulatory authorities and industry stakeholders with a basis for practical discussions on how to integrate scientifically sound alternatives into regulatory testing.

I-1-672

The consistency approach in lot release testing of vaccines

C. Hendriksen[1], B. Metz[2], J. van der Gun[1] and G. Kersten[2]

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Vaccine lot release testing is characterised by extensive use of laboratory animals, particularly to demonstrate product safety and potency. Successes have been achieved in replacing existing animal models by Three R methods; cell-based and serology or humane endpoints to remove lethality parameters. However, progress is tedious, time consuming and costly.

A new paradigm in lot release testing of established vaccines (e.g. Tetanus and Diphtheria toxoid) is the consistency approach. This approach starts from the idea that subsequent lots of vaccine produced can be compared to an earlier (reference) lot (clinical-, historical batch) with a thoroughly tested and well defined profile of safety and efficacy/potency. Consistency for lot release has come within reach since vaccine starting material is better characterised (quality by design), production processes have been optimised and standardised, a tight protocol for in-process testing has been implemented, quality monitoring systems such as GMP, QA and pharmacovigilance are now state-of-the-art and, last but not least, new physicochemical and immunochemical techniques have become available.

Consistency testing may lead to a significant reduction in animal use, since a narrow set of animal tests performed on each final lot, with potentially limited power to predict vaccine behaviour in the target populations, may be replaced by a battery of meaningful physicochemical-, immunochemical- and eventually *in vitro* functional tests with enhanced capacity to measure equivalence with batches of proven safety and efficacy.

The paradigm of consistency is an interesting strategy for vaccine manufacturers as it might allow for a reduction in testing costs and a shortening of the testing period. The concept of consistency testing was recently adopted by the European Partnership on Alternative Approaches to Animal Testing (EPAA) as a promising strategy to animal reduction.

This presentation will provide an introduction of the approach and discuss advantages and limitations. An outlook will be given of remaining research questions and implementation strategies.

I-1-148

Alternative safety testing strategy for acellular pertussis vaccines

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The mouse histamine sensitization test (HIST) is the currently accepted regulatory method used to monitor for residual pertussis toxin (PTx) activity in acellular pertussis vaccines. This lethal end-point test is technically challenging and raises ethical concerns, thereby making the development of alternative methods highly desirable. Various *in vitro* assays have been developed which each monitor different biochemical or cellular functions of PTx. These include PTx binding activity, enzymatic activity, and its ability to agglutinate or induce a signal transduction event in cultured cells. As the mechanism of PTx toxicity is complex, a panel of these *in vitro* assays will most likely be required as an alternative to the HIST. Several scientific meetings have been held recently to discuss how to proceed with the validation and adoption of these alternative assays. Major questions to be addressed include the sensitivity and specificity required of each assay, identification of assay limitations, effects of vaccine formulation on assay outcome, and the relation of *in vitro* data to HIST. This presentation will provide a review of the outcomes from these scientific meetings and the potential path forward to adopt these alternative assays to meet regulatory requirements.

I-1-326

Alternatives to animal use for the LAL-assay

I. Spreitzer, C. Bache, J. Hermanns, B. Loeschner, B. Becker, C. K. Schneider and T. Montag

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The Limulus Amebocyte Lysate (LAL)-assay is frequently performed to quantify endotoxin (LPS), the most effective pyrogen (fever inducing substance) known. Due to its persistence despite the use of cleaning/inactivation procedures, the impact of LPS on the pharma, life sciences and medical devices sectors is enormous. The LAL-assay has replaced the Rabbit Pyrogen Test (direct animal test) for most products, and is obligatory for many source materials, intermediates and final products. However, the "alternative" LAL is an "indirect animal test": the *Limulus* lysate is obtained from wild populations of horseshoe crabs (mainly *Limulus polyphemus*). To harvest the hemolymph, the crabs are captured, partially bled and released. Despite immense efforts by the LAL-industry, the mortality caused by this procedure is approximately 10-15%. In 2009, 512,552 horseshoe crabs were caught, bled, and released in the US, of which approximately 60,642 died (official estimation). All horseshoe-crab species are endangered (IUCN Red List), mainly due to loss of habitats, pollution (oil spills, etc.) and commercial use. The US *Limulus* population is controlled by fishery management plans. The Asian *Tachypleus* population (especially China) is threatened severely. The still growing demand for lysate cannot be satisfied by these wild populations, which could result in a major drug safety problem. The Three Rs could be applied immediately, in particular replacement and reduction alternative methods (validated alternatives like the monocyte activation Test / MAT, recombinant LAL-assays) and refinement (reduced lysate volume). These opportunities will be discussed in the presentation. Furthermore, we describe a more sensitive and accelerated MAT-version to make a feasible alternative available.

Session I-1: Poster presentations

I-1-060

Monocyte activation test (MAT) reliably detects pyrogens in parenteral formulations of human serum albumin

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The monocyte activation test (MAT) was performed to detect pyrogens in human serum albumin (HSA), and results were compared with those obtained with the rabbit test and Limulus assay (LAL). All batches were contaminated with (1,3)-β-glucans as assessed by conventional LAL, however endotoxin-specific LAL was not suitable to test HSA due to unacceptable endotoxin recoveries in the interference test. Three batches failed the rabbit test and were clearly detected with the MAT using IL-1β and IL-6 response as readout. Experiments combining polymyxin B and the MAT demonstrated that pyrogenic batches were contaminated with endotoxins, but the endotoxin-specific LAL failed to detect one of them, thus LAL did not offer a high security level to test pyrogens in HSA. (1,3)-β-glucan enhanced the IL-6 response to endotoxin, but not IL-1β. In addition, endotoxin concentrations obtained with IL-6 readout were usually higher than with IL-1β, likely related to a direct IL-6 response of monocytes to (1,3)-β-glucans. Contaminating (1,3)-β-glucans produced no pyrogenic reactions in rabbits. Hence, IL-1β readout resembles better the typical pyrogenic response. Nevertheless, IL-6 can be a useful readout to assess glucan contamination and its immune-modulating effect, which are potentially deleterious and not necessarily evidenced through a febrile reaction. The MAT correlates with the rabbit test while providing a higher safety level for pyrogenicity testing in HSA and probably other therapeutic proteins, so it can be a useful alternative method to detect any pyrogenic contamination in these products.

I-1-106

Comparison of the bioactivity for pertussis toxin by the histamine sensitization test and *in vitro* assays

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Pertussis toxin (PT) in its detoxified form is an important antigenic component of acellular pertussis vaccines. The specific toxicity test for acellular pertussis vaccines, the histamine sensitizing (HIST) assay, is still the only assay used, and involves many experimental animals. Currently, the enzyme coupled-HPLC assay and carbohydrate binding assay for pertussis toxin have been developed for potential replacement of the current *in vivo* HIST. In this study, we compared the bioactivity for pertussis toxin by the HIST and the *in vitro* assay. Although some questions still need to be answered in relation to the development of suitable replacements for *in vivo* tests of pertussis vaccines, the prospects for further improvements are promising.

I-1-138

Endotoxin and non-endotoxin pyrogens trigger inflammatory cytokine release in the Monocyte-Activation Test with cryopreserved human blood

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Pyrogen tests for products of the pharmaceutical industry so far require the utilization of animal-based test methods, the Rabbit Pyrogen Test (RPT) or the Limulus Amoebocyte Lysate (LAL) test. As the LAL test is limited to endotoxins, the RPT must be used for the detection of non-endotoxin pyrogens. With the development of the Monocyte-Activation Test (MAT) using cryopreserved human blood, there is now a convenient, validated *in vitro* alternative. In 2010 the European Pharmacopoeia declared that "the MAT is suitable after a product-specific validation as a replacement for the rabbit pyrogen test" (Chapter 2.6.30). Here we show that the MAT with cryopreserved whole human blood is a useful tool to study details of the fever reaction pathway in the innate human immune response. Different inflammatory cytokines are induced and regulated by a number of ligands for Toll-like receptors (TLRs) or intracellular nucleotide-binding oligomerization domain (NOD) proteins. Whereas TLR 4 is known to be activated by endotoxin, TLR 2 is a receptor for non-endotoxin bacterial cell wall components and TLR 7 and 8 show specificity for nucleic acid pyrogens. The cytokine induction with a collection of well characterized pyrogens thus reflects the effects expected for the human body. In contrast to the animal model the MAT allows differentiation between the activities of the substances and allows the kinetics of the reactions to be analyzed individually. IL-1β and IL-6 were found to be equally useful for a sensitive and specific readout of the inflammatory reaction.

I-1-149

Characterization of binding assay components used to detect residual pertussis toxin in vaccine preparations

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We previously described a modified binding assay for the detection of residual pertussis toxin (PTx) in acellular pertussis vaccines as a potential alternative to the mouse histamine sensitization test (HIST). The aim of the current study is to further understand two critical steps of the binding assay which confer specificity for PTx: 1) the use of fetuin to capture the PTx and; 2) the monoclonal antibody (7F2) used to detect the captured PTx. Enzymatic HPLC, CHO cell agglutination, inhibition binding assays, and surface plasmon resonance (SPR) were used to characterize the binding properties of PTx and chemically-inactivated pertussis toxoid (PTd) to 7F2, fetuin, and asialofetuin. 7F2 did not affect PTx enzymatic activity, but did inhibit both PTx-induced cell agglutination and fetuin binding activities. However, inhibition of agglutination required a far lower 7F2:PTx ratio than inhibition of fetuin binding, suggesting a divergence between cellular response and binding activities. SPR studies showed that PTx binds strongly to both 7F2 and fetuin with B50 of 2.3 and 2.0 μg/ml, respectively. Interestingly, fetuin and asialofetuin both inhibited the binding of 7F2 to PTx, suggesting a structural link between the binding domains of PTx subunits S2/S3 and the non-binding S4 subunit, which is recognized by 7F2. Despite this apparent competition between fetuin and 7F2 for PTx binding, the use of both agents provides very high sensitivity in the detection of residual PTx in vaccine samples which contain high concentrations of PTd.

I-1-222

Alternate *in vitro* methods for detection of pertussis toxin in component pertussis vaccines

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Current requirements for component pertussis vaccines include testing for residual pertussis toxin (PTx). Histamine sensitization tests in mice, currently used for this purpose, are problematic due to the high variability, ethical concerns and issues with non-specificity.

Alternate *in vitro* methods that measure the binding and enzymatic activities of PTx have been proposed as replacement tests. Based on these methods, we have developed and validated an enzyme-linked immunosorbent assay (ELISA) method for the carbohydrate-binding activity of the B-oligomer of PTx and a high-pressure liquid chromatography (HPLC) method for the ADP-ribosyltransferase activity of the A-protomer in a pertussis combination vaccine. The ELISA was validated as a limit test and as such LOD, LOQ, specificity and robustness were assessed. The method was specific for detection of PTx, with an LOD of 0.004 μg/ml and an LOQ of 0.008 μg/ml.

The multi-step gradient elution HPLC method was converted to isocratic separation employing a temperature controlled, solvent-saver octadecyl silane column, which resulted in enhanced peak resolution, specificity and 70% lower acetonitrile consumption. Method parameters validated included system suitability, specificity, accuracy, linearity, range, LOQ, repeatability, intermediate precision, robustness and stability of test solutions. An r2 = 1.00 was obtained for the linear regression of area response versus PTx spiking concentration in a mock vaccine sample from 1 μg/ml (LOQ) to 30 μg/ml. CVs <7% and <8% were obtained for repeatability and intermediate precision.

A significant positive relationship between HIST activity (either dermal or lethal endpoint) and ELISA binding activity was determined by statistical modeling.

I-1-229

Interferon genes regulated by pertussis toxin: potential for an *in vitro* pertussis vaccine safety assay

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Pertussis vaccines have proven very effective in decreasing the incidence of whooping cough. Since these vaccines are routinely administered to healthy infants and contain inactivated bacterial components, tests to ensure their safety are of critical importance. The histamine sensitization test (HIST) is currently the standard assay to test for the absence of active pertussis toxin (PTx). HIST is a lethal animal test that is difficult to standardize, therefore, replacement of the HIST is a priority. Moreover, the exact mechanism of the test is undefined, and it is not clear whether the assumed underlying mechanism, i.e. PTx-mediated ADP-ribosylation of G proteins, is the only relevant effect of PTx. Therefore, we decided to perform micro-array experiments in a relevant human cell line to analyze PTx-induced quantitative gene expression to gain insight into PTx mechanisms. The selected human EA.hy926 cell line is a hybrid of endothelial and epithelial cells, i.e. cell types that are involved in *in vivo* PTx effects. Unexpectedly, exposure of EA.hy926 cells to 250 ng/ml PTx differentially regulated only a limited number of genes with modest changes. Gene set enrichment analysis revealed that most affected genes were within interferon signaling pathways. We are currently investigating the validity of these pathways by quantitative PCR using other cell lines and sources of pertussis toxin. The ultimate aim is to develop an *in vitro* safety test for pertussis vaccines that does not require the use of experimental animals and that may be more informative than current *in vivo* testing.

I-1-260

Immunological response of MUTZ-3 dendritic cells to the different components of conjugated *Haemophilus influenzae* type B vaccine: potential *in vitro* assay for vaccine immunogenicity

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The whole suite of immune responses to vaccination that occur *in vivo* in humans cannot be tested *in vitro* using a single cell type. Nonetheless, dendritic cells (DC) form an important candidate cell type, since they are pivotal in inducing and orchestrating immune responses. We used the human cell line MUTZ-3, the cell line that most closely resembles *ex vivo* human DC, and compared its response to monocyte-derived DC (moDC). Haemophilus influenzae type B (Hib) vaccine was chosen as model vaccine because its components exert different effects *in vivo*: while the Hib antigen, poly ribosyl phosphate (PRP) fails to induce sufficient protection in children below 2 years of age, conjugation of this sugar antigen to outer membrane protein (OMP) of *Neisseria meningitides*, results in sufficient protection. PRP induced little or no effects on cytokine production and surface marker expression. OMP induced high levels of IL-6, IL-8, IL-12p40, and TNF-α in MUTZ-3 cells, and of IL-6, IL-10, IL-12p40, IL-12p70, IL-23, and TNF-α in moDC. In MUTZ-3 cells decreased expression of CD34, CD209 (DC-SIGN), and CD86 was seen, while CD1a, CD80 and CD83 expression was increased. In moDC decreased expression of CD209 (DC-SIGN) was seen, while CD80 and CD83 expression was increased. Conjugated PRP-OMP induced a considerably smaller response in both cytokine production and surface marker expression than OMP alone. PedVax showed a similar response compared to PRP-OMP. In conclusion, we have developed an assay that is able to measure immunogenicity of the different Hib vaccine components.

I-1-327

The Pertussis-ATP-Test to replace the animal experiments for testing acellular pertussis vaccines

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Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*. One of its most eminent virulence factors is Pertussis Toxin (PTx). PTx is also of utmost importance as antigen for immunization via so called Acellular Pertussis Vaccines (ACV). To guarantee non-hazardous application for all recipients, the pertussis toxin (PTd) needs to be detoxified. The success of this detoxification has to be controlled to exclude residual or reversed active toxin. The animal test used for that purpose, the Mouse Histamine Sensitisation Assay (HIST) – required by the pharmacopoeias – is hard to standardize, since it is affected by inconsistencies, which make repetitions and accompanying use of high resources of animals inevitable. An alternative testing method is needed.

The so called Pertussis-ATP-Test (PAT), which utilizes PTx enzymatic activity such as NAD-glycohydrolase and ADP-ribosyltransferase, was developed by our group. Inside the cell, PTx transfers ADP-ribose onto an inhibitory G-protein and thereby interferes in the signal transduction pathway. This leads to an increase of cAMP and a decrease of ATP. We implemented decreasing ATP levels caused by PTx as indicator for the activity of the latter. Freshly isolated peripheral blood mononuclear cells (PBMCs) as well as the permanent human lymphocyte cell line Jurkat were used as human indicator cells. Their ATP levels were monitored by luciferin-luciferase mediated bioluminescence.

I-1-328

Improved Monocyte Activation Test (MAT) for accelerated and sensitive pyrogen detection

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Testing for the absence of pyrogenic (fever-inducing) substances is of utmost importance for safe administration of various pharmaceuticals. If administered intravenously, pyrogens can lead to complications with life-threatening consequences within minutes. Therefore, all Pharmacopoeias demand the Rabbit Pyrogen Test (RPT) as standard animal test for pyrogens. This commonly accepted test was established over 50 years ago, and still requires an intensive use of animals. Great efforts during the last 15 years have led to animal-free alternative methods. One of these methods, the Monocyte Activation Test (MAT), uses human monocytes from different sources such as fresh blood, peripheral blood mononuclear cells (PBMCs), monocytic cell lines or cryopreserved human whole blood. The MAT in its diverse variants involves an overnight-incubation followed by detection of pro-inflammatory cytokines (mainly IL-1β, IL-6 or TNF-α) via ELISA. The MAT has been implemented in the European Pharmacopeia. This test provides reliable and reproducible results for many final products, but might be too time-consuming in several cases, e.g. for inline-production-testing. Confronted with the need for a faster and more sensitive test, a variant of the MAT was developed measuring intracellular accumulated cytokines in individual cells by flow cytometry. Here, monocytes in fresh human whole blood are chemically impaired regarding the export of cytokines, and then incubated with samples (e.g. lipopolysaccharide / LPS). The accumulated cytokines are detected intracellularly by fluorescent-labelled antibodies. This method is capable of detecting 6.25 pg/ml LPS within 2 hours with a strong potential to be further improved time- and sensitivity-wise.

I-1-346

Application of 454 pyrosequencing technology on the detection of adventitious agents in vaccines

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Next generation sequencing (NGS) technology, also known as "deep sequencing" is a novel cutting edge technology which has the capacity to discover and determine genetic sequences with high sensitivity. This technology may revolutionize the method for adventitious agents (AVA) detection, which is required for release of commercial products and clinical trial materials. Currently, animals are used in these *in vivo* tests to ensure that the products do not contain any AVA that might be harmful to humans. Each year, animals such as mice, rats, ferrets and guinea pigs are used for vaccine release. Yet, animal tests may not be sensitive enough to identify all AVA in the vaccine materials. A recent report (Victoria et al., 2010) demonstrated that adventitious agents, such as non-pathogenic porcine circovirus (PCV-1), were detected in commercial rotavirus vaccine materials by the deep sequencing technology. However, PCV-1 was not readily detected by the existing *in vivo* tests. Thus, genetic sequencing may be more sensitive than the animal test in AVA detection and this technology may eventually eliminate or substantially reduce the numbers of animals used in these tests. A proof of concept study was conducted to sequence live attenuated influenza vaccine (LAIV) by 454 pyrosequencing and the data will be presented to illustrate the application of 454 pyrosequencing on AVA detection.

Reference

Victoria, J. G. et al. (2010). *J. Virol. 84*, 6033-6040.

I-1-361

Comparison between rabbit pyrogen test (RPT) and human whole blood cytokine release assay (IPT)

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Pyrogenic contamination is the most important problem of injectable products. Since LAL has the disadvantage of detecting only endotoxins, the rabbit is still used, despite interferences. In 2010 the European Pharmacopoeia introduced the Monocyte Activation Test (MAT) as a third method to detect pyrogenic contamination. In the routine of quality control of injectable products, the National Institute of Quality Control in Health performs RPT in large volume parenterals (LVP), biologicals and medical devices. In order to start a comparison between RPT and IPT, some samples were tested by both methods. Thirty LVP and biologicals were tested. Twenty-six samples were non-pyrogenic in both methods while 5 samples needed to repeat the assay with new animals. One anti-rabies serum presented sum of 8 rabbits equal to 4.2°C being pyrogenic in both methods. One Ringer solution presented sum of 8 rabbits of 3.0°C, being negative in RPT and positive in IPT. Two anti-scorpion venom sera presented only one rabbit above 0.5°C and the sum of 8 rabbits was 1.9°C, being also negative in IPT. It shows that IPT is sensitive enough to detect pyrogenic contamination early while in case of RPT it is necessary to repeat the assay, using 8 rabbits at all. It can be also seen that, when the summed temperature is low enough to be considered pyrogenic, IPT showed it was not contaminated, while it was necessary to repeat the assay in case of RPT. These partial results strongly indicate that IPT may, in the future, replace RPT.

I-1-379

The re-use of rabbits in pyrogen testing (RPT) of hyperimmune sera and vaccines contributes to the reduction of animal use

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Rabbits are still used for determining pyrogenicity of samples in the quality control of injectable products, since up to now, there is not a method for replacing this method. In case of biological products it is not recommended to re-use rabbits, due to possible cross-reactions. The National Institute of Quality Control in Health (INCQS) analyzes between 200 and 300 injectable products and medical devices per year, around 50% of which are hyperimmune sera and vaccines. For assaying these products, 1200 rabbits are used per year. For this study, 5 IU/kg LPS-spiked and non-spiked Anti-Bothrops venom, Anti-Rabies and Anti-Tetanus sera and Anti-Miningitidis C vaccine were assayed. Five rabbits per group were used, following the 48 hours-interval administration schedule: I – only spiked sample; II – one non-spiked and one spiked; III – two non-spiked and one spiked; and IV – three non-spiked and one spiked. The result of the last injection of each group (spiked sample) was compared to response of the group I in order to verify if there was any influence of previous non-spiked injections. There was no statistical difference among the four spiked responses for any of the products assayed. It is possible to re-use a rabbit that received non-pyrogenic hyperimmune sera or vaccine up to four times in a one-week period without jeopardizing the animal's response. By re-using rabbits, it is possible to reduce the number of animals used in the Rabbit Pyrogen Assay by 70%.

I-1-443

Development of xMAP technology for the control of multicomponent vaccine bioactivity

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Vaccines are often composed of three, four, five or six different antigens, for example tetanus toxoid (T), diphtheria toxoid (D) and Bordetella pertussis (Bp) proteins. Usually, the vaccine bioactivity is determined using lethal or paralysis challenge on animals for tetanus, dermal or lethal challenge on guinea pigs for diphtheria and a serological method on mice for pertussis. For these antigens, serological methods on guinea pig are now available and referenced in the Ph. Eur. They are based on the antibody detection in serum from immunized animals using a single antigen ELISA. Recently a new technology has been developed: xMAP technology, a multiplex bead-based assay that can analyse simultaneously up to one hundred different analytes in the same sample.

In this context, the objective of our study is to develop this technology for the control of the vaccine bioactivity. The same animals will be used for the analysis of the three vaccine components' bioactivity. Consequently, the animal use will be reduced and refined. The guinea pigs were immunized with a tetravalent vaccine and blood sampled 35 days later. The antibody titers for tetanus, diphtheria and pertussis were determined in the serum using single antigen ELISA and xMAP technology.

Our first results show quite a good correlation between the different methods for the vaccine bioactivity analysis. Tetanus and Diphtheria vaccine titres have been determined and compared with the results obtained by challenge method in our laboratory. In conclusion, this method may be a good alternative method to replace the challenge method.

I-1-462

Identification and characterization of monoclonal antibodies for use in *S. pneumoniae* vaccine characterization assays

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The objective of Sanofi Pasteur's pneumococcal vaccine is to treat *Streptococcus pneumonia*, a leading cause of serious illness in children and adults throughout the world. Since the *S. pneumoniae* vaccine is aimed at inducing a protective antibody response, it is desirable that the potency of the vaccine can be evaluated with monoclonal antibodies (mAbs) that can recognize protective epitopes. To this end, a panel of mAbs against an *S. pneumoniae* protein was extensively screened as potential reagents for assay development. Multiple approaches have been utilized in characterizing these reagents including *in vivo* protection models, surface accessibility assays, Western blots, affinity binding and epitope specificity assessment by ForteBio Octet®. The data demonstrates a progression from the initial screening of mAbs in animal models to *in vitro* testing of the attributes of the applicable protein by using a selected panel of mAbs. The ultimate goal of the project is to effectively assess vaccine integrity and potency by monitoring availability of protective epitopes in *in vitro* assays and replace *in vivo* potency assays.

I-1-504

International validation and evaluation of an alternative to HIST for pertussis containing vaccines

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Pertussis vaccines are commonly used worldwide for the prevention of the disease pertussis (whooping cough). Currently a significant number of animals are routinely used in the lethal challenge histamine sensitisation test (HIST) for control of pertussis toxin presented in pertussis and pertussis based combination vaccines. Thus, there is an urgent need to develop an alternative to the HIST. A refined detection strategy of an *in vitro* test system has been developed to examine both the functional domains of PTx, based on a combination of enzyme coupled-HPLC and carbohydrate-binding assays. Preliminary in-house validation of the developed assay system with the *in vivo* HIST showed that by using a mathematical equation linking the multi-functions of carbohydrate binding and enzymatic activities, there is a good correlation between the *in vitro* and *in vivo* tests. The acceptance of this system as an alternative to the current *in vivo* HIST by regulatory authorities would depend on its transferability between laboratories. An international collaborative study between national control laboratories (NCLs) and manufacturers for method transfer as well as for further validation of this *in vitro* assay system has been carried out. Three types of products representing the major types of ACV products currently in the worldwide market are included in the study and this study involves a total of 17 laboratories from 9 countries including vaccine manufacturers and NCLs.

I-1-592

Implementation of the *In Vitro* Pyrogen Test (IPT) for replacement of the rabbit

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The *In Vitro* Pyrogen Test (IPT) was adopted into the European Pharmacopoeia in 2010 (EP 6.7, chapter 2.6.30, Monocyte Activation test MAT). With this final step, the IPT is a fully validated and, under the name of PyroDetect, commercially available replacement method for the rabbit pyrogen test. As it is now vital to implement the test for routine use and as a service to industry, our laboratory in Tübingen, Germany, will acquire good manufacturing practice (GMP), at the same time building on the already existing GLP system for hemocompatibility. With the creation of a GMP laboratory, a signal is sent to national and international parties to adopt the *in vitro* test instead of the former "gold standard" rabbit pyrogen test, which has been in use for injectables since the 1940's. All in all, the IPT has the capacity to save 200,000 rabbits each year in Europe alone and, unlike the animal test, it is suitable for a broad range of applications.

I-1-675

Characterisation of antigen adsorbed to aluminium salts

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Suspensions of aluminium salts are still the most used vaccine adjuvants. The antigen is adsorbed to the surface of the particles. This hampers characterisation and demonstration of batch comparability as well as effects between adsorbed and non-adsorbed antigen. Nevertheless there are physico-chemical techniques like spectroscopy and calorimetry that allow the characterisation of proteins adsorbed to solid phases. The development and use of physico-chemical techniques to characterise adsorbed antigens, notably diphtheria toxoid, will be presented. It is shown that adsorbed toxoid has a different conformation as compared to non-adsorbed antigen.

I-1-676

Pertussis toxin content of acellular vaccines assessed by *in vitro* cAMP responses

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Current safety testing for pertussis toxin (PT) content of acellular pertussis vaccine batches relies on the histamine sensitisation test (HIST). This *in vivo* test is based on the empirical finding that pertussis toxin sensitises mice for histamine. Studies into the mechanism of this test demonstrated that decreased contractile properties of resistance arteries and increase of blood pressure significantly contribute to PT induced histamine sensitisation. In addition, sensitisation was not limited to histamine but could also be induced by the vasodilating substance sodium nitroprusside. This knowledge was used to develop the functional *in vitro* cAMP assay. The assay is based on a rat vascular smooth muscle cell line and centres on the enzyme adenylate cyclase. This enzyme converts ATP into cAMP and its activity is regulated by specific $G_{inhibitory}$ and $G_{stimulatory}$ proteins.

The majority of the biological effects of PT are attributed to its ability to ADP-ribosylate Ginhibitory proteins. This leads to ineffective proteins unable to inhibit adenylate cyclase. Consequently, intracellular cAMP may increase, especially in the case that stimulatory proteins are active. In our assay, activation of $G_{stimulatory}$ proteins is realised by isoprenaline stimulation. Aim of the study was to determine the sensitivity and the specificity of the cAMP method for PT. Treatment of the cells with different amounts of PT resulted in a dose-dependent cAMP increase. In addition, treatment with other acellular vaccine components including filamentous hemaglutinin, pertactin, fimbriae, $AlPO_4$ and the components diphtheria toxoid, tetanus toxoid and inactivated polio virus did not significantly affect cAMP levels. Current activities focus on optimisation of the assay procedures and identifying and reducing sources of variation. Subsequently we aim for technology transfer. Our ultimate objective is to replace the *in vivo* HIST by a functional *in vitro* method.

I-1-698

The consistency approach for diphtheria and tetanus toxoid vaccines

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Tetanus and diphtheria toxoid vaccines are among the safest and most successful vaccines produced in decades. According to the requirements of the European Pharmacopoeia, the World Health Organization and the US Food and Drug Administration each individual lot of diphtheria and tetanus vaccine should be tested for potency in an animal model based on measuring the vaccine induced protection against a lethal challenge with the subsequent toxin or by bleeding the animals followed by an *in vitro* or *in vivo* antibody titration. In general the potency of both diphtheria and tetanus vaccine are far above the minimum protective level. Diphtheria and tetanus vaccine are produced worldwide and the annual animal usage for potency testing is extensive. In contrast with diphtheria and tetanus vaccine, HiB and HepB vaccines are relatively new vaccines that can ultimately be released on the basis of an *in vitro* characterization test after demonstration of consistency in production. For inactivated poliomyelitis vaccine the *in vivo* potency test can be waved after demonstration of the predictability of the D antigen ELISA.

At the NVI a comparable approach is explored for tetanus and diphtheria vaccine. For various lots of routinely produced D and T vaccines, in process quality indicators, batch release QC results and results of additional testing on intermediate and final products were collected. Based on the historical data of individual lots passing the *in vivo* potency test, pass and fail criteria for each parameter were set.

The results show that in a strict GMP setting and after demonstration of consistency in production the combined results during manufacturing (in process controls) and release testing are good quality indicators and safeguards for product consistency. It is concluded that diphtheria and tetanus vaccines produced by a manufacturer with a demonstrated consistency in production and using a panel of tests to obtain additional quality information, can safely be released on the basis of *in vitro* tests only.

Session I-2: Addressing systems toxicology

Session I-2: Oral presentations

I-2-460

Integrated testing strategies and *in vitro-in vivo* extrapolations: their role in chemical risk assessments

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There is increasing need for efficient approaches to assess the environmental or human health risk for many chemicals. Today's heavy reliance on approaches using animal models increasingly meets criticism for ethical, economical as well as scientific reasons.

A shift in the paradigm on assessing risk focusing more on the mechanisms of toxic action rather than apical endpoints in animal studies is emerging. This approach integrates: 1) *in silico* methods for relating chemical structure and physico-chemical properties to biological activities (QSARs), 2) modern cell biology techniques for *in vitro* measurements of data related to the toxic mechanisms by applying systems biology methods, "omics", imaging techniques, etc., with 3) computer-aided kinetic modelling (PBBK). This allows the use of these data in a quantitative *in vitro-in vivo* extrapolation (QIVIVE) by relating the *in vitro* concentration-effect relations to an *in vivo* dose-effect relationship, which then can be used as a point-of-departure in risk assessments.

A limited number of studies have been performed using this approach. In general these have shown many possibilities and some limitations for risk assessments not requiring animal toxicity studies.

I-2-218

Human multi-organ chips – a possible solution for animal free systemic ADMET testing

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Animal free systemic toxicity and ADME testing predictive of human exposure is currently the major challenge for science, regulatory bodies and the industry involved. On the one hand, modern test system engineering focuses mainly on single organ equivalents, rather than the systemic combination of organs. On the other hand, none of the currently available systems ensures long-term homeostasis of the respective tissues over months. This is primarily caused due to the lack of *in vivo*-like vasculature.

On the basis of three design pillars – device, micro-architecture and culture process – we have designed a "multi-organ-chip" (MOC) platform technology. The MOC platform is

based on a self-contained sensor-controlled dynamic micro-tissue-bioreactor, the shape of a standard microscope slide. A fast and flexible standardized prototyping procedure has been established allowing continuous improvement of the MOC design. The current fourth MOC generation supports the continuous maintenance of either human micro-scale liver tissue or miniaturized human full skin organoids (epidermis-dermis) optionally containing micro hair follicles. A micro-channel circulation system, which can be covered with human endothelial cells, ensures the circulation of nutrients through the micro-organoid growth chambers. The MOC design supports relevant substance exposure routes. Data on organoid self-assembly, indicators for equivalence to human *in vivo* performance and evidence for long-term tissue behaviour will be presented. The next generation of MOC prototypes combining human liver and skin organoids within a common vasculature will be discussed. Finally, the challenges and opportunities of this platform technology in comparison to the existing dynamic bioreactors will be addressed.

I-2-231

Long term Cyclosporin A increases transepithelial electrical resistance in renal proximal tubule monolayers: a possible role of claudin rearrangement

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The immunosuppressive compound Cyclosporine A (CsA) is known to be a chronic nephrotoxin. CsA has been shown to have diverse adverse effects on renal cells in culture including induction of reactive oxygen species, enhanced glycolysis rates and decreased cell proliferation. Here we provide evidence that CsA can also effect proximal tubular epithelial barrier function by inducing alterations in claudin isotypes.

The human renal proximal tubule cell line RPTEC/TERT1 was cultured on microporous growth supports for 2 weeks. Cells were treated with 5 and 15 μM CsA every day for 14 days. Trans epithelial electrical resistance was measured every day using the ENDOHM/EVOM system and cells were harvested on day 1, 3 and 14 for RNA isolation and subsequent transcriptomic analysis.

CsA caused a mild induction of TEER by day 2 at both concentrations. At day 7, 15 μM CsA had induced TEER to 7.8 fold and remained heavily induced until experiment termination. Transcriptomic analysis revealed a CsA induction of claudin 1, 4, 12, 15 and 23 with a decrease in claudin 2, 10 and 16. Additionally, E-Cadherin, cingulin and ZO-3 were decreased by CsA, whereas alpha catenin and ZO-1 were induced. The majority of the claudins induced by CsA are sealing claudins, while those decreased are all pore forming. Interestingly, we have also demonstrated that CsA impacts negatively on dome formation, which may be related to the fact that claudin 2 has recently been demonstrated to be able to form paracellular water channels.

Long term treatment of human renal proximal tubular cells with CsA induces TEER dramatically, which is most likely due to a loss of pore forming claudins in the tight junctions and an increase of sealing claudins. Such a dramatic alteration in proximal tubule function could have serious consequences for homeostasis.

I-2-237

Cardiac safety testing of human pharmaceuticals using fresh human tissue

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Despite the rigid regulatory framework, advances in preclinical modelling and the emergence of *in silico* technologies, approximately 25% of human pharmaceuticals are still failing Phase II trials or being withdrawn from the market due to off target effects on the heart. Using fresh, functional cardiac tissue we have attempted to bridge the gap between the currently utilised cell-based assays, animal models and the clinical situation.

The effects of various compounds on the resting tone and contractile properties of both small (<500 μm) and large (500-1000 μm) isolated functional human coronary arteries were assessed using standard myography methods. Strips of endocardial muscle were isolated from the left ventricle of functional human hearts and mounted in an organ bath. Using electrical field stimulation we assessed the effects of drugs on the contractility of the muscle. In addition, artificial arrhythmias were induced in the tissue by increasing the frequency of the field stimulation. The effects of drugs on the threshold for producing an arrhythmia were investigated.

Using the approaches described above we investigated the use of fresh, functional cardiac tissue in assessing the safety of human pharmaceuticals. Each assay was shown to confirm the observed off-target clinical effects of drugs such as cisapride and sumatriptan as well as highlighting potential mechanisms for issues observed with tegaserod and mephedrone. The data demonstrates that human tissue can be used for translational endpoints which may not be detected in whole animal or cell-based models.

I-2-110

Challenges and solutions associated with transfer of *in vitro* cellular monolayer techniques to 3D tissue models

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Although three dimensional (3D) reconstructed tissue models theoretically can provide numerous advantages to the field of *in vitro* toxicology, it is not always trivial to directly utilize techniques developed for monolayer cell culture with the appropriate 3D models. Even methods as basic as cellular viability assays may prove difficult to apply because of the difficulty in evenly treating the 3D tissue surface, targeting relevant cell types within the construct or extracting colored marker chemicals from the often complex matrix of 3D models. Other difficulties may occur during the exposure phase, e.g. adequately modeling the evaporation of volatile test materials, understanding whether topical doses applied *in vitro* are similar to those obtained *in vivo*, determining penetration of the test material, or assuring that containment of the 3-D model is sufficient to permit chemical exposure only via the intended biological barrier. Examples of solutions to these and similar challenges will be presented.

Session I-2: Poster presentations

I-2-049

Development of *in vitro* panel of assays for rapid toxicological assessment of novel munition compounds

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The U.S. Army Environmental Quality Technology and Ordnance Environmental Program is dedicated to finding replacements for substances causing environmental and/or occupational risks to health. This includes recent efforts to find a less hazardous replacement for the explosive 1,3,5-trinitrohexahydro-1,3,5-triazine (RDX) used since World War II. There are 35 energetic candidates under development. No toxicity data, however, exist for these novel formulas. Fast, high-throughput methods are needed to assess relative toxicity of new compounds proposed for use. Toxicological tests can be conducted *in vivo* and *in vitro*. *In vitro* cellular assays have the advantage of being relatively inexpensive, high-throughput and capable of addressing many mechanistic issues. This project developed an *in vitro* panel of assays to rapidly evaluate RDX and 35 novel replacement formulations for their aquatic toxicity in *Vibrio fischeri* and basal cytotoxicity in human liver cells, neurotoxic function in human neuroblastoma cells, and metabolic fate in human and animal liver tissues. These studies address an urgent need for toxicity information to assess the risks of environmental and human exposure to these new compounds. All these results will assist munition scientists in making health-based decisions regarding the design and selection of new formulas and guide toxicologists in conducting further animal studies for those formulas in development. In addition, the large number of compounds run through the "robust tests" improved the usefulness and practicability of these methodologies for fast screening of a broad range, increased volume of new munition compounds and toxic industrial chemicals/materials for their relative toxicity prior to conducting intensive animal studies.

I-2-076

Preliminary study of the revision of Japanese Pharmacopoeia test for rubber closure for aqueous infusions

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With a view to revising the Japanese Pharmacopoeia General Tests, Session 7.03, specifically for assessing rubber closures for aqueous infusions, we performed a preliminary study of a cytotoxicity test as an alternative to acute toxicity testing using mice. Twelve types of additives (rubber accelerators and antioxidants) that may be used in the manufacture of rubber closures for aqueous infusions were evaluated. The cytotoxicity test involved determination of IC_{50} values in a colony-forming assay using Chinese hamster V79 cells. The additive-derived solutions, prepared based on greater or equal IC_{50} concentrations, were compared to the sensitivity results of acute toxicity studies. Cytotoxicity assay sensitivities for all additives were found to be at least equal to the sensitivities derived by current acute toxicity testing methods. Thus, cytotoxicity assay is a valid option for assessing rubber closures for aqueous infusions.

I-2-082

Toxicogenomics to assess pesticide mixture toxicity

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Pesticides are chemical substances which include fungicides, herbicides and insecticides, some of which pose significant risks to human health. Regulatory authorities mandate submission of toxicity data (e.g. Directive 98/8/EC) prior to marketing approval of such chemicals, particularly with respect to their toxicological and ecotoxicological profiles. Such data are normally obtained on the basis of short term (repeat dose), subchronic and chronic toxicity involving a rodent and non-rodent species (e.g. dog) for mammalian toxicology. For the purposes of environmental toxicology, aquatic species are normally used. Whereas toxicity data have traditionally relied on exposure of test animals to single chemicals, the health risks posed by pesticide combinations have become increasingly evident. The large number of pesticide chemicals in use today in various combinations has created a logistical challenge for manufacturers and regulators alike, with respect to toxicity testing. The advent of toxicogenomics in combination with high throughput robotic systems could provide a replacement to animal use and cope with the large number of pesticide mixtures. The results of a pilot study will be presented to illustrate the principle of using toxicogenomics as part of a tiered testing strategy to replace the use of animals in toxicity testing of pesticide mixtures.

I-2-089

Non-animal approaches for consumer safety risk assessments: Unilever's scientific research programme

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Assuring all aspects of human safety associated with the inclusion of new ingredients into consumer products without the generation of any new animal data on these ingredients currently poses a considerable challenge. To meet this challenge, Unilever initiated a research programme in 2004 to critically evaluate the feasibility of a new conceptual approach for assuring consumer safety without animal testing (Westmoreland et al., 2010).

Unilever's current approach for safety assessment is risk-based, meaning that all available data on a new ingredient (including predicted levels of consumer exposure during product use) are used to assess the level of risk posed by its proposed consumer use. The scientific challenge we are investigating is how, in the future, novel *in vitro* and *in silico* data may be used within this risk-based framework. Our areas of focus are:

1. Risk-based approaches to assuring safety in the area of skin allergy (underpinned by a systems approach to understanding the mechanistic basis of skin sensitisation).

2. A case study (DNA damage-induced carcinogenicity) to evaluate the potential application of a toxicity pathways-based approach (TT21C) (Krewski et al., 2010) to risk assessments for repeat dose toxicity.

It is a significant scientific challenge to understand how safety may be assured for such complex endpoints, using data derived from a pathways-based approach that is rooted in mechanistic understanding of the underlying biology. An equally important challenge is how, if successful, such a pathways-based approach to safety assessment could ultimately receive regulatory acceptance.

References

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I-2-098

Accelerating the transition to 21st century toxicology: outcomes of a workshop organized by the Human Toxicology Project Consortium

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In 2007 the U.S. National Research Council (NRC) published "Toxicity Testing in the 21st Century: A Vision and a Strategy". The report calls for a fundamental shift in the way that chemicals are tested for human health effects and are evaluated in risk assessments. The new approach would decrease the current reliance on animal studies and move towards *in vitro* methods, typically using human cells in a high-throughput context. The *in vitro* methods would be designed to detect significant perturbations to "toxicity pathways", i.e., key biological pathways that, when sufficiently perturbed, lead to adverse health outcomes. To explore progress on the report's implementation, the Human Toxicology Project Consortium hosted a workshop entitled "Accelerating Implementation of the NRC Vision for Toxicity Testing in the 21st Century" on November 9-10, 2010 in Washington, DC. The goal of the workshop was to identify ways to accelerate implementation of the NRC vision for toxicity testing in the 21st century. The workshop format consisted of plenary presentations, break-out group discussions, and concluding commentaries. The speakers and session chairs were drawn from industry, academia, government, and public interest organizations. The workshop identified a number of recommendations for accelerating implementation of the NRC vision, including the need for overarching strategic planning, coordination, and communication, as well as the development of pilot projects with more direct approaches to implementation. The principal recommendation was the urgent need to establish a steering group to help manage the planning, coordination, and communication needed to make the NRC vision a reality.

I-2-099

Assessment of cardiac toxicity of doxorubicin on the rat electrocardiogram and its prevention by drugs

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The therapeutic usefulness of doxorubicin (Dox), an anthracycline antibiotic used as an anticancer agent, is limited by its cardiotoxicity. Dox-induced cardiotoxicity is mainly attributed to accumulation of reactive oxygen species and interaction of Dox with cellular iron metabolism. In this study, the potential protective effects of the free radical scavenger, proanthocyanidins (Pro) and the iron chelator, deferiprone (Def) against Dox-induced cardiotoxicity were investigated using rat electrocardiogram (ECG). Cardiotoxicity was induced in rats by single i.p. injection of Dox (15 mg/kg). The effect of Pro (70 mg/kg, orally) or Def (10 mg/kg) on Dox-induced cardiotoxicity was examined. Three days after Dox injection, rats were anesthetized with urethane (1.8 g/kg, i.p.) and electrocardiograms were recorded from standard lead II limb leads using a single channel ECG (Fukuda ME Kogyo Co. Ltd., Model: 501-B III, Tokyo, Japan). Then, the jugular vein was cannulated for aconitine infusion to induce ventricular tachycardia. Aconitine was infused in a concentration of 2.5 μg/ml and a flow rate of 0.5 ml/min. Dox caused significant increase in heart rate (40.76%), elevation of ST segment (95.87%), prolongation of QT interval (23.15%) and increase in T wave amplitude (56.55%). Moreover, Dox caused a significant decrease in the threshold dose of aconitine producing ventricular tachycardia (34.61%). Administration of Pro prevented these alterations except QT interval prolongation, while Def administration prevented change in all the measured parameters. These results suggest that both Pro and Def might be potential cardioprotective agents against Dox-induced cardiotoxicity.

I-2-102

Prototype pathway research for Toxicity Testing in the 21st Century (TT21C) – a case study using DNA damage characterisation

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The NAS/NRC report on TT21C offers an attractive future paradigm for toxicologists based on toxicity pathway perturbations and human exposure, rather than apical end points measured in experimental animals. In the context of a risk-based approach to the safety assessment of ingredients in consumer products, we are exploring the practical application of this paradigm through a collaborative research programme with the Hamner Institutes. Work investigating the individual elements of: (a) exposure and consumer use assessment, (b) fast, high-content-information *in vitro* assays in human cells, (c) dose-response assessments, (d) computational models of the circuitry of relevant toxicity pathways, and (e) pharmacokinetic models supporting *in vitro* to *in vivo* extrapolations, are being brought together to craft novel risk assessments for putative "genotoxic" case-study chemicals, maintaining exposure below the levels that significant pathway perturbations occur. A combination of techniques are being employed and combined, from Cellomics high content imaging for dose-response assessments of DNA damage and underlying threshold characteristics, to bioavailability measures derived from bloodspot and/or micro-dosing determinations in human subjects. We anticipate that this prototype toxicity pathway research will provide scientific evidence to support the future application of the TT21C principles, and will foster greater development of these much-needed methodologies.

I-2-175

Comparative cytotoxicity evaluation of essential oil from *Minthostachys setosa* in CHO cells, NIH/3T3 cells and human keratinocytes

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Minthostachys is a genus of shrubs of the Lamiaceae family, traditionally used as condiments or preservatives in food storage by the communities of the Andes in Peru. Aromatic and antimicrobial properties of the essential oil from *Minthostachys setosa* confer a potential use in cosmetics, leading to the goal of this work to evaluate the toxicity of the essential oil from *Minthostachys setosa*. The use of alternative methods for new ingredients toxicity determination is commended in the 7th Amendment of the European Cosmetics Directive 2003/15/EC. The essential oil from *Minthostachys setosa* was obtained from the aerial parts of the plant through hydrodistillation. The three cells types – NIH/3T3, CHO and human keratinocytes - were seeded in 96-well plates and incubated at 37ºC on 5% CO_2. Eight concentrations – 0.0028 mg/ml to 0.28 mg/ml – dispersed in culture medium with the surfactant Polysorbate 20 were added after 24 h. After one day, MTS was added to the cells and analyzed by spectrophotometer at 490 nm. All the tested concentrations except the last one presented cell viability higher than 90% compared to the control. The 0.28 mg/ml concentration showed cell viability of 30% for human keratinocytes, 87% for CHO and 79% for NIH/3T3. It is worth mentioning that the antimicrobial minimal concentration of *Minthostachys setosa* oil is 0.028 mg/ml, which is in the non-toxic range; therefore, the results suggest that essential oil can be safe to use as a natural preservative in cosmetic formulations.

I-2-195

Alternative *in vitro* phototoxicity test using reconstructed skin model KeraSkin™

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The reconstructed human skin model, KeraSkin™, has similar morphology, characteristics, and even biochemical marker expressions to native human skin. These similarities offer usefulness as an alternative testing method for skin irritation and corrosion, which has been proven by several validation studies. Therefore, this study was conducted to validate the *in vitro* phototoxicity test method using KeraSkin™. Nine phototoxic or non-phototoxic test chemicals were topically applied onto KeraSkin™. After 24 h incubation, the KeraSkin™ was exposed to 20 J/cm^2 of UVA. The test chemicals were removed, and cell viability was quantified by MTT assay after incubation for another 24 h. Phototoxicity was determined by viability (>20%), photo-irritation factor (PIF; >2), and mean photo effect (MPE; >0.1). When only viability and PIF criteria were applied, accuracy was 88.9%, showing 85.7% of sensitivity and 100% of specificity. But adding MPE criteria increased the overall accuracy to 100%, showing 100% sensitivity and specificity. In conclusion, a good *in vitro* alternative phototoxicity test method using KeraSkin™ was successfully established.

I-2-232

Comparison of xenobiotic metabolizing enzyme activities in normal human skin and reconstructed human skin models from skinethic laboratories

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Skin represents the major protective barrier of the body to its environment. Also, skin is an organ involved in the metabolism of xenobiotics, and its ability to metabolize xenobiotics can become consequent when considering its total surface area (2 m^2). Consequently, research on skin metabolism would need a real scientific effort to characterize skin metabolizing enzymes and their activities. In addition, the 7th European amendment to the Cosmetics Directive forbids the use of animal testing to assess the efficacy and safety of new cosmetic ingredients. This policy has forced the cosmetic industry to develop *in vitro* tools such as reconstructed human skin models as alternative methods to animal experiments. For these reasons, these skin models need to be characterized and compared with normal human skin (NHS) samples in terms of metabolic capabilities. This work presents the mRNA expression of several enzymes (CYP450, esterase, ADH, ALDH, NAT, GST, UGT, SULT, etc.) and their apparent catalytic parameters (apparent K_m, V_{max} and the ratio V_{max}/K_m) in skin models compared with NHS. Results showed that all these enzymes involved in the metabolism of xenobiotics are expressed and effective in the NHS and skin models. Also, apparent ratio V_{max}/K_m (estimating the metabolic clearance) and the metabolic abilities were often comparable between skin models and NHS. These results indicate that the skin models can substitute for NHS to select cosmetic ingredients on the basis of their metabolism, efficacy and/or safety.

I-2-233

Skin metabolism

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Skin is mainly considered to be a physical barrier. However, skin is also recognized to be an important site of extrahepatic metabolism and requires to be characterized in terms of metabolic abilities. Moreover, the 7th European amendment to the Cosmetic Directive will totally ban, in 2013, the use of animal testing to develop and trade new cosmetic ingredients in the European Community. For more than twenty years, L'Oréal has been producing and improving reconstructed human skin models (skin models) as *in vitro* alternative methods to animal experimentation. It uses them to predict different toxicological endpoints such as skin corrosion and skin irritation on finished products and cosmetic ingredients in development. For these reasons, it is essential to assess and compare these skin models with normal human skin (NHS) in terms of metabolic abilities.

In this work, the apparent metabolizing enzyme activities of three SkinEthic™ skin models were determined and compared with NHS for the main cytochrome P450-dependent monooxygenases involved in drug metabolism, esterases, alcohol and aldehyde deshydrogenase, glutathione S-, N-acetyl-, glucuronyl- and sulfo-transferases. Results show that the skin is much better equipped in conjugating enzymes and esterases than in P450-dependent monooxygenases and define it as a much more detoxifying organ than a bioactivating one. They demonstrate as well that the skin models are equivalent to NHS in terms of metabolic capabilities. Consequently they are useful as substitutes of human skin samples to assess the local efficacy and safety of new cosmetic ingredients.

I-2-238

Deciphering the mechanisms of action of potassium bromate in human and rat renal proximal tubular cells

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Potassium bromate (KBrO3) is an oxidising agent and is widely used in the food industry as a maturing agent for flour and as a dough conditioner. However, it has shown to be both a nephrotoxin and a renal carcinogen.

Within the carcinoGENOMICS project we conducted a genome-wide transcriptomic screen in NRK-52E and RPTEC/TERT1 cells treated with a sub-toxic concentration of KBrO3 (0.5 and 1 mM, respectively) for 6, 24 and 72 h. Analysis of altered gene expression revealed "NRF2-mediated oxidative stress", "Glutathione metabolism", "Tight junction signaling", and "DNA damage, p53 signaling and cell cycle" as the most enriched pathways. These gene alterations were reflected by increased expression of heme oxygenase 1 and NQO1 proteins, glutathione depletion, cytosolic occludin accumulation and decreased trans-epithelial electrical resistance.

The characterized biological responses to KBrO3 exposures indicate oxidative damage as a primary mechanism leading to DNA damage and the involvement of p53 and cell cycle alterations. A novel finding of this study is that sub-lethal oxidative damage induced by KBrO3 affects the expression of a number of tight junction proteins. The data elaborated here will be useful for screening other compounds for oxidative damage potential.

I-2-262

Alternative approaches for the evaluation of repeated dose toxicity and its use for quantitative risk assessment of cosmetic ingredients

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including repeated dose toxicity, in view of the full marketing ban foreseen in 2013 for cosmetic products and ingredients tested on animals in Europe. For repeated dose toxicity, current animal studies provide information on many endpoints. They allow evaluation of an integrated response and its quantitative aspects, making its replacement very challenging. Alternative methods have been developed mainly as standalone methods for predicting effects in specific target organs. Initial attempts of computer-based modelling/techniques suggest the feasibility of developing models providing meaningful predictions of chronic toxicity. "Omics" technologies have been applied recently to *in vitro* models for the purpose of understanding and ultimately predicting toxicity. However, a major challenge is to develop approaches for combining and interpreting data on multiple endpoints, obtained from several alternative methods. The experts concluded that, for quantitative risk assessment, enhanced scientific knowledge on exposure, toxicokinetics, dose response, mechanisms of toxicity, and extrapolation between exposure routes is needed. Better understanding of mode of action and key events associated with repeated dose toxicity endpoints would support the development of alternative approaches. Biokinetic models need to be improved to support dose response extrapolation from *in vitro* to *in vivo*. Additionally, optimal use of existing data by the Threshold of Toxicological Concern concept, read-across and integrated testing strategies can provide opportunities to avoid *in vivo* testing. Although full replacement will not be available by 2013, possibilities for reduction/refinement for repeated dose toxicity testing were identified and will be described.

I-2-273

Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including toxicokinetics, in view of the full marketing ban foreseen in 2013 for cosmetic products and ingredients tested on animals in Europe. Toxicity risk assessment is moving away from descriptive animal toxicity studies towards mechanistic *in vitro* screening systems and their integration with various types of computational approaches, including modelling and simulation. The use of omics techniques and high-throughput screening are some of the emerging tools to ensure predictive toxicity risk assessment.

Toxicokinetics (TK) is the endpoint that provides information about the penetration into and fate within the body of a toxic substance, including the possible emergence of metabolites, and their absorption, distribution, metabolism and excretion (ADME). Currently, when there is an increasing reliance on non-animal testing approaches, toxicokinetics has been identified as a key element to integrate the results from *in silico*, *in vitro* and *in vivo* studies. There are several crucial contributions from TK knowledge in integrated risk assessment. TK is needed to estimate the range of target organ doses that can be expected from realistic external exposure scenarios. This information is crucial for determining the dose range that should be used for *in vitro* testing. TK is necessary to convert the *in vitro* results, generated at tissue/cell or sub-cellular level, into dose response or potency information relating to the entire target organism, i.e. the human body.

For the optimal use of TK knowledge it is imperative that in all *in vitro* toxicity testing systems the behaviour of the compound

under study is investigated, to produce data on disposition (biotransformation and transport) and concentration-response relationships. *In silico* approaches, such as QSARs, read across, etc., allow further quantitation of specific processes needed for the prediction of TK *in vivo*.

Physiologically based toxicokinetic modelling (PBTK) is currently regarded as the most adequate approach to simulate the fate of compounds in the human body and can be used to estimate the relevant exposure at the organism level from measured *in vitro* concentrations, and *vice versa*. However, validation is currently a bottleneck and novel approaches are needed to ensure that mechanistic and integrative risk assessment can be employed to the full extent.

I-2-290

The Integrated discrete Multiple Organ Co-culture (IdMOC) technology as an *in vitro* model for systems toxicology

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A major limitation of *in vitro* toxicity testing is the use of single cell-type cultures for the evaluation of organ-specific toxicity. Such systems ignore the critical interactions between different cell types within an organ or between multiple organs. The multiple cell type/organ interactions include paracrine signaling, endocrine signaling, as well as xenobiotic metabolism (e.g., hepatic metabolism of a xenobiotic with toxic metabolites exerting effects on extrahepatic tissues). The Integrated discrete Multiple Organ Co-culture (IdMOC) technology is developed in our laboratory to overcome this major deficiency of most single cell type *in vitro* systems. In the IdMOC, multiple cell types are co-cultured as physically separated cultures interconnected by an overlying medium. IdMOC employs the wells-in-a-well concept, with shallow inner wells situated within a larger containing outer well. The dimensions of the inner wells are designed to minimize dilution of critical extracellular signals (e.g. metabolites; cytokines) by the overlying medium. The IdMOC system has been applied to evaluation of toxic potential of xenobiotics towards key human organs including liver (hepatocytes), kidney (renal proximal tubule cells), lung (airway epithelial cells), nervous system (neurons/astrocytes), and vascular endothelium (aortic endothelial cells). The toxicants evaluated include pharmaceuticals, pesticides, environmental pollutants, and cigarette smoke condensates. Endpoints used successfully with the IdMOC include cellular ATP content, MTT metabolism, caspase activation, and gene expression using real-time PCR for the evaluation of cell-type specific functions. Specific examples of the application of IdMOC to evaluate organ-specific toxicity, metabolism-dependent toxicity, paracrine signaling and endocrine signaling will be described. The IdMOC system represents an improved *in vitro* experimental system modeling complex multiple organ interaction in an intact animal/human. The IdMOC system has the potential to be used routinely as a replacement of whole animal studies for the evaluation of xenobiotic properties.

I-2-294

Implementation of a redesigned preclinical biocompatibility testing program to support personal lubricant medical device products

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A traditional biocompatibility testing battery for a personal lubricant medical device consists of *in vitro* cytotoxicity testing, systemic toxicity testing in mice, irritation testing in male and female rabbits (RPI and RVI, respectively), and evaluation of contact sensitization potential in guinea pigs. A redesigned preclinical biocompatibility testing program to support personal lubricant medical device development has been developed in the spirit of the 3Rs; reduction of the number of animals used, refinement of the data derived from the animals used, and replacement of a non-relevant model. The RVI and RPI tests have been refined by expanding the scope of the protocols to evaluate both genital irritation potential and systemic toxicity endpoints concurrently, thereby making more efficient use of the animals on study, exhibiting greater relevance than mouse IV or IP exposure, and still yielding some degree of exaggerated exposure relative to intended consumer use. Furthermore, the standard

agar overlay cytotoxicity assay in monolayers of L-929 mouse fibroblast cells will be replaced with the Epi-Vaginal assay model, a human 3D vaginal-ectocervical tissue construct, to measure cytotoxic potential. Additionally, there is greater reliance on raw material selection and paper toxicology assessment when possible, to support the progression of the personal lubricant directly to human clinical RIPT testing instead of conducting the guinea pig sensitization testing. In summary, this redesigned testing program is being implemented with the intention of providing more predictive results with greater suitability to the test material, while reducing and refining the use of animals for biocompatibility testing.

I-2-310

Taking a mode-of-action approach to designing a hepatotoxicity screening strategy using the HepaRG cell model and high content imaging

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The liver is central to the metabolism of xenobiotics and faced with harmful effects of toxic substances. Evaluating the risk of liver toxicity is a major issue and there is still no established *in vitro* screening strategy to reliably identify potentially hepatotoxic chemicals. In the approach described here, a mode-of-action targeted analysis of the literature has been used to identify toxicity pathways and the key biological events associated with them. This knowledge has then been used to design a multiparametric HTS experiment to classify chemicals based on their likely association with a specific mode-of-action.

We used a metabolically competent cellular model, HepaRG, and high content imaging implemented on a HTS platform. The HepaRG cell line expresses the major liver functions, including P450s, phase II enzymes, transporters and nuclear receptors at levels comparable to those found in primary hepatocytes.

The high content screening approach we adopted is based on automatic analysis of image-sets acquired with an epifluorescent microscope, for the quantification of immuno-fluorescently stained biomarkers expressed by treated HepaRG cells. A quantitative high throughput screening format was employed using a 96-well plate format, which facilitated the testing of a set of 92 reference chemicals and drugs with known hepatotoxic activity. Multiple cellular phenotypic changes were analysed by staining with fluorescent dyes for identification and quantification of response parameters. A biostatistical model was then developed to associate the test chemicals with different mode-of-action based categories. A systematic comparison of the classification results with literature findings allowed a preliminary validation of the approach.

I-2-325

Toxicity of unfiltered and filtered diesel exhaust under various loads

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Diesel engine exhaust contains numerous gas pollutants and particulate matters which may pose adverse health risks. The use of diesel engines for powering passenger cars has gained more popularity due to diesel fuel containing more energy per litre than petrol. In addition, combustion inside a diesel engine is more complete, hence there is less emission of pollutants such as CO and HC.

The aim of this study was to develop and validate the use of a direct dynamic method for exposing human cell lines (A549 and HepG2) directly to filtered and unfiltered diesel exhaust at a range of exposure of 7.5 to 15 min, and post-incubation periods of 0-24 h. In summary, cells were grown on porous membranes and placed inside dynamic exposure chambers connected to a diesel engine exhaust. The cytotoxicity of the exhaust was analysed using ATP, MTS and NRU assays. The exhaust was also analysed for pollutants such as CO, CO_2, NO_x, HC and diesel particulate.

Results of this study indicated that human cell lines (A549 and HepG2) were sensitive to diesel exhaust pollutants at all exposure

times and loads. The 0 h post-incubation period assay showed greater reproducibility, while there was no statistical significant difference in cell viability between filtered and unfiltered diesel exhaust. The results suggest that the gaseous component of diesel exhaust causes cell death rather than the particulates.

I-2-339

A virtual liver for pharmacological and toxicological investigations: multiscale, location dependent, xenobiotic hepatocyte response mechanisms

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We describe experiments on iteratively improved, multizone *in silico* livers (ISLs) (e.g., Park et al., 2010) that are designed to improve instantiated, predictive, mechanistic insight into hepatic disposition, enzyme induction details, and hepatotoxicity patterns (Sheikh-Bahaei et al., 2010) that influence safety and efficacy testing of new xenobiotics (XBs). ISLs are examples of a new class of biomimetic, discrete event, object and agent oriented, multilayered, multiscale, physiological models (Hunt et al., 2009). Hepatocytes within liver lobules are quasi-autonomous; the mechanistic details of XB clearance and enzyme induction are location dependent. A project goal is to make the same true within ISLs: intralobular, XB-specific metabolic clearance, enzyme induction, and biliary elimination patterns quantitatively mimic literature data. ISL experiments use independent XO objects that carry a list of physicochemical properties (PCPs) of the referent compound. Upon dosing, XOs percolate through ISL spaces. Most events are stochastic. An XO that encounters an enzyme within an ISL hepatocyte (Hc) may be metabolized, and that can initiate enzyme induction events that are specific to that Hc and location. Absent XO exposure, enzyme number per Hc is within a "normal", zone dependent range. An XO-enzyme interaction can initiate a metabolic event and/or an induction event. A metabolite that maps to a toxic counterpart can subsequently initiate a toxicity event. There is a location signal dependent enzyme removal rate (rule) that maps to a blood oxygen gradient. Rules are placeholders for more fine-grained mechanisms. Simulations were conducted on Amazon's EC2 cloud platform using a validated protocol (Ropella and Hunt, 2010).

The AR&D Foundation provided support.

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I-2-368

COSMOS – A new European project to develop computational models for the repeat dose toxicity of cosmetic ingredients to humans

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The COSMOS (Integrated *In silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety) Project is funded by the European Commission and COLIPA. It is part of the Seurat Cluster (start date 1 January 2011), which aims to assess the safety of cosmetic ingredients to humans using non-test methods. The focus of the COSMOS Project is to develop an integrated suite of open source and open access computational models to predict human repeated dose toxicity for cosmetic ingredients. This suite of models will form a flexible and transparent tool within an integrated computation-

al workflow. The *in silico* workflows will allow for the prediction of repeated dose toxicity to humans through the integration of models based on threshold of toxicological concern (TTC), innovative chemistry and physiologically based pharmacokinetics (PBPK). The workflows will be adaptable and form a set of building blocks, allowing users to incorporate their own data and search existing data compilations. The specific objectives of the COSMOS Project are to:

Collate and curate new sources of toxicological data and information from regulatory submissions and the literature.

– Create an inventory of known cosmetic ingredients and populate with chemical structures.
– Establish thresholds of toxicological concern for endpoints relating to human repeated dose toxicity.
– Develop innovative strategies based around categories, grouping and read-across to predict toxicity and relate to adverse outcome pathways where possible.
– Establish kinetic and PBPK models *in vitro* and *in silico* and other relevant data to predict target organ concentrations and long term toxicity to humans.
– Integrate open source and open access modelling approaches into adaptable and flexible *in silico* workflows using the KNIME technology.

The funding of the EU COSMOS Project (Health-F5-2010-266835) is gratefully acknowledged.

I-2-426

The platform on science within the European Partnership for Alternative Approaches to Animal Testing (EPAA)

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative between the European Commission and industry, created in November 2005 to promote the development and implementation of novel 3Rs approaches to regulatory testing. The partnership focuses on identifying research needs, developing novel approaches and strategies, promoting communication, validation and acceptance of alternative approaches. Its activities are coordinated by three platforms: Science, Regulation and Communication/Dissemination. Current activities within the EPAA Platform on Science include:

– Building up a research consortium bringing together computational chemists and system biologists to evaluate whether special aspects of liver toxicity can be identified without animal studies as was recommended during the 2010 workshop "Harnessing the chemistry of life".
– A gap analysis in current stem cell research with focus on toxicological pathways and applications for systemic toxicity testing. It is planned to stimulate collaboration of experts who would not traditionally apply their work to toxicology.
– An *in vitro* and *in silico* ADME project addresses one of the greatest unmet challenges for complete replacement.
– Methods used in different sectors/companies are being compared to optimize the current toolbox and identify gaps.

Full replacement of animals with alternative approaches is not achievable in the short term and unpredictable in the long term. However, as new *in vitro/in silico* approaches become available, they are considered for inclusion in integrated testing strategies (ITS). Therefore, all the EPAA projects are being considered for their possible impact in integrated testing strategies.

I-2-450

Data integration and analysis approaches for toxicogenomics applications in the 3Rs: McDSA, ConXbase and ToxProfiler

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To reduce, refine and replace animal testing in toxicology, it is increasingly important to integrate information from heterogeneous data sources. The toxicity of a substance may be learned from integration of data from animal or non-animal studies, which can be improved with increasing availability of omics data, allowing for understanding of mechanisms of toxicity. In addition, information improving chemically and biologically-based grouping of chemicals contributes to the 3Rs. This complexity of different data resources necessitates a system that allows integration of this information. To this end, a framework for Metadata Capture and Data Storage and Analysis (McDSA) to integrate toxicological knowledge with toxicogenomics data is implemented. Metadata capture entails the systematic description of the experimental setup in a database. Besides metadata, project data need to be brought together with the actual measurements, as well as with the biological context of the results. To achieve this, we have developed ConXBase. ConXBase is a web-based tool that connects projects, researchers, studies, biological source, experimental conditions, chemicals, chemical groupings, genes, pathways, and experimental results. ConXBase is integrated with ToxProfiler, a data analysis and database tool for the analysis of toxicogenomics data at the level of biologically relevant gene sets. The benefits of this infrastructure will be illustrated using datasets from the Netherlands Toxicogenomics Centre (NTC), the EU Project Carcinogenomics, as well as the ASAT dB project, in which human disease mechanisms are explored to improve animal-free chemical hazard assessment.

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I-2-470

The FP7 Project AXLR8 – Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally coordinated research and technology development

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The EU FP7 coordination support action project AXLR8 (=accelerate) aims to support the transition to a toxicity pathway-based paradigm for quantitative risk assessment. To reach this goal, AXLR8 is conducting the following activities: 1) organize a series of annual workshops to map research progress, gaps and needs in the FP6/FP7 program on alternative testing strategies; 2) provide a forum for enhanced interdisciplinary and international communication, coordination and collaboration in order to maximise the impact of available resources; 3) work to improve acceptance procedures to provide for the uptake of validated 3Rs methods, including the transition to 21st century systems as they become available; 4) produce annual progress reports on the state of the science, including recommendations on priority research and funding targets to ensure a prominent role for European science in this rapidly developing global research area.

In 2010 and 2011 the AXLR8 workshops (AXLR8-1 & AXLR8-2) have focused on progress made in the EU FP6/FP7 projects funded by the health theme of the DG Research and Innovation "Alternative Testing Strategies: Replacing, reducing and refining use of animals in research". The results of the discussions and recommendations of the AXLR8 Scientific Panel at the AXLR8-1 workshop 2010 have been published in the AXLR8 Progress Report 2010. The results and the recommendations of the 2011 AXLR8-2 workshop on a "Roadmap to innovative toxicity testing (ITT)" will be presented.

I-2-545

Displacement of test chemicals from serum constituents in mixtures and possible effects on free concentrations and *in vitro* assay results

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In vitro assays may be used to estimate toxicity of mixtures of chemicals. The concentrations of chemicals in these assays are normally expressed as nominal concentrations. However, the freely available concentration may be much lower than the nominal concentration because the chemical may bind to serum constituents in the culture medium. When chemicals are exposed to an *in vitro* assay in a mixture, one chemical that is normally bound to serum protein (and thus has a low free concentration) may be displaced from serum protein by another chemical that binds more strongly to protein, thus increasing the free concentration and response of the first chemical in the assay. When nominal concentrations are used, one could falsely attribute this increase in response as being a direct effect of the second chemical. Therefore, the aim of this study was to measure the free concentration of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), individually and in a mixture, in a CAFLUX and a cytotoxicity (AlamarBlue) assay, using solid phase microextraction (SPME). Results indicate that the extent of synergistic and antagonistic effects attributed to non-AhR agonists may in part change when considering displacement of AhR agonists from serum constituents by more lipophilic non-AhR agonists.

I-2-546

In vitro kinetics of chlorpromazine after repeated exposure in primary rat hepatocytes and human HepaRG cells

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In the extrapolation of *in vitro* data to the *in vivo* situation, it is important to take the *in vitro* kinetics of a compound into account. In this project, part of the FP7 project Predict-IV, the *in vitro* kinetics of chlorpromazine hydrochloride (CPZ) were determined in two different liver cell systems. Primary rat hepatocytes (sandwich cultured) and human HepaRG cells were exposed daily to two concentrations of CPZ for 14 days. Samples were taken from medium, cells and plastic at five different time points after the first and last day of treatment. These samples were analyzed by HPLC-UV to determine the total concentrations of CPZ.

The concentration of CPZ in the supernatant decreased over time. This decrease was more pronounced in the primary rat hepatocytes. CPZ was taken up by the cells, as shown by an initial increase in the amount of CPZ inside the cells, while at later time points, the amount of CPZ decreased. More CPZ was found on the last day of treatment, indicating accumulation of CPZ inside the cells. Plastic binding of CPZ was only found in the HepaRG cell cultures. After 24 h, all CPZ had disappeared from the rat hepatocyte culture compared to only half in the HepaRG cell culture.

The *in vitro* kinetics of CPZ were different in the two *in vitro* liver systems. Furthermore, a difference was seen between the first and last day of exposure. Therefore, the kinetics of a compound in an *in vitro* cell system should be taken into account for a reliable interpretation of toxicity results.

I-2-577

Experiences with cytotoxicity assays to select starting doses for acute oral toxicity testing

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With the objective of reducing and refining animal experiments for determination of acute oral toxicity, we have implemented cytotoxicity data to estimate acute oral starting doses in rats. We used the neutral red uptake (NRU) method in Balb/c 3T3 fibroblasts (ICCVAM, 2006) to determine the cytotoxicity of about 120 test substances including chemicals and formulations. The estimated predicted starting doses were then used in rat acute oral toxicity studies.

Comparing the predicted LD_{50} and the *in vivo* determined GHS classification, the cytotoxicity assay showed good prediction only for low toxic substances (83%, GHS Cat. 4, >300-2000 mg/kg body weight). The overall concordance was rather low (36%), mainly because 76% of the tested substances were classified as low toxic *in vitro*, but only 34% *in vivo*. Expanding the prediction +/- one category greatly enhanced the overall concordance to 82% with only 8% overpredicted (*in vitro* Cat. 3, >50-300 mg/kg, *in vivo* Cat 5, >2000 mg/kg) and 10% underpredicted test substances (*in vitro* Cat. 4, *in vivo* Cat 1-2, ≤50 mg/kg).

The use of cytotoxicity data to predict starting doses did not sufficiently contribute to the refinement and reduction in acute oral toxicity testing. As the predictivity of the *in vitro* test highly depends on specific properties of the tested substances, further analysis considering metabolic breakdown and mode of action are necessary. Using this data the applicability domain of the cytotoxicity assay for the prediction of acute oral toxicity in rats might be refined.

I-2-594

Autonomous virtual hepatocyte micromechanisms learn to respond to compound physicochemical properties (PCPs): clearance from simulation experiments, given new compound PCPs, predicts *in vitro* clearance

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We demonstrate the feasibility of using *in silico* hepatocyte cultures (ISHCs) to provide predictions of the intrinsic clearance (CL) of compounds in hepatocyte cultures. We compare results to predictions obtained using a multiple linear regression method. Our expectation is that the method can influence safety and efficacy testing of new xenobiotics by, for example, being extended to predict *in vivo* clearance of new compounds in humans. Within ISHCs, mobile "compounds" carry referent compound's physicochemical properties (PCPs). We used an Iterative Refinement Protocol for ISHC refinement and development of parameterization methods. Quasi-autonomous "hepatocytes" and their components (including "transporters" and "enzymes") use a small, event-specific subset of PCPs to interact with mobile "compounds" each simulation cycle. The probability of occurrence for each event type is specified by a rule based on a subset of PCPs known to influence that event counterpart *in vitro*. ISHC experiments mimic *in vitro* counterparts. *In silico* clearance is measured the same as *in vitro* and used to predict a corresponding CL value. For 39 of 73 compounds having calculated CL standard deviations (SDs), 79% of ISHC predictions and 23% of regression predictions were within CL ±2 SD. For all 73 compounds, 38% of ISHC predictions and 32% of regression predictions were within a factor of two of the referent CL values. ISHC details during simulations stand as a mechanistic hypothesis of how clearance phenomena emerge during *in vitro* experiments.

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I-2-639

Effect of Trichostatin A on miRNA expression in cultures of primary rat hepatocytes

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In the present study, the effect of Trichostatin A (TSA), a histone deacetylase inhibitor, was investigated on the microRNA (miR, miRNA) expression profile in cultured primary rat hepatocytes by means of microarray analysis. Simultaneously, albumin secretory capacity and morphological features of the hepatocytes were evaluated throughout the culture time. In total, 25 out of 348 miRNAs were found to be differentially expressed between freshly isolated hepatocytes and 7-day cultured cells. Nineteen of these miRNAs were connected with "general metabolism". MiR-21 and miR-126 were shown to be the most up and down regulated miRs upon cultivation and could be linked to the proliferative response triggered in the hepatocytes upon their isolation from the liver. MiR-379 and miR-143, on the other hand, were found to be the most up and down regulated miRs upon TSA treatment. Together with the higher expression of miR-122 observed in TSA-treated versus non-treated cultures, we hypothesise that the changes observed for miR-122, miR-143 and miR-379 could be related to the inhibitory effects of TSA on hepatocellular proliferation.

I-2-642

Detection of endpoints and biomarkers of repeated dose toxicity using *in vitro* systems

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DETECTIVE is part of an integrated research strategy towards the replacement of animal testing set up by the European Commission within the FP7 Health Programme and supported by the European Cosmetics Association. Within this collaborative project, 15 partners address the development of biomarkers of long-term toxicity in human target cells.

As from 1 January 2011 and for a duration of 5 years, emphasis will be put on the systematic exploitation of a battery of complementary functional and "-omics" readouts, including high content and high throughput screening platforms to identify and investigate human biomarkers in cellular models for repeated dose *in vitro* testing. While functional parameters give more insights into the effects of toxicants on specific cell functions of interest, "-omics" techniques will deliver data on the entire cellular situation at the molecular level. Importantly, DETECTIVE will perform for the first time an in-depth investigation of repeated dose effects on epigenetics and microRNA (miRNA) expression, thus exploring whether such analyses deepen our understanding of toxic modes of action.

Upon combination and subsequent integration of the various readouts, biomarkers of optimal predictivity for human long-term toxicity *in vitro* can be obtained. Based on integrative statistical analysis, systematic verification and correlation with *in vivo* data, the most relevant, highly specific, sensitive and predictive biomarkers will be selected. DETECTIVE concentrates on hepatoxic and cardiotoxic, and – to a smaller extent – nephrotoxic effects, representing three common target organs of repeated dose toxicity. Ultimately, developed concepts will also be applicable to other organs or organ systems affected by systemic toxicants such as the nervous system. Furthermore, it is expected that DETECTIVE will be able to define human toxicity pathways relevant for all organs.

I-2-722

Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals – COACH

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The European Commission and the European Cosmetics Association (COLIPA) have launched a research initiative to improve current safety assessments and to accelerate the development of alternatives for the complex human health endpoint "systemic repeated dose toxicity testing" by making a total funding of € 50 million available. As a 1st step towards a vision of a "Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)," six individual research projects SCR&Tox, HeMiBio, DETECTIVE, COSMOS, NOTOX and ToxBank kicked off in January 2011 in order to develop technologies and to gain necessary scientific knowledge relevant for assessing repeated dose effects of cosmetic ingredients.

The collaboration within the cluster of six research projects (SEURAT-1) is facilitated by the coordination action COACH ("Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals") which will constitute the secretariat of the cluster in order to:

– monitor progress of the cluster towards the goals of the SEURAT initiative
– facilitate the information exchange and collaboration between the different cluster projects
– disseminate results of the cluster via annual strategy books, leaflets, in meetings, etc.
– cooperate with other international research teams
– prepare a strategy for future research activities in an international context

The poster will provide an overview of the objectives of COACH in order to facilitate the activities of the SEURAT-1 cluster.

Session I-3: Biological and biotechnology-based therapeutics

Session I-3: Oral presentations

I-3-066

Consideration of alternative approaches for the purpose of reducing animal numbers in the preclinical development of biotherapeutic products

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As the development of biotherapeutics becomes a more advanced science-based challenge, the selection of relevant animal models, utility of traditional species and alternatives to traditional safety approaches are becoming more accepted and in fact, necessary. The last ten years has seen a significant advancement of our knowledge and development of biotechnology-derived products for the treatment of debilitating, life-threatening diseases. As the therapies being developed are more sophisticated and generally more specific, the need to establish safety in relevant models has become more and more of a challenge. Alternatives to the traditional safety approach include the use of homologous proteins, transgenic animals, animal models of disease as well as state of the art non-invasive, non-terminal technologies, such as high resolution imaging and scanning methods. In addition, a science-based approach to rational study design has allowed for a better use of animals through the development process. Study design considerations must be addressed in order to most effectively utilize animals and wherever possible reduce the need for large numbers and multiple studies. The opportunities and challenges for these approaches as well as the approach to implementing these areas to help reduce animal use and advance the science of biotechnology drugs will be discussed.

I-3-656

Minimising non-human primate use in monoclonal antibody (mAb) development

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The changing environment of monoclonal antibody (mAb) development is impacting on the cost of drug development and the use of experimental animals, particularly non-human primates (NHPs). The drive to reduce these costs is huge and involves rethinking and improving nonclinical studies to make them more efficient and more predictive of man. As our knowledge base on mAbs expands, the information can be used to improve drug development and maximise the output of experimental data. Cross-company data-sharing of nonclinical study decisions and designs in a non-competitive way can establish an evidence base to influence regulatory change. This presentation will focus on the data collected and analysed by an NC3Rs/industry

consortium of 21 pharmaceutical and biotechnology companies, contract research organisations and regulatory bodies on mAbs currently in development. Analysis from two rounds of data collection has shown that there are opportunities to design novel studies that use rodents for chronic studies, fewer dose groups and less recovery animals. These opportunities have been developed into practical guidance and recommendations on the use of science-based rationale to design studies using fewer animals. The aim is to give an overview of approaches that companies are currently using to develop mAbs and how novel approaches can be translated into practice.

I-3-480

Informal communication with US FDA: pre-preIND approach to reduction of animal use in translational research and product development

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In research and development for cell therapy (CT) and gene therapy (GT) products, preclinical studies are essential for supporting safe clinical trials and eventually effective use in humans. According to 21 CFR 312.23 (a)(8), adequate information derived from pharmacology and toxicology studies is needed to support a trial that is reasonably safe and scientifically feasible. The Pharmacology/Toxicology Branch in OCTGT/CBER/FDA has established an informal pre-preIND process to provide an opportunity for sponsors to engage in a scientific discussion with OCTGT pharmacology/toxicology reviewers regarding development of appropriate preclinical paradigms to evaluate the safety and rationale for administration of novel CT and GT products prior to a formal preIND meeting with the CBER/FDA. The goal of this pre-preIND process is to provide guidance in preclinical study designs that is based on advanced science and technology, as well as applicable regulations, and to ensure judicious use of animals in assessment of these novel products. The current thinking and approaches applied toward the reduction of animal use in the preclinical assessment of CT and GT products are described. This process is one example of the FDA's support and application of the principle of the 3Rs to regulation of investigational products.

I-3-356

Better prediction of immunogenicity of biopharmaceuticals in humans: is it possible?

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Due to the exponential growth of biotechnology, the total number of new biopharmaceuticals has increased rapidly. A major drawback of these products is the possible induction of immunogenicity upon clinical use that may result in a safety issue and/or a reduction of drug efficacy. Current preclinical models have proven difficulties with correlation to predict clinical immunogenicity. Therefore, there is a need for better methods to predict which drugs are likely to induce immunogenicity in clinical trials without the use of non-human primates (NHP).

TNO is developing a multidisciplinary toolbox for prediction of relative immunogenicity based on historical data of structurally, therapeutically and/or "mode-of-action" similar compounds. Since there is not one validated model available and various assays are being used, the toolbox will be an integrated approach including different *in silico*, *in vitro* and *in vivo* tools, preferably including human data. Data will be gathered and combined in an (self-learning) algorithm to perform a structured analysis of the potential immunogenicity. Therefore it is cost and time efficient to eliminate candidates that present a high risk of provoking anti-product immune response.

Since the use of NHP is under increasing societal pressure, the second approach is an alternative animal model for safety evaluation of biopharmaceuticals, the minipig. We explored a.o. the possibilities for immunogenicity testing of 3 known biopharmaceuticals and it was concluded that comparable results were obtained in respect to the immunogenicity in minipigs and NHP. These approaches enable us to use minimal testing, reduce the use of animals, reduce costs and provide good predictive data for induction of potential immunogenicity.

I-3-380

In vitro MABEL approach for nonclinical safety assessment of MEDI-565 (MT111), a novel CEA/CD3-bispecific single-chain BiTE antibody

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MEDI-565 (MT111) is a novel bispecific single-chain antibody of the BiTE® (bispecific T cell engager) class that transiently links carcinoembryonic antigen (CEA; also called CEACAM5, CD66e) on cancer cells with CD3 on T cells. Binding of MEDI-565 to CEA and CD3 results in T-cell-mediated killing of cancer cells expressing CEA. MEDI-565 specifically binds to human and cynomolgus monkey CEA with high affinity but not to any other member of the CEACAM family; rodents do not express CEA. MEDI-565 binds to human CD3, but does not bind to cynomolgus monkey or mouse CD3. Consequently, no pharmacologically relevant animal species exists for testing the toxicity of MEDI-565. In an effort to introduce a pharmacologically relevant model, two surrogate antibodies were made, cyS111 and hyS111, with specificity to monkey or mouse CD3, respectively. However, the characteristics of these two antibodies were different from those of MEDI-565 to an extent that it was determined that hyS111 and cyS111 would not have utility in nonclinical toxicity studies. Hence, no *in vivo* toxicology studies were conducted in a relevant animal model with either MEDI-565 or with the two surrogate antibodies. Rather, MedImmune implemented a strategy which utilized an *in vitro* approach to assess nonclinical safety instead of performing *in vivo* toxicity studies which would have required the use of nonhuman primates. Results from these studies were used to select an appropriate starting dose for Phase 1 clinical studies of MEDI-565 for the treatment of patients with cancers expressing CEA.

I-3-620

Pharmaceutical testing of follicle stimulating hormone (FSH): a new cell-based assay for the replacement of the Steelman-Pohley *in vivo* assay

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We will present a new cell based assay (CBA) for the replacement of the animal based Steelman-Pohley *in vivo* assay for pharmaceutical testing of follicle stimulating hormone (FSH). The Steelman-Pohley *in vivo* assay (SPA), first published in 1953, is mandatory for pharmaceutical batch release testing according to the European (Ph. Eur.) and US Pharmacopoeia (USP). The *in vivo* assay is based on rat treatment. During 3 days of treatment the immature female rats are exposed 3 times to FSH. Then the animals are killed and the ovaries are prepared and weighed. The FSH bioactivity is related to the ovarial growth. The animal test is designed as a confirmatory assay to verify an expected FSH sample bioactivity. The SPA is also applied to analytics in R&D, lead optimisation, process development and in-process controls.

For a single batch release testing, 3 different dosages have to be tested on at least 5 animals each. In combination with the application of an additional 15 rats for reference material testing and 5 rats for negative control, 35 animals are required in total.

In addition to the ethical concerns, the *in vivo* assay has several scientific and technical limitations: no full-dose-response analysis, few doses only, limited relevance for recombinant human FSH products, concerns about reproducibility of ovary preparation and weighing and extensive logistics, e.g. sample shipment to animal facility and prearrangement of animal breeding and treatment.

The new cell based assay (CBA) is based on the FSH-sensitive human granulosa cell line KGN. In KGN cells the progesterone production and secretion is induced specifically by FSH.

The progesterone concentration in culture supernatants is quantified by diagnostic-grade ELISA. The CBA is designed in the 96-well format for screening and full-dose-response analyses. Up to 10 different sample dilutions (1:3 steps) can be applied in quadruplicates. The assay is successfully validated for pharmaceutical batch release testing according to the USP (Chapter 1033; Biological assay validation) and was part of a ring test for the evaluation of a new FSH WHO standard coordinated by the NIBSC (2010; data unpublished).

The new CBA for pharmaceutical FSH testing will help to reduce the number of test animals and is intended to replace the Steelman-Pohley *in vivo* assay. The *in vitro* assay is improving experimental data by full dose-response curves and is suitable for comparability testing of biosimilars. The assay could also be used for clinical monitoring of immunogenicity (formation of drug neutralising antibodies, ADA). The new format reduces experimental costs and time.

Session I-3: Poster presentations

I-3-050

Critical selection of reliable reference genes for gene expression study in the HepaRG cell line

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The human HepaRG cell line has proven to be a valuable *in vitro* tool for repeated exposure to chemical compounds and to evaluate their potential toxic outcome. Seeing the importance given by the current EU legislation of cosmetics and chemical substances to the use of *in vitro* methods in human safety evaluation, one can expect that HepaRG cells will gain importance as a human-relevant cell source. At the transcriptional level, RT-qPCR assays are often used to obtain quantitative results. The choice of internal control is important as it may affect the study outcome. Indeed, it is well known that expression levels of traditional reference genes can vary across tissue types and across experimental settings within one specific tissue type. As limited information exists with respect to S18, often used as internal control in HepaRG cell experiments, we aimed to select the most optimal reference genes for gene expression studies in HepaRG cells and to check whether S18 is a suitable reference gene. The expression stability level of 12 candidate genes was analyzed by three algorithms (geNorm, BestKeeper, Normfinder). These identified TBP as the optimal single reference gene and TBP, UBC, SDHA, RLP13, YHWAZ, HMBS, B2M and HPRT1 as the most suitable set of reference genes for HepaRG transcriptional profiling. This study provides a new set of reference genes to be tested whenever RT-qPCR data for HepaRG cells are generated. The most stable ones can then be selected for further normalization.

I-3-178

The effect of three different types of extract of *Viscum album* in two squamous cell carcinoma cell lines of the tongue

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Head and neck squamous cell carcinoma is the 6th most frequent malignant neoplasia worldwide. The carcinogenesis is a multifactorial process in which respective proteins are the result of several molecular events from oncogenes or tumor suppressors. The treatment of these tumors is mostly surgical excision. Occasionally radiotherapy or chemotherapy can also be used. Even with advances in adjuvant therapies, the survival rate has not significantly changed over the last 20 years. Extracts of *Viscum album* (VA), a *Loranthaceace* family plant, have been used in adjuvant cancer therapy in European countries with promising results.

Three VA extracts (Iscador Qu Spezial, Iscador P and Iscador M) in two squamous cell carcinoma cell lines of the tongue (SCC9 and SCC25) were investigated and compared. The VA extracts at concentrations of 0.3 mg/ml (IC_{50}) were added into the culture medium, and after 24 and 48 h the Annexin V and FITC/propidium iodide assay was performed to evaluate apoptosis rate. A Western blot was also performed to verify the expression levels of pAkt, PTEN and Cyclin D1. The proteins for the Western blot analyses were obtained after the cells had been incubated for 24 and 48 h with the respective drugs.

The VA extracts presented positive results in apoptosis induction. Both cell lines presented different behavior in the presence of the drug. The quantitative protein analysis by SDS-PAGE showed different expression levels, especially of Cyclin D1. The Iscador Qu Spezial and Iscador M possess higher cytotoxic potential on both cell lines compared to Iscador P.

I-3-219

Reduction in the number of animals needed for immunogenicity studies by improved analysis of biopharmaceutical-specific antibody responses

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Immunogenicity is a continuous efficacy and safety issue in the development of biopharmaceuticals. Preclinical models for prediction of immunogenicity in humans, as well as new biomarkers to reveal potential anti-drug reactions, are still needed. A sensitive, robust and specific immunogenicity assay has therefore been developed that can detect anti-drug antibodies of all five Ig classes in plasma or serum. The production of drug-induced antibodies in mice injected with a recombinant human protein has been measured by using a modified version of a multi-parametric bead analysis technique (Luminex). Competitive binding was included to verify drug-specificity of the antibodies. Additionally, validation was performed to evaluate reproducibility and specificity.

Results showed that the murine response against the recombinant human protein was IgG1- and IgG2b-specific, suggesting that the drug-induced response was driven by both Th1/Th2 cells, a finding confirmed by results from cytokine profiles. The assay requires considerably lower volumes of plasma or serum in order to screen for the presence of drug-specific antibodies of different classes and sub-classes in one single sample. Altogether, 1 μl plasma from each animal was required for the analysis, while a conventional ELISA measurement would have required 20 times more material for the same analysis. Thus, this refined test system allows for a reduced number of animals needed, due to the possibility of "piggy-backing" on the analyses of other studies.

With the described assay, anti-drug antibody class and subclass screening may be executed in one step, potentially facilitating immunogenicity screening in clinical trials also.

I-3-364

Correlation of *Erythrina velutina* biological activities: behavior *in vivo* and toxicity *in vitro*

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Erythrina velutina is used for its effects on the central nervous system as an anxiolytic and sedative. The aim of this study was to obtain *Erythrina velutina* (bark and leaves) powder samples of different particle sizes and evaluate its biological activity in animals and cells. The behavioral studies were conducted through the elevated plus maze assay, using four groups of adult Swiss male mice, weighing 25-40 g. The cytotoxicity studies were conducted using the neutral red uptake assay using NCTC clone 929 cell culture. The particle size of the obtained powders was in the range of 710.0 to 355.0 μm and 150.0 to 75 μm, which were determined by granulometry test and scanning electron microscope. The samples (1000 mg/kg) from infusion (MUC01 and MUC02) and infusion (MUF01 and MUF02) of *Erythrina velutina* showed different behavioral activity in the number of visits and time spent in the open and closed arms of the maze. The DL_{50} values obtained for MUC01 and MUC02 were: 72.47 mg/ml and 53.01 mg/ml, respectively; and for MUF01 and MUF02 were 50.08 mg/ml and 37.38 mg/ml, respectively. The data derived from the *in vivo* behavioral assay for *Erythrina velutina* herbal medicine powder samples with larger and smaller particle size showed considerable correlation. The *Erythrina velutina* leaves powder with smaller particle size showed good correlation between the *in vivo* and *in vitro* data.

I-3-412

A single dose subcutaneous injection efficacy study followed by a 14-day observation period for reduction of subcutaneous fat in female Göttingen minipigs

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The objective of the study was to determine the efficacy of parenteral formulations containing different Merz test articles following a single occasion of dosing by subcutaneous injection in the fat tissue of female Göttingen minipigs followed by a 14 day observation period.

The test and control/vehicle articles were administered by subcutaneous injection in the fat tissue in pre-determined regions on the dorsal and ventro-lateral side of the pig. The dorsal region of the pig was divided into 6 treatment areas, while the ventral region was divided into 2 regions, for a total of 8 treatment regions per pig. A permanent skin marker was used to delineate a 3 cm x 3 cm square (for a total surface area of 9 cm^2) in each of the 8 treatment areas. The different treatment areas were separated by at least 6 cm from each other to be able to separate the effects of the different products. Each treatment region received five 1 ml subcutaneous injections using a 27 G needle attached to a syringe. The injections were so that a square pattern was formed (the 5 injections formed the four points of the square and a center point). Subcutaneous fat thickness was measured using ultrasonography prior to treatment, and on days 1, 3, 7, 10 and 14. A 5 Mhz linear probe was used to obtain an ultrasonographic image perpendicular to the skin surface including the subcutaneous fat layer. The ultrasound images was used to measure the thickness of the subcutaneous fat layer and the screen image with anatomic markers and fat thickness values were printed and kept in the raw data.

I-3-420

The value of non-human primates in the development of therapeutic monoclonal antibodies

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The pharmaceutical industry is increasingly focusing on the development of biological therapeutics. These molecules generally cause no off-target toxicity and are highly species specific. Therefore, non-human primates (NHPs) are often the only relevant species in which to conduct regulatory safety testing to support clinical trials. However, species specificity and immunogenicity may negatively impact the predictive value of these ethically contentious animals and thus limits their value as a test species for drug development.

To study what the value has been of 30 years of NHP testing in drug development, we investigated the drug registration files of all therapeutic monoclonal antibodies (mAbs) which were approved in the European Union to date. We analysed 30 mAbs of which 5 were diagnostic agents. As the industry moved towards the development of more human proteins, we observed that the average use of NHPs also increased. 16 registration files described studies in which anti-drug-antibodies caused increased clearance of the therapeutic and potentially confounded the study. Post mortem analysis in repeated-dose toxicity studies rarely revealed new or unexpected findings nor did embryo-fetal and peri-postnatal developmental toxicity studies. These issues limited the value of NHPs in the safety assessment of new monoclonal antibodies. To reduce the use of less relevant NHP studies in the development of new biological drugs, regulatory demands might be decreased, and manufacturers should be given incentives for successfully evaluating the safety of biological therapeutics using alternative technologies.

Session I-4: Regulatory testing paradigms and validation of alternative test methods for detecting estrogen active substances; impact on the Three Rs

Session I-4: Oral presentations

I-4-530

Use of Tox21 tools from screening and prioritization to risk assessment: When, how and what?

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EPA's Office of Pesticide Programs (OPP) is committed to improving and transforming its approaches to pesticide human health and ecological risk assessment and management by increasing the efficiency and effectiveness of testing for risk assessment to inform decisions and to reduce the cost of the process both in dollars and animals. OPP is building on the ambitious NAS vision for Toxicity Testing in the 21st Century which calls for a shift toward the avoidance of significant perturbations of normal cellular pathways in exposed populations by using cell based assays to measure these perturbations, dose-response modeling organized around computational systems biology models of the circuitry underlying each toxicity pathway, and *in vitro* to *in vivo* extrapolations based on pharmacokinetic models to predict tissue concentrations under specific exposure conditions. OPP's long-term goal is to move from a paradigm that involves requiring *in vivo* testing for "every possible adverse outcome" toward a hypothesis-driven paradigm where *in vivo* testing is targeted to the most likely hazards and risks of concern. Thus, rather than taking a one size fits all approach to toxicity testing OPP proposes a progressive, tiered-testing approach that starts with hazard-based hypotheses about the plausible toxicological and fate potential of a pesticide or group of pesticides based on their physical-chemical properties (e.g., using read-across and structure activity relationships [SARs] to examine toxicological potential). Existing exposure and toxicity information is then combined with refined exposure models, computational toxicological models (e.g., quantitative SARs or QSARs [(Q)SARs]), and diagnostic *in vitro* assays to narrow requirements for *in vivo*. Consistent with this view is the consideration of time and cost efficiencies associated with the generation and interpretation of toxicity and exposure data and the sound and responsible use of animals in testing. As the science evolves so too must the process to apply this information to predict the effects of concern in humans and non-humans well. Toxicity Test Validation in the 21st Century requires a hierarchical approach tailored to the purpose desired and based on an improved understanding of chemically-induced adverse outcome pathways to make the linkage between the molecular initiating event and an adverse outcome at the individual or population level. Approaches to accomplish this will be described in this talk with a particular emphasis on the evaluation of endocrine disruption.

I-4-130

BG1Luc ER TA test method: results of an international validation study and proposed performance standards

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The 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) recently convened an international peer review panel to assess the validation status of the BG1Luc ER TA test method (also known as the LUMICELL™ assay)

The BG1Luc ER TA test method uses transactivation of an estrogen responsive luciferase reporter gene in human ovarian cancer cells to assess compounds for *in vitro* estrogen agonist and antagonist activity. This test is intended to be used as one component of a multi-test screening strategy as described in US EPA's Endocrine Disruptor Screening Program (EDSP) and offers potential benefits over the existing method, OPPTS 890.1300. BG1Luc ER TA is the only method validated to assess ER TA *in vitro* activity up to the 1 mM limit currently required in the US EPAs EDSP and is the only ER TA method to be validated for the detection of anti-estrogenic substances.

We will provide an overview of the validation report and discuss performance standards that may be applicable to the OECD concept of developing a Performance Based Test Guideline.

I-4-054

H295R cells: an *in vitro* model for the risk assessment of single fungicides and mixtures of them modulating estrone biosynthesis

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Over the past years multiple pesticide residues have been detected in or on fruits and vegetables. The aim of this study was to determine whether an *in vitro* cell culture model could be used to assess the biological effects elicited by pesticide mixtures. Fungicides are among the most frequently detected pesticide residues, the top five often being cyprodinil, pyrimethanil, procymidon, myclobutanil and azoxystrobin. Based on the fact that the above-mentioned compounds are known to or supposed to modulate the biosynthesis of sexual steroid hormones, H295R cells were used to test their effect on estrone biosynthesis, individually and as binary mixtures. When tested individually cyprodinil, pyrimethanil and procymidon enhanced estrone biosynthesis in H295R cells, while myclobutanil and azoxystrobin reduced it, and myclobutanil was a much stronger inhibitor than azoxystrobin. The extent of the effect induced by the combinations cyprodinil + pyrimethanil and cyprodinil + procymidon (stimulation of estrone biosynthesis) was mainly determined by the most potent compound of the mixture. Depending on the concentration of the compounds used an additive effect was observed, but in no cases was a synergistic effect observed. In the case of the combinations cyprodinil + myclobutanil and cyprodinil + azoxystrobin and myclobutanil + azoxystrobin the estrone biostimulating effect of ciprodinil was antagonized in a concentration-dependent manner, myclobutanil being by far the most potent antagonist (as expected from the tests with the individual compounds). In conclusion, the data presented show that H295R cells can be used to test *in vitro* the combined effects of fungicides modulating estrone biosynthesis.

I-4-155

3Rs alternatives for detection of endocrine disruptors: broadening our possibilities

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Endocrine disruptors (EDs) are a group of natural or synthetic compounds that have the capacity to interact with the endocrine system of living organisms. Due to the impact that this interaction could have on human health and wildlife, there is an increasing interest in assessing the risk of the exposure to EDs. The US EPA developed the Endocrine Disruptor Screening Program (EDSP), which has been recently implemented. With it, a large number of experimental animals will be used, even for testing some of the *in vitro* assays of the Tier 1 approach.

Here we present an alternative testing paradigm which includes two novel and robust test systems, with which the detection of EDs can be achieved in a way that includes both replacement as well as reduction through a refinement concept. The first test system is based on the transcriptional activation of transgenic yeast for detection of compounds that interact with the human estrogen or androgen receptor, and is a full *in vitro* method. The second approach involves the identification of EDs metabolic profile by means of metabolomics tools. The metabolome profile is derived from a small blood sample obtained during regulatory testing (e.g. from a OECD 407 – 28 day rat study), identification is achieved using a database (MetaMap®Tox) which contains profiles of reference compounds. The combination of these methods will not only contribute to refinement and reduction of animal testing, but also allows for a sound assessment of the endocrine disruption potential of compounds.

I-4-272

Supporting the implementation of the EU Community Strategy on Endocrine Disrupters

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The regulatory challenge posed by endocrine disrupting chemicals cuts across several major pieces of EU legislation, either already in force (such as REACH, the Plant Protection Products Regulation (PPPR) and the new Cosmetics Regulation), or still at the stage of proposal, such as the recast of the Biocides Directive. Although it was conceived before these Regulations or legislative proposals were adopted, the EU Community Strategy on Endocrine Disrupters provides a valuable coordination framework in order to meet this challenge, by developing a systematic approach for the identification and assessment of endocrine disruptors, which can be applied across the different pieces of legislation. Set out by the European Commission in 1999, the Strategy focused on short, medium and long term actions to be undertaken to address the potential environment and health impacts of endocrine disruption. These included the establishment of a priority list of substances for further evaluation of their role in endocrine disruption, the development and validation of internationally agreed test methods to assess endocrine disruption in people and wildlife, the co-ordination and funding of international research into the underlying mechanisms of endocrine disruption, and, last but not least, the adaptation and/or amendment of EU legislative instruments as necessary/appropriate in order to account for endocrine disrupting effects. This presentation reviews aspects of the implementation of the EU Community Strategy so far, describes some on-going European Commission activities on endocrine active substances in which the Joint Research Centre is involved and presents some future prospects in the light of recent developments in the research and regulatory fields.

I-4-287

A strategy for reducing animal use in the U.S. EPA's endocrine disruption screening program

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The current U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) uses a two-tiered approach to evaluate chemicals for possible effects on the estrogen, androgen or thyroid hormone systems. In Phase 1 of the program, orders for Tier 1 testing were issued for 67 chemicals, most of which already have a wealth of data associated with them. In lieu of conducting some or all of the Tier 1 tests, EPA was directed to allow the submission of Other Scientifically Relevant Information (OSRI) that was directly or functionally equivalent to the data gathered in Tier 1. Of the 47 OSRI submittals EPA has reviewed to date, the agency has denied all OSRI for 20 of those chemicals, meaning that the test order recipients must conduct the full Tier 1 battery for these chemicals, which will result in the use of more than 10,000 animals. EPA accepted some OSRI for the remaining 27 chemicals and issued waivers for 45 *in vitro* and 48 *in vivo* Tier 1 tests. Typically, accepted OSRI was either identical to the Tier 1 test it satisfied, or it indicated a positive response by the chemical in question. While the *in vivo* waivers will save about 2,500 of the nearly 25,000 animals required for 47 chemicals, applying an iterative decision-making process along with a weight-of-evidence evaluation, as shown here, has the potential to further reduce the perceived need for testing and save an even greater number of animal lives.

I-4-308

Detection of endocrine activity *in vitro* – current status of tests developed in the framework of the EU project "ReProTect"

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From 2004-2009, the EU-FP6 Integrated Project "ReProTect" brought together reproductive toxicology expertise from more than 30 academic, public and industrial partners throughout Europe with the aim to develop new *in vitro* methods relevant for reproductive toxicity assessments. In order to address the complexity of mammalian reproduction, ReProTect made use of promising *in vitro* models with the aim to convert them into defined toxicological *in vitro* tests. Various toxicological targets and mechanisms of reproductive toxicants, such as gametogenesis and developmental toxicity, were addressed. The optimization of *in vitro* methods aiming at the detection of endocrine active compounds was a major activity within ReProTect.

The presentation will provide an overview on ReProTect and its achievement. The current status of four tests for assessing (anti) estrogenic and (anti) androgenic compounds will be addressed in detail. Two recombinant receptor binding assays, the ERa and the AR binding assay, were optimized in ReProTect and are presently on the way to undergoing formal validation under the umbrella of the OECD. Moreover, data on the performance of two reporter gene assays based on the estrogen-sensitive MELN cell line and androgen-sensitive PALM cells will be presented and an update on their (pre)validation will be given.

Session I-4: Poster presentations

I-4-288

Identification of endocrine disruptors using an organotypic normal human cell based vaginal tissue model

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Environmental or occupational exposure to a broad variety of chemical agents can alter normal endocrine function and have serious health implications including effects on reproductive capacity, fetal development, the immune system, and carcinogenesis. Animal tests are expensive, are not necessarily applicable to humans, and were banned by the EU Cosmetics Directive for studies involving a broad variety of cosmetic and personal care products. In this study, we investigated the potential use of an organotypic tissue model, EpiVaginal^{TM}, for Tier 1 screening of chemicals that may be agonists or antagonists of the estrogen receptor (ER). H&E stained tissue cross-sections showed thinning of the basal and parabasal layers following 72 h exposure to ER antagonists when compared to the control tissues; exposure to ER agonists resulted in thicker basal and parabasal layers, indicating stimulation of cellular proliferation. RT PCR analysis showed an increase in progesterone receptor b (PRb) levels for 3 of 3 agonists and a decrease or no change for 6 of 8 antagonists when compared to negative controls. Furthermore, ER-α expression increased following exposure to the ER agonists and decreased following exposure to ER antagonists. ELISA assays showed increased estrone release by ER agonists but not ER antagonists. Based on estrone release (n = 22 test articles), a prediction model (PM) for ER agonists was established. The PM identifies ER agonists with a high sensitivity (85.7%), specificity (100%), and accuracy (95.5%). In conclusion, the EpiVaginal^{TM} tissue appears to be a useful *in vitro* model to screen for chemicals with endocrine disrupting potential.

I-4-454

Results of the validation study of the stably-transfected estrogen receptor alpha transcriptional activation antagonist assay using the HeLa9903 cell line

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The HeLa9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs, i.e. estrogen receptor alpha (ERα) and the luciferase reporter plasmid, has been developed to detect the ERα (anti-)agonist activity of chemicals. The Stably Transfected Human Estrogen Receptor (ER) Transcriptional Activation Assay (STTA) using the human ERα-HeLa-9903 (HeLa9903) cell line for the detection of estrogenic agonist activity of chemicals was established as OECD Test Guideline 455 in 2009. To evaluate the reliability and reproducibility of the HeLa9903 cell line for detecting anti-estrogenic activity, a validation study of the STTA anti-estrogenic assay was initiated and is being coordinated by JaCVAM, under the auspices of the OECD Validation Management Group: Non Animal, with membership of the study management group including representatives from ECVAM, EFSA and the US EPA. Six participating laboratories have demonstrated sufficient skills to conduct the STTA assay by completing the agonist assay according to TG 455 in Task-1. In Task-2, the testing of

un-coded reference chemicals based upon provisional performance standards for the antagonist assay was performed by four laboratories. The protocol, including the performance standards for the antagonist assay, has been revised based upon the Task-2 results from all the laboratories that demonstrated proficiency in Task-2. Repeat testing of 20 coded chemicals was conducted in Task 3. Preliminary results from Task-3 are promising with respect to reproducibility in the intra- and inter-laboratory tests, and indicate that the STTA assay is an appropriate *in vitro* assay to screen for ERα antagonist activity of test chemicals.

I-4-473

Comparison of alternative screening approaches for potential estrogenicity

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Several approaches are used for screening endocrine activity of chemicals. These range from QSAR models for estrogen receptor binding to the Endocrine Disruptor Screening Program (EDSP) of tiered short-term *in vitro* and *in vivo* tests, and the US-EPA's ToxCast Program that integrates information from *in vitro* assays, pathway analysis, and bioavailability into a Toxicological Priority Index. In this analysis, we compared estrogenic activity for six pesticidal compounds included in ToxCast for which we had preliminary EDSP data. Four compounds had no indication of estrogenicity using QSAR, ToxCast (at concentrations <50 μM) or in the rat uterotrophic assay included in EDSP. One compound showed a minor prediction in one ToxCast assay, but was not supported by QSAR, EDSP, or the other ToxCast assays. The remaining compound showed minimal activity in at least one ToxCast assay, with a prediction of weak ER binding, but no activity in the uterotrophic assay or other ToxCast assays. Neither QSAR nor *in vitro* ToxCast assays account for disposition in the whole body, and ToxCast does not assess the endocrine activity of metabolites. None of the six compounds showed activity in the *in vivo* uterotrophic assay. These results indicate that the minimal predictions of some activity for two compounds using QSAR and/or ToxCast are not predictive of *in vivo* effects and, therefore, can be misleading. This limited analysis suggests opportunities for increased use of *in vitro* and *in silico* methods in endocrine screening, although additional research is needed to establish the predictive capabilities of these alternative approaches.

Session I-5: Nanotoxicology and the Three Rs

Session I-5: Oral presentations

I-5-648

Alternative *in vitro* assays in nanomaterial toxicology

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Nanomaterials are acclaimed for their novel properties, for which broad new uses are being discovered with increasing frequency. It is obvious that, as the properties change, unwanted properties (toxicity) are to be expected as well. Today's toxicology, however, is already overwhelmed with the challenge of addressing new chemicals, not to mention the enormous number of old chemicals never properly assessed. Limitations of traditional approaches range from animal welfare issues, which were a strong driving force for alternative approaches (the 3Rs concept) over the last two decades, to aspects of throughput and accuracy of the predicted toxicities. The latter has prompted discussion about a revolutionary change in chemical safety assessment, now known as Toxicology for the 21st Century (Tox-21c). The multitude of possible formulations of nanomaterials to be assessed for novel toxic properties makes these alternative approaches especially attractive, given the well-recognized limitations of traditional animal-based approaches – limitations that might be even more pronounced for nanomaterials, which have notably altered biokinetics.

I-5-053

Three Rs and the safety assessment of nanotech drugs

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Many drug products are nanoparticles. Some were originally designed as nanoparticles, including monoclonal antibodies, and others were originally larger size particles that were later milled to nanosize to improve bioavailability. Drug nanosize products may include base particles, carriers or encapsulators, therapeutics, contrast agents, targeting molecules, chelators, diagnostics, or a combination of all of these. Judicious use of *in vitro* data can reduce the use of animals to assess the safety of nanodrugs. Before any studies are conducted, the material needs to be made reproducibly and be carefully characterized for critical attributes. *In vitro* assays can assess comparability across particle size ranges and stability in various media, and dermal penetration. Nanoparticles may distribute to tissues or organs that larger particles do not, such as crossing the placenta or the blood-brain barrier. Mouse stem cell assays might be used to bridge embryofetal effects of larger particles to nanosize particles, and various *in vitro* systems can assess penetration of the blood-brain barrier. Small bridging studies may be used to assess nanoparticles milled from larger particles.

I-5-351

Development of an integrated aerosol measurement system in the i-Lung

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Respiratory research can be divided into medical device testing and research of particle deposition in lungs, like aerosol inhalation. The necessity of testing the effects of aerosols on human health gained even more importance when the topics of fine dust, nano-particles and the safety of chemicals emerged. Amongst others, these issues have to be tested by using realistic models of the respiratory tract. Different kinds of mechanical lung simulators and numerical simulation models have been derived in the course of time.

The presented aerosol application and measurement system, using the novel lung simulator (i-Lung) as the core element, can be employed for the reduction of necessary laboratory animals according to the EU Cosmetics Directive. Additionally it allows the realisation of extensive test series, as demanded by the EU REACH regulation, with an appropriate number of animals. The simulation of a physiologically or pathologically breathing human lung can be performed by using different lung equivalents, like latex bags of different sizes or primed porcine lungs. In the presented test setting, the simulation device has been synchronised and combined with an aerosol spectrometer with a white light aerosol sensor in order to detect in- and exhaled particles in a size range of 0.2-10 μm. The used lung equivalents respired a DEHS aerosol produced beforehand. Particles entering and exiting the lung were counted. The measurement results show a remarkable degree of separation of the aerosols during in- and exhalation, if a primed porcine lung is used as human lung equivalent.

I-5-425

A simple method for testing the toxicity of nanomaterials on 3D air-liquid interface human airway epithelia (MucilAir™)

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We developed a simple method to deliver nanoparticles to air-liquid interface (ALI) culture systems. This patented method (PCT/IB2010/053956) uses Dextran as carrier, which allows testing a wide range of doses of nanoparticles. Briefly, the nanoparticles were diluted and mixed with the Dextran powder; small pellets were made and then applied onto the apical surface of the ALI culture. We tested the toxicity of several nanoparticles, such as ZnO and $Fe(IO_3)_3$, on an *in vitro* cell model of the human airway epithelium (MucilAir™). MucilAir™ closely mimics the morphology and functions of the normal human airway epithelium. Moreover, it has a unique shelf-life of one year, allowing chronic/long term toxicity testing. Using multiple endpoints, like trans-epithelial electrical resistance (TEER), cell viability assay (LDH), cilia beating frequency, morphology, cytokine release, etc., we determined the dose response curve of ZnO and $Fe(IO_3)_3$ nanoparticles on MucilAir™ . Toxicity of ZnO (9 nm) was observed at doses higher than 0.1% (9 $\mu g/cm^2$). Interestingly, at 0.1% of ZnO, the epithelia had the potential to recover/repair after the exposure, while at 0.5% (45 $\mu g/cm^2$) of ZnO, this was not the case. Effects of two forms of $Fe(IO_3)_3$ from 10 to 20 nm were also compared, namely spheroids and nanoneedles.

Our results showed that the Dextran-carrier method is an easy and efficient way to deliver the nanoparticles *in vitro*. MucilAir™ is a relevant *in vitro* system for assessing the toxicity of nanoparticles.

I-5-432

Use of normal human 3-dimensional (NHu-3D) tissue models (EpiDerm, EpiAirway) for nanotoxicology applications

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Nanomaterials are increasingly utilized in numerous commercial applications where dermal contact, inhalation or oral ingestion is likely. However, their toxicological properties are largely unknown. Potential adverse effects of nanoparticle exposure include allergenicity, cytotoxicity and genotoxicity. Nanomaterials may enter the body by interacting with, and eventually crossing, epithelial barriers including skin, airway and intestinal epithelium. Once inside the body, additional interactions with internal organs, such as heart, liver, brain, kidney and others, are possible. Therefore, there is an urgent need for animal alternative tissue models that can be utilized for toxicological evaluation of nanoparticle materials. This poster summarizes use of *in vitro* NHu-3D skin (EpiDerm, EpiDerm-FT) and airway (EpiAirway) models for nanotoxicology applications. Notable applications to date include the use of: 1) the EpiDerm model to study potential health implications of cerium oxide nanoparticulates (SafePharm Laboratories, UK), 2) EpiDerm to investigate skin irritation/toxicity potential of nanosilica particles (Korea University College of Medicine), 3) the EpiDerm-FT skin model to evaluate skin penetration of quantum dot nanoparticles (Korea University College of Medicine), 4) EpiDerm-FT for investigations of single-walled carbon nanotubes (NIOSH, USA), 5) EpiDerm-FT to investigate the effect of nanoparticle formulations on wound healing (Free University Berlin) and 6) the EpiAirway model for *in vitro* determination of nanoparticle translocation through airway epithelium (Procter and Gamble, USA). These studies demonstrate that *in vitro* NHu-3D models are useful tools for the study of nanoparticle interactions and potential toxicologic effects on epithelial tissues.

Session I-6: Advances in alternative methods for ecotoxicology

Session I-6: Oral presentations

I-6-172

Fish cell lines as alternatives to fish toxicity tests

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Fish are the dominant vertebrate species for the regulatory evaluation of ecotoxicity and are generally afforded the same legal protection as mammals. It is for this reason that the establishment and validation of cell culture assays as alternatives to fish tests, as initially suggested almost 40 years ago, is a desired and urgent societal goal. On this background, we establish novel fish cell cultures models with a particular focus on cell lines and develop strategies to overcome common limitations in the application of cell culture assays as substitutes for fish. The most recent development is a cell line from rainbow trout intestine; we are currently working to establish this cell line as an intestinal barrier model. To elucidate active transport mechanisms for chemical and toxicant distribution, we identified mRNA expression and activity of nine selected ABC transporters belonging to the ABCB, ABCC and ABCG families in seven rainbow trout cell lines. Finally, improved procedures for exposure of a rainbow trout gill cell line led to toxicity results that are well comparable to those obtained in the acute fish toxicity test. Thus, fish cell lines hold great potential for deciphering the molecular mode of action of chemicals and, provided the right choice of *in vitro* model and exposure conditions, may supplement or even substitute fish toxicity tests. Our vision is to build an *in vitro* fish test which not only provides knowledge on cell type interactions in physiology and toxicology but also supports the development of computational models toward a virtual fish.

I-6-340

Quantitative and comparative analysis of alternatives to *in vivo* tests for endocrine disrupting chemicals (EDCs) in fish and amphibians

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Endocrine disruption plays an important role in environmental safety assessment of chemicals. Therefore, appropriate screening assays have been developed, such as the US EPA Tier 1 screening assay in fish and frog species (adopted in 2009). Both assays are closely aligned with the OECD test guidelines 229 and 231. However, these assays use a large number of animals. Furthermore, they are costly and quite long in duration relative to an ideal screening assay. A shorter-term and alternative to an-

imal tests would be advantageous in order to reduce the number of animals used. A literature search was conducted to identify potential alternatives and a database with alternatives to fish and frog testing methodologies was assembled. Data from 1995 to the present were collected related to the detection/testing of estrogen-, androgen-, and thyroid-active chemicals in the following test systems: cell lines, primary cells, fish/frog embryos, yeast, bacteria, cell free systems, and "omics" technologies. A critical analysis was performed to (1) determine the strengths and limitations of each assay and (2) present conclusions regarding chemical specificity, sensitivity, and correlation with *in vivo* data. Due to the relatively large amount of data available for estrogenic compounds comparative analyses were performed specifically for this group of EDCs. For example, a high correlation was observed between ligand binding and reporter cell assays and between fish and frog estrogenic data. Furthermore, alternative assays appear to be able to detect specific hormone receptor binding. A summary of these and other data on alternative assays for EDCs will be presented.

I-6-423

Adverse outcome pathways and extrapolation tools to advance the Three Rs in ecotoxicology

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Adverse outcome pathways (AOPs) are conceptual frameworks for identifying and organizing predictive and causal linkages between cellular-level responses and endpoints conventionally considered in ecological risk assessment (e.g., effects on survival, growth/development, and reproduction). The proposed paradigm for "Toxicity Testing in the 21st Century" advocates the use of mechanistically-based, high-throughput *in vitro* assays as a potential cost effective and scientifically-sound alternative to some whole animal hazard testing. To support the development of this approach, there is a recognized need to (1) identify and catalog common adverse outcome pathways (AOPs) and (2) based on these pathways, strategically develop appropriate batteries of alternative assays. Furthermore, there is a need to develop a variety of extrapolation tools which can translate *in vitro* assay data into credible predictions of *in vivo* outcomes, preferably for a wide range of organisms. This presentation will highlight the utility of the AOP concept and discuss extrapolation tools needed to define and expand the applicability domains of mechanistic high throughput *in vitro* assay data, with specific emphasis on how these approaches can support the reduction, refinement, and/or replacement of animal use in ecotoxicology and ecological risk assessment.

I-6-536

What reductions in fish use can be made employing alternatives for wastewater effluent assessment?

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Fish testing has many applications which may include new chemicals screening, product safety assessment, and water supply safety. The majority of fish testing use can be attributed to compliance and investigational testing of treated wastewaters throughout the world. Permitted wastewater discharges to surface waters are routinely tested in North America for effluent toxicity to determine compliance to state and national water quality standards. Similar mechanisms or goals exist in certain parts of Europe and Asia. Larval, juvenile and adult fish are employed for acute and/or chronic toxicity determinations. A major advantage of effluent toxicity testing is the integration of mixture toxicity and bioavailability. This is unlike chemical-specific analysis which identifies only individual constituents and can make it difficult to determine if receiving waters are protected. We suggest that a 3Rs approach for effluent testing will lead to adequate or new alternative solutions that are better designed

for their purpose and enable fast, cost-effective equivalents.

Alternatives for fish effluent testing include streamlining existing tests to use fewer fish, *in vitro* methodologies and fish embryo testing. We will review several options to reduce numbers of fish using techniques such as: combining control treatments, test designs using fewer effluent concentrations and possible development of fish embryo tests yielding equivalent results to standard assays using swim-up/larval forms and chronic toxicity endpoints. A program has been initiated to test assumptions and assay conditions using fathead minnow and zebrafish. Comparisons of embryo tests and classical effluent survival and growth assays for both species are planned using chemicals and representative effluents. Successful results in the pilot experiment would prompt a more extensive program. Lastly, these assays may have additional utility to develop fish toxicity perspectives on complex mixtures and metabolites using bench top wastewater treatment plant models (e.g., CAS units) during the development of new or existing chemical assessments.

I-6-447

Product stewardship "incorporating the 3Rs while improving bioaccumulation assessments"

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Product stewardship is an integral, on-going aspect of environmental programs at major corporations. Animal welfare concerns, as exemplified by the 3Rs, are an important consideration in product stewardship testing and the evolution of regulatory testing guidelines. Intelligent testing strategies that incorporate tiered testing and evaluation schemes help guide efficient use of limited resources, including test organisms, and facilitate scientifically-based chemical evaluations to ensure achievement of product stewardship and environmental protection goals. An example of a tiered assessment framework for bioaccumulation assessment is presented that incorporates data mining, modeling, physical-chemical property measurements, *in vitro* assays, and *in vivo* toxicity tests to provide an assessment of bioaccumulation potential in fish. Examples of the application and limitations of the framework for bioaccumulation assessments will be presented including an evaluation of the animal welfare benefits of the framework and suggestions for additional research to improve the framework.

I-6-114

Harmonizing and optimizing fish testing methods: The OECD framework project

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The Organisation for Economic Cooperation and Development (OECD) serves a key role in the international harmonization of testing of a wide variety of chemicals. An integrated fish testing framework project was initiated in mid-2009 through the OECD with the US as the lead country. The objectives of the project were to review regulatory needs and data requirements for fish testing in the context of existing OECD Test Guidelines. One goal was to support animal welfare concerns by identifying unnecessary test methods and ensure the optimal use of data derived from *in vivo* studies. A September 2010 workshop with participation from over 40 international experts was organized with the goal of producing a guidance document that provides a detailed discussion of technical issues, relevant endpoints, and specific recommendations for a harmonized testing framework for fish. In addition to detailed reviews of individual OECD fish test guidelines, topic areas included general testing issues, regulatory needs and data requirements for fish testing, statistical issues, animal welfare considerations and alternative approaches to testing. General guidance on possible strategies for approaching hazard testing with fish was developed by identifying broad principles to guide testing sequences which can then be adapted for specific circumstances and types of chemicals. This presentation will highlight the conclusions and recommendations of the workshop and discuss the resultant framework document.

Session I-6: Poster presentations

I-6-095

Statistical power of the OECD 210 chronic fish early life stage test and what this suggests for future animal alternative approaches

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The Fish Early Life Stage Test (OECD 210) is the most common chronic fish ecotoxicity assay in use for assessing industrial chemicals, biocides, pharmaceuticals, and agrochemicals. The assay encompasses exposure from egg through juvenile life stages accompanied by determinations of hatchability, survival, growth (length and weight) and developmental abnormalities. The guideline is flexible with respect to species and key aspects of experimental design. With cooperation from several industry partners, we gathered over 100 OECD 210 studies (82 compounds, 16 laboratories). Fathead minnow, zebrafish, and rainbow trout comprised 71%, 16%, and 13% of the studies, respectively. Experimental design, water quality, and test endpoints were summarized. Information was collected at the level of individual chambers to allow determination of the statistical power of the reviewed studies to detect biologically meaningful effects. Statistical overdispersion in tests indicated that extra bionomial variation was frequently present, consistent with the presence of significant chamber-to-chamber variability. In order to increase the sensitivity of the assay, we recommend maximizing numbers of test chambers rather than the number of fish per chamber. Power analysis indicated that most endpoints could detect a 20% change relative to controls when using at least four replicate chambers, although to detect a 10% change would often require as many as 20 replicates for some species and endpoints. The complexity of chronic tests with respect to organism development and execution make replacements and refinements difficult; however, future alternatives could consider this as a "bar" against which they can be measured.

I-6-161

Development of a mechanistically informative, genome-wide, *in vitro* chemicals screening technology

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The testing of chemicals is an important regulatory activity which, in many cases, requires the use of animals. Reducing the number of animals for testing depends on the development of alternative test procedures that do not use living animals but yield information relevant to the health and environmental impacts of the chemicals in questions. Two methods coming into use are permanent fish cell lines and early stage fish embryos. We want to increase the information content of these alternative test methods by applying refined microarray technologies, alongside powerful statistical technique, and utilising the experimental power of well-controlled experimental designs to define a large number of genes responding to toxic exposure. These transcriptomic signatures will describe the distributed nature of gene responses, which can be modelled as affected processes and pathways, and as a network of regulatory effects. We want to know whether this system-wide view of toxic effects, which relates to the full complexity of the system, can be used as a classifier to predict the toxic behaviour of novel materials, and which alternative test method offers the best prospects for discrimination between toxic compounds. Moreover, we are interested in whether ecologically relevant doses or those that relate to the dose response curve are most appropriate for testing purposes and how the results are affected by dose. We will compare and contrast the performance of fish cells and zebrafish embryos, and demonstrate how this system-wide view of toxicological effects generates more understanding and predictive power than a candidate gene-centered view.

I-6-197

Predicting acute toxicity in fish using the rainbow trout gill cell line RTgill-W1

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The OECD test guideline 203 for the determination of fish acute toxicity requires a substantial number of fish and uses death as an integrative but crude endpoint. Therefore, the development of appropriate alternative methods is timely. One promising approach is the use of fish cell lines; however, several studies indicated that fish cell lines appear less sensitive than fish. We optimized the fish cell line approach using several steps to increase the sensitivity of a fish gill cell-based assay. These steps included the modification of the exposure medium and the determination of the chemical bioavailability. We further showed that chemical toxicity is dependent on the solvent and dosing procedure. Based on these findings, we designed dosing and exposure protocols that account for factors otherwise compromising the *in vivo-in vitro* correlation. The optimized cell line approach was now used to determine the toxic potential of 34 organic chemicals towards the RTgill-W1 cell line. The selected chemicals have a wide range of mode of action and physico-chemical properties. Results reveal a good agreement of *in vivo* and *in vitro* values. Outliers from the correlation can be explained by certain modes of action. Compounds that give the greatest deviation are either neurotoxicants or chemicals that need to be metabolized into more toxic compounds, target sites or processes that are not mimicked by the gill cells. Based on this knowledge we are developing a strategy to use fish cell lines as surrogates for acute fish toxicity studies.

I-6-382

Transepithelial electrical resistance to monitor epithelial cell integrity for *in vitro* toxicity testing of water samples

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Current methods for testing water pollution are expensive and do no test for a wide variety of toxicants. In terms of testing new toxicants the OECD has set guidelines which involve exposing live fish to test toxicants for up to 96 hours and testing how many fish have died at intervals of 24, 48, 72 and 96 hours.

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

A simple fluidics system for epithelial cell culture has been developed. It includes a double flow PDMS chamber, with upper and lower compartments separated by an ultrathin microporous silicon nitride membrane for cell growth. Integrated electrodes allow regular measurements of the TEER of the epithelial cell layer.

The chosen cell line for use in the model is the caco-2 (C2BbE1) cell line. This is a polarized colon cell line forming functional tight junctions after 21 days in culture. First results have shown that those cells grow well in the double flow PDMS chamber and that TEER can be monitored.

Future work is ongoing to optimize the system and to compare TEER measurements with classical toxicity endpoints.

I-6-478

Assessment of mitochondrial toxicity of environmental chemicals using a quantitative high-throughput screening approach

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As part of the U.S. Tox21 initiative, the NCGC is developing and optimizing cell-based and biochemical assays suitable for quantitative high throughput screening (qHTS) in a 1536-well format. This effort will generate pathway profiles for environmental compounds that will facilitate the evaluation of mechanisms of toxicity and prioritization for more extensive testing, as well as the development of predictive models for *in vivo* toxicity. In this study, we optimized a mitochondrial membrane potential (MMP) assay using a water-soluble fluorescent MMP sensor to detect mitochondrial depolarization in HepG2 cells and we used this method to evaluate the mitochondrial toxicity of 1353 environmental compounds provided by the NTP. In response to mitochondrial depolarization, the ratio of the mitochondrial red fluorescent aggregates to the cytosolic green fluorescent monomeric form of the dye decreases. Of the 1353 compounds screened over a 14-point concentration curve (0.59 nM to 92 μM), 107 (8.9%) compounds disrupted the mitochondrial potential in HepG2 cells after treatment for one hour and 104 (7.6%) did so after five hours, with 88 (6.5%) compounds active at both exposure durations. To evaluate the structure-activity relationship of these potential mitochondrial disruptors, we clustered these 88 compounds by structural similarity. This analysis resulted in seventeen structural clusters and 26 singletons. We selected 39 compounds for further studies, including high-content imaging and sensitivity to different energy sources. Our results confirm the robustness of this assay for identifying MMP disruptors in qHTS format.

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I-6-537

EUROECOTOX – European network for alternative testing strategies in ecotoxicology

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EUROECOTOX is a project funded by the Seventh Framework Programme (FP7) of the European Union. It aims at mapping and reviewing the current status of alternative testing approaches for environmental risk assessment. The main objectives of the project are

– to contribute to the advancement of alternative methods of eco-
– toxicity testing in Europe;
– to promote the validation and regulatory acceptance of new alternative ecotoxicity methods;
– to facilitate the networking of research groups working in the field of alternative approaches in ecotoxicology;
– to interact with stakeholders involved in the development, validation, regulatory acceptance and final use of alternative ecotoxicity testing strategies;
– to identify bottlenecks for the implementation of alternative testing approaches and
– to propose research needs for the promotion of alternative testing.

EUROECOTOX is organising a couple of events to address these objectives, such as an expert meeting, a conference and a report. Furthermore, a website has been launched (www.euroecotox.eu) which will serve as a resource centre and a database on primarily European activities on development and validation of alternative methods for ecotoxicological testing. At the 8th World Congress on Animal Alternatives and Animal Use in the Life Sciences, a first overview on the mapping results and database implementation will be given.

I-6-585

An educational program for the use of alternative methods to animal experimentation and testing

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Alternative methodology is one of the scientific fields that have been growing exponentially during the last years. Because of the robust quality, validity and human relevance of the data collected with alternatives these methods are increasingly adopted for safety testing purposes in the field of regulatory toxicology.

The continuing progress in the field of *in-vitro* methods, e.g. high throughput and high content imaging methods and advanced knowledge in the field of systems biology, protein interactions and gene expression patterns (-omics) opened up new prospects, especially in the field of toxicology, to investigate pathway related and human relevant targets with methods alternative to animal testing. Thus, today the field of alternatives is multidisciplinary, encompassing moral philosophy as well as genomics, transcriptomics, proteomics, systems biology and also regulatory toxicology. Because of the resulting complexity of this diverse field it is inevitable that the segmental knowledge has to be shared between many experts.

However there are very few relevant educational programs in the field of alternatives, i.e. programs which are comprehensive and embedded within the framework of scientific curricula. CAAT-Europe brought together key teachers in the field of alternative methods and engaged them to complement their expertise. The synergistic outcome with regard to specified target groups resulted in an advanced and comprehensive educational setup including different modules for different audiences with different demands.

I-6-586

Center for Alternatives to Animal Testing – Europe (CAAT-Europe)

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Two universities renowned for their experience in the area of alternative methods in Europe and the US have joined forces to complement the 30-year-old Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University with a corresponding Center for Europe (CAAT-Europe) at the University of Konstanz. The "Excellence University" of Konstanz has twenty years of experience in alternatives to animal experiments, employing five professors in pharmacology and toxicology along with numerous coworkers researching human-relevant alternative methods. CAAT-Europe brings together industry and academics to address the needs for human-relevant methods, to use strategic funds to fill gaps in the development and implementation of alternative methods, to coordinate information days and workshops in Europe on relevant developments in the area of alternatives, to develop strategic projects with sponsors for the promotion of humane science and "new toxicology" and to set up a joint education program between Johns Hopkins and the University of Konstanz.

I-6-591

Application of the 3Rs in the field of ecotoxicology

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Despite constituting 11% of total UK animal usage in 2009, fish are often overlooked with respect to the 3Rs. Here we describe several initiatives to address this issue.

Reduction: For evaluating endocrine disruptors, fish full life-cycle tests are considered the "gold standard," but use many animals. By utilizing all available information (e.g. preclinical data), however, it is possible to focus testing on sensitive life-stages and endpoints. We recently employed this strategy to design a "targeted" chronic study that used fewer fish than the standard test.

Refinement: Although there is pressure to improve environmental enrichment for fish, most current approaches are based on mammalian research. Consequently, we aimed to identify enrichments that are suitable for fish and compatible with prescriptive regulatory protocols. In addition, little information is available on pain alleviation in fish, so we are developing alternative procedures that are both scientifically and ethically preferable to existing approaches to anaesthesia and analgesia.

Replacement: Cell culture is a promising alternative to fish use, although questions remain around *in vivo* translation. We assessed a fish hepatocyte culture for endocrine disruptor (ED) testing that proved sensitive for certain biomarkers, and exhibited good metabolic capability. We have also evaluated algae and crustaceans as surrogates for fish genotoxicity assessment, the data generated suggesting effective metabolic activation and measurable induction of DNA damage.

In summary, various initiatives have been undertaken, some of which have the potential to improve the 3Rs in fish, provided appropriate levels of comparability with *in vivo* studies can be demonstrated.

I-6-599

OECD validation study on the transferability, intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test

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The OECD Fish Acute Toxicity Test Guideline (TG 203) is an integral component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuffs, and biocides. One of the most promising alternative approaches to the fish acute toxicity test is based on the use of zebrafish embryos. In 2005, the German Federal Environment Agency submitted the draft TG "Fish embryo toxicity (FET) test" to the OECD Test Guideline Program and a supportive Background Paper. Subsequently, OECD established the *Ad hoc Expert Group on the Fish Embryo Toxicity Test*. Based on the outcome of expert meetings, OECD decided to perform a validation study (coordinated by ECVAM and steered by a validation management group). The validation study aims to evaluate the transferability and the intra/interlaboratory reproducibility of the Zebrafish FET (ZFET) test for different chemicals newly fertilised zebrafish embryos are exposed to for up to 96 h. Four apical endpoints are recorded daily as indicators of acute lethality in fish: coagulation of the egg, lack of somite formation, non-detachment of the tail bud from the yolk sac and lack of heart-beat. LC_{50} values are calculated for 48 h and 96 h exposure. A total of 20 chemicals will be tested at five different concentrations in three independent runs in at least three laboratories with appropriate controls. Stock solutions and test concentrations of at least one laboratory are analytically confirmed. This presentation will give an overview on the study design and discuss the preliminary results.

Disclaimer: The opinions expressed and the arguments employed herein are those of the authors and do not necessarily reflect the official views of the OECD or of the governments of its member countries.

Session I-7: Potency and safety testing of veterinary vaccines

Session I-7: Oral presentations

I-7-257

The reduction of animal-based safety testing of veterinary vaccines

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Veterinary vaccines in North America are regulated to assure that they are safe, pure, potent and effective. The veterinary biologics industry conducts a number of *in vivo* and *in vitro* assays as part of the vaccine development and manufacturing process to assure that vaccines conform to these requirements. The batch or serial release safety test is one such assay. The serial release safety tests are conducted using a combination of target and/or laboratory animals for every batch of vaccine prior to its release for commercial distribution.

In an effort to reduce the number of animals required for the production of veterinary vaccines, industry and regulators are working together to evaluate alternatives to *in vivo* safety testing for batch release. Factors under consideration in developing a regulatory framework that would reduce these *in vivo* tests include: consistency of the production process (GMP or equivalent), review of the safety profile during vaccine development (field and laboratory trials), historical *in vivo* batch safety test results, vaccinovigilance programs, the impact of future production process changes, the potential for additional *in vitro* testing requirements, and the implementation of additional production constraints.

In addition to the required changes in domestic regulations, to effectively reduce the numbers of *in vivo* tests required by industry to assure batch safety in the current global marketing environment, these regulatory changes will also require harmonization across all markets requiring the *in vivo* testing.

I-7-677

Successful development and validation of an *in vitro* replacement assay for Leptospira potency tests

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The standard requirement for serial release potency testing of Leptospira bacterins in the United States is the hamster vaccination-challenge test. It is a test that uses a large number of animals experiencing pain or distress, takes weeks to conduct, can be expensive and requires that laboratory personnel handle a viable zoonotic pathogen. In an effort to address these concerns, the United States Department of Agriculture (USDA) developed an *in vitro* method for potency testing of four Leptospira serovars. This enzyme-linked immunosorbent assay (ELISA) was subsequently validated in the target species. USDA informed their biologics licensees, permittees and applicants of the availability of reference bacterins and the regulatory acceptance regarding this alternative test method in notices issued in 2007 and 2009. This presentation describes how the initial research and subsequent development and validation work were accomplished.

I-7-472

Major challenges in the development of potency tests for fish vaccines

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Research of fish immunology is minimal in comparison to research of avian and mammalian immunology. This has a enormous impact in the development of *in vitro* assays and the identification and production of specific and sensitive reagents for batch release testing. Furthermore, difficulty to replicate in the laboratory stressor factors commonly seen in commercial fish farming, such as transport stress, accelerated smoltification and change in environment, represents one of the major challenges in the development of alternative testing for vaccine potency determination.

Fish are, evolutionarily, the first vertebrates to develop both innate and acquired immune systems. These are quite distant from avian and mammalian immune systems in terms of the organization of the immune tissue and the molecules that participate during the immune response. For instance, fish do not have lymph nodes, which are important lymphoid organs in mammals and birds where most immune responses occur. Immunoglobulin M (IgM) is the most common Ig isotype found in fish blood. Therefore, in fish it is difficult to observe class switching, a phenomenon that occurs in the lymph nodes and that is easy to determine in mammal serum by the presence of IgG.

Differences between fish species are so broad that the use of reagents, such as antibodies, cannot be used across species. The difficulties of accurately detecting an specific immune response in combination with the physiological and anatomical differences between fish species and other species and the lack of reagents pose a problem for the development of an *in vitro* potency test.

I-7-449

In vitro detection of tetanus toxicity by a combined assay taking into account binding and enzymatic activity

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Tetanus neurotoxin (TeNT) is a potent toxin produced by the bacterium *Clostridium tetani*. It consists of two disulfide-linked protein subunits: The heavy chain mediates the binding and uptake by neurons, whereas the light chain cleaves the neuronal protein synaptobrevin and thus causes a spastic paralysis. Chemically inactivated TeNT (tetanus toxoid) is used for the production of veterinary and human vaccines. In order to exclude the presence of residual active toxin, toxicity tests in guinea pigs are prescribed by the European Pharmacopoeia for each toxoid bulk. Our aim is to replace these animal tests by an *in vitro* method.

Most *in vitro* assays for the detection of tetanus toxicity described to date have solely been based on the proteolytic activity of TeNT. According to our experience, however, such methods often generate false-positive results. In particular, free toxin light chains (which are proteolytically active, but non-toxic) can interfere with these assays. In order to better reflect the *in vivo* situation of a tetanus infection, we are developing a combined assay which takes into account additional determinants of tetanus toxicity. In this assay, only TeNT molecules which display both receptor binding and synaptobrevin cleaving features on separate, disulfide-linked subunits will finally generate a signal. The presentation will outline the current state of the assay development project and highlight some recent results.

Session I-7: Poster presentations

I-7-354

Preliminary study of development of an *in vitro* potency test for black disease vaccine using vero cells

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Black disease is one of the infectious animal diseases caused by *Clostridium novyi* that produce alpha toxin, which damages the liver causing death. Vaccination is an effective method to protect animals from clostridial disease and the potency test is one of the most important quality control tests requiring a very high number of laboratory animals.

The aim of the present study was to develop an *in vitro* method to replace the *in vivo* toxin neutralization test for potency testing of black disease vaccine. The sensitivity of vero cells to alpha toxin was first measured. The alpha toxin was purified from a 48 h culture of *C. novyi* at 37°C by adding 40% ammonium sulfate. After centrifugation, the pellet was redissolved in PBS and dialyzed against distilled water. The concentrated toxin was purified by sephadex column chromatography.

The vero cells were cultivated in DMEM medium with 10% FCS. Different dilutions of toxin in a microplate containing vero cells were examined and finally in dilution 1/1500, 10-15% viable vero cells were detected by MTT staining method after 3 days.

Sensitivity of vero cells to alpha toxin can be used as an alternative toxin neutralization test to test the potency of black disease vaccine.

I-7-457

Application of the Three Rs to challenge assays used in vaccine testing

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This presentation will introduce and summarise the recommendations from a recently published expert Working Group report on the implementation of the Three Rs in the testing of vaccines for regulatory and other purposes. The principles described are widely applicable to all situations that involve experimental infections of animals, but the focus is on identification of reduction and refinement opportunities in the challenge assays used to assess batch potency of certain vaccines, since it is these tests that cause most suffering. The report encourages a practical approach, including the review of all aspects of experimental design and test procedures, and of the animals' life time experiences.

Guidance is provided on: preparation, maintenance and storage of materials and equipment; selection of animals; animal housing and care; numbers of animals and statistical design; administration of substances; vaccination and challenge schedules; humane endpoints; animal monitoring and staffing issues.

The report also aims to help interpret the requirements of the European Pharmacopoeia with regard to the use of alternative tests, humane endpoints and other refinements, and discusses the need for international harmonisation of requirements, taking two specific worked examples, for batch potency testing of *Clostridium chauvoei* and canine leptospira, as examples.

Session I-8: Safety testing for chemically-induced eye injuries: Recent Three Rs advances

Session I-8: Oral presentations

I-8-673

Chemical injuries and the corneal response

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Chemical injuries represent one of the most difficult clinical entities to treat due to the variety of chemicals and their actions on corneal tissue. The risk is of course the loss of vision which may result in corneal blindness. The most frequent injuries are from encounters with extreme pH conditions (<4 or >10); however, there are also unusual or exotic chemicals used in industrial solutions and processing for which the response would not be well understood. As the chemical penetrates into the corneal tissue it has access to other ocular tissues, such as the iris and lens. Damage to the endothelium is one of the most damaging effects as the endothelial cells in humans do not regenerate and a corneal graft is the only realistic therapeutic approach. Chemical splashes also will take in the conjunctival tissue surrounding the cornea and may damage the stem cell for the cornea at the limbus. This type of injury can be approached by the use of regenerative limbal or conjunctival stem cell transplants, or by oral epithelium transplants or by use of amniotic membrane patches. A common outcome of chemical injuries is also the invasion of blood vessels into the cornea with a subsequent loss of visual acuity. Additionally, damage to the conjunctiva can lead to exteme dry eye. The access of chemicals to the cells and cellular structure is critical to understand for both awareness and avoidance of potential injury and to understand the outcome for restoration of vision.

I-8-444

Human eye exposure to surfactant solutions; *in silico* determination

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For the last three decades we have been dedicated to the development and use of *in vitro* alternatives to replace and reduce animal testing. The trans-epithelial permeability (TEP) assay has been validated internally as an alternative to the Draize rabbit eye test and has been used as part of our safety assessment program for 20 years. Over the last two decades we have compiled a large data set of human ocular testing with end points of eye stinging, bulbar conjunctive redness, palpebral conjunctive redness, and lacrimation. This data set includes ocular testing on approximately 20,000 human eyes with 170,000 end points.

From this data set, ingredient effects of surfactant solution are modeled, and human clinical results are predicted.

With over 19,000 ingredients available in personal care, a structure-activity relationship-type approach is used to compress the many ingredients into fewer compound families. The experimental clinical data sets are also augmented with surfactant theory and high throughput physical chemistry testing to create a structural model of the ingredient effects on clinical results. This modeling of clinical results based on the constituent ingredients creates insight into the effects of different ingredient types (e.g., anionic surfactants create harsher surfactant systems, non-ionic surfactants make systems milder). This large data set enables the evaluation of variations in ocular response among subject populations; we observe differences in the age dependence of ocular redness between males and females. Also, the neuronal eye sting response is not observed to be correlated with eye redness.

I-8-363

Measuring Depth of Injury (DoI) in the Bovine Corneal Opacity and Permeability (BCOP) Assay

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Past studies have shown that the severity of ocular irritation in the Low-Volume Eye Test is dependent on DoI regardless of chemical type, and that DoI measuring biomarkers for cell death and viability can be used to assess irritation potential in an *ex vivo* rabbit eye test. An ongoing project in the COLIPA eye irritation program builds on this earlier DoI work using the standard BCOP assay. This study is evaluating the ability of DoI to discriminate between different Globally Harmonised System (GHS) categories in the BCOP test. Bovine eyes were obtained from a slaughterhouse and processed for standard BCOP protocol (minimum 3 eyes/irritant) using chemicals with different irritation potential having GHS (Draize) classifications of Category 1 (7 severe irritants), Category 2 (5 irritants) and Not Classified (4 non-irritants) compared to water as control. All corneas received 10 minute exposure and 3 h recovery and were then fixed in 2% paraformaldehyde, frozen in liquid nitrogen, sectioned and evaluated by fluorescent staining with phalloidin. DoI was then assessed using an epifluorescence microscope to measure dead and viable corneal epithelial and stromal thickness. Percent DoI for epithelium, stroma and cornea were then calculated. Results indicate that stromal DoI was different between different GHS Classifications with Category 1 producing >50% compared to Category 2 (0-40%) and Not Classified (0%). These preliminary data suggest that measuring DoI in the BCOP assay can correctly identify Category 1 from Category 2 ocular irritants.

I-8-055

A procedure for application of eye irritation alternative methods to cosmetic ingredients

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Alternative methods to replace the Draize rabbit eye test for evaluation of eye irritation by cosmetic ingredients have been studied by many in the cosmetics industry and independent organizations in several countries for many years, such as ECVAM, ICCVAM, IRAG, etc. Alternative assays have been extensively researched, and some have undergone formal validation. However, to date, there is no single *in vitro* assay that has been validated as a full replacement for the Draize rabbit eye test. The isolated rabbit eye test (IRE) and the hen's egg test-chorioallantoic membrane (HET-CAM) assays are accepted for specific and limited regulatory purposes. Both have already been validated by ICCVAM. In light of the deadlines established in the EU Cosmetics Directive for cessation of animal testing for cosmetic ingredients and the regulation for the least amount of animal testing for safety assessment on chemicals in the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), we evaluated the applicability and predictive capacity of IRE and HET-CAM, and established the prediction model with both of the alternative methods by discriminant analysis. Furthermore, we proposed and evaluated a procedure for application of eye irritation alternative methods (PEIAM) to cosmetic ingredients. The PEIAM had a good predictive capacity when compared to the results of animal tests, indicating potential for *in vitro* screening of chemicals for eye irritation.

I-8-503

Development of a non-animal testing strategy for ocular hazard labeling of some specific EPA-regulated products

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Changes in toxicological testing strategies within a regulatory agency can occur through several pathways. In 2009, the EPA's Office of Pesticide Programs instituted a pilot program which utilizes results from specific non-animal testing methods for assessment of eye irritation potential, as long as the testing methods and testing results are deemed by EPA to be adequate and appropriate to support labeling decisions. Prior to this, cleaning products making an anti-microbial claim (and thus considered pesticides) normally had to be tested using the Draize rabbit eye irritation test to provide data for hazard labeling. However, several years ago a suggestion was made by the Pesticide Program Dialog Committee (a diverse group of stakeholders selected by the EPA to provide feedback to the pesticide program on various pesticide regulatory, policy and program implementation issues) that non-animal methods should be investigated for their ability to provide satisfactory information to determine an EPA hazard category for eye irritation. Subsequently seven AMCP manufacturers agreed to share data from non-animal, *in vitro* ocular studies (along with historic animal data) which they had individually generated. This allowed the creation of an extensive database describing the performance of several *in vitro* methods for assigning eye irritation hazard categories for AMCPs, relative to that of the traditional rabbit eye test. A testing strategy involving up to three *in vitro* methods (Bovine Cornea Opacity and Permeability assay, EpiOcular™ tissue (MatTek Corp., Ashland, MA), and the Cytosensor Microphysiometer (Molecular Devices, Sunnyvale, CA) was devised and subsequently evaluated by EPA staff. It was determined that this *in vitro* strategy for labeling of ocular hazard was sufficient to propose its further prospective evaluation in a pilot program. Cooperation among the seven companies was essential for this success, since it is unlikely that the amount of information that any one company possessed would have been sufficient to allow the EPA to initiate the pilot program.

Session I-8: Poster presentations

I-8-057

Comparative study of five *in vitro* tests as an alternative method for eye irritation testing

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The purpose of this study was to evaluate the applicability and predictive capacity by comparing five *in vitro* tests for eye irritation, to try to find better testing methods for eye irritation of cosmetics. 27 cosmetic products were assessed in the study, they were tested by five alternative methods for eye irritation tests, i.e. HET-CAM, CAM-TBS, FLT, 3T3-NRU cytotoxicity assay and red blood cell (RBC) haemolysis assay. The cosmetics were tested *in vivo* by the Draize test. *In vivo* and *in vitro* test results were compared and analyzed with SPSS software. It was shown that ranking correlation and class concordance existed between the five alternative methods and the Draize test by applying 27 cosmetic products, the relationship between HET-CAM, CAM-TBS, FLT and MMAS is better, a predictive model was developed of Y from X, subject to the maximum possible correlation between the MMAS and HET-CAM, CAM-TBS, FLT from X. It is suggested that the three *in vitro* assays, HET-CAM, CAM-TBS and FLT have good predictive capacity, reproducibility and reliability when compared with the Draize test. In addition, a predictive model has valuable application as a screening test in cosmetics safety evaluation.

I-8-091

Extracellular acidification and changes in bioimpedance of L929 cells

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Monitoring of extracellular acidification from L929 mouse fibroblasts with the cytosensor microphysiometer is a non-animal method to identify non-irritant water-soluble substances in the field of eye irritation (Hartung et al., 2010). No other non-animal methods exist for this category. However, the cytosensor microphysiometer is no longer commercially available. Other instruments (e.g. from Bionas GmbH or cellasys GmbH) for determining extracellular acidification rate may fill the gap (Scott et al., 2010).

Besides the extracellular acidification, cellular respiration and changes in bioimpedance have the potential to classify substances correctly. In this work, extracellular acidification was compared with changes in bioimpedance of L929 fibroblasts using the IMOLA-IVD technology which was developed in the group of Prof. B. Wolf at the Technische Universität München (Wiest et al., 2006). The results show that changes in bioimpedance are also useful to investigate toxicological effects. However the mechanistic background is so far not completely understood. A prediction model has to be established to allow bioimpedance measurement to enter the validation process.

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I-8-127

In vitro eye irritation assessment using the SkinEthic HCE test method applied to ingredients used in cosmetics

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To comply with the EU Cosmetics Directive, predictive alternative methods are required to evaluate eye damage potential of chemicals. Linked to the complexity of both eye irritation mechanisms and the diversity of chemicals, the *in vitro* assessment of eye irritation potential is a complicated issue. The corneal epithelium is crucial and represents the first line of defense against injury. 3D *in vitro* tissues sustained by adapted technologies allow the testing of substances in conditions similar to *in vivo* exposure. In this study, we have used the standardized SkinEthic reconstructed human corneal epithelial (HCE) model to evaluate *in vitro* eye irritancy. A specific protocol was developed aiming to match chemicals' properties with adapted exposure steps (1 h exposure, 16 h post exposure incubation) in particular for cosmetic ingredient families. Analyses were performed according to the Globally Harmonized System (GHS) classification. The Prediction Model, using a 50% viability cut-off, allowed the drawing up of 2 categories: Irritants (grouping Cat1 and 2) and No Category. Applied to a broad set of 435 cosmetic substances the SkinEthic HCE test method showed good and balanced prediction performances (81% sensitivity; 82% specificity). Furthermore by using appropriate controls the applicability domain of the method can be extended to the MTT reducers and/or dye substances by using additional controls. Severe or irreversible irritant chemicals were not specifically differentiated from reversible and mild irritants. This test method is part of the ongoing ECVAM eye irritation validation. It could be part of a specific tiered test strategy for hazard assessment of test substances in regulatory schemes.

I-8-150

Assessment of eye irritation potential using the reconstructed human corneal tissue LabCyte CORNEA-MODEL

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In vitro eye irritation testing alternative to animal testing such as Draize eye test using rabbits is required from an animal welfare standpoint. We have developed the new reconstructed human corneal epithelium model, LabCyte CORNEA-MODEL, and we investigated a test method to evaluate eye irritation using this model. Claudin-1, a component of tight junction; E-cadherin, a component of adherence junction; and Desmogrein-3, a component of desmosome, were strongly expressed in all layers of LabCyte CORNEA-MODEL. Mucin-1 and Mucin-16 were expressed in the super-facial layer. These results suggested that this model was correlated with the tissue structure of normal human corneal epithelium. New methods were also established for eye irritation testing using LabCyte CORNEA-MODEL. The application period of chemicals was set to 1 min for liquids and 24 h for solids, and the post-incubation period was set to 24 h for liquid or none for solid. If the viability was less than 50%, the chemical was judged to be an eye irritant. Sixty-one chemical substances were applied to this new *in vitro* eye irritation test and the correlation between *in vivo* class and the *in vitro* prediction of eye irritation was evaluated. Since *in vitro* results using LabCyte CORNEA-MODEL were highly correlated with *in vivo* eye irritation (sensitivity 100%, specificity 80.0%, and accuracy 91.8%), it is suggested that the eye irritation test using this model could be useful for a variety of chemicals with irritant potency as an alternative method to the Draize eye test.

I-8-151

A tiered approach combining the STE test, the EpiOcular assay, the HET-CAM assay and the BCOP assay for predicting eye irritation potential of chemicals

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The predictive potential of a tiered approach using the results from the STE test, the EpiOcular assay, the HET-CAM assay and the BCOP assay, was examined for assessing GHS eye irritation. Around 50 chemicals with a balance of GHS eye irritation categories and a wide range of chemical classes were selected. The chemicals were evaluated in either the STE test or the EpiOcular assay. In addition, we adopted the evaluated classification of the HET-CAM data and BCOP data from the background review document (BRD). The first step in our approach was to evaluate the chemicals in the STE test, depending on chemical solubility. For non-soluble chemicals, EpiOcular results or adopted HET-CAM data for tested chemicals from the BRD were used. If the chemical was classified as a "non-irritant" by first phase tests, it was considered to have a GHS ranking of "not classified." If the chemical was classified as an "irritant" in first level tests, the classification was subsequently confirmed by reviewing the results from the BCOP data adopted from the BRD. If the classification was "severe", the chemical was considered a GHS "Cat.1". For those chemicals classified as "non-severe", these were considered to be GHS "Cat.2". The tiered (bottom-up) approach combining either the STE test or the EpiOcular assay with the HET-CAM assay and then the BCOP assay allowed the evaluation of GHS eye irritation category with an accuracy of more than 73%.

I-8-180

Inter-laboratory phase II validation study of *in vitro* eye irritation test; Short Time Exposure (STE) test

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The Short Time Exposure (STE) test is an easy-to-use *in vitro* eye irritation test using the cell viability of SIRC (rabbit corneal cell line) cells as an end point following one 5 min treatment. The Executive Committee of the Japanese Society for Alternatives to Animal Experiments conducted a validation study with five laboratories from 2008 to 2009. These data showed good transferability. Assignment of 25 blinded chemicals to the STE irritation categories "Non-Irritant" (NI) or "Irritant" (I) showed good inter-laboratory reproducibility and predictive capacity for predicting the GHS category of "Not classified" (NC) or I (category 1 or category 2). In this study, we selected 40 additional blinded chemicals to optimize the balance of GHS category and re-evaluate the predictive capacity of the STE test.

The results showed that the STE test was not only easy to acquire and implement among three laboratories, but it also had a high intra- and inter-laboratory reproducibility, and a high ability to predict the GHS category (NC or I). However, a predictive ability of the STE rank for predicting GHS categories was not good compared with that of STE irritation categories (NI or I). Therefore, the STE test can assess not only the severe/corrosive ocular irritant (correspond to GHS category 1) but also the mild or moderate ocular irritants (correspond to GHS category 2). However, a predictive ability of the STE rank was insufficient for identification of GHS categories. The STE test was recommended for use as part of a tiered testing strategy for regulatory classification.

I-8-302

Development of IRR-IS®, an Episkin® based model for quantifying chemical irritation potency using an algorithm based on analysis of magnitude of gene expression of selected biomarkers

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Although a number of assays for irritation potency classification using 3D reconstructed epidermitis and MTT measurement have been developed, their ability to quantify irritation potency is limited. To formulate and address new and future regulatory demands, the ability to measure and quantify the skin irritation potential of chemicals without animals is of high importance. Moreover the ability to quantify skin irritation can be valuable information when measuring eye irritation. We developed IRR-IS®, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®). The selection of biomarkers was done by analysis expression profiles in 3D reconstructed epidermis with several irritants. Test chemicals were applied for 30 minutes then washed and the tissues were further incubated for 6 h. Tissues were teased, total RNA purified with Trizol and expression of genes measured by quantitative PCR after reverse transcription. We selected 25 biomarkers and developed an algorithm based on analysis of magnitude of gene expression. We will present here the results of these studies and will show the quantitative capacity of this approach on a set of 40 chemicals

I-8-314

Comparative studies for three *in vitro* methods to evaluate the eye irritation potential of disinfectants

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In Brazil, the health legislation requires the Draize test for registering household products. However, the global trend has been the replacement of *in vivo* tests by *in vitro* assays. Thus, there is a need for studying *in vitro* methods to develop effective predictive models to detect the irritation potential. This study aims to compare three *in vitro* assays to the method described by Draize to evaluate the ocular irritation potential. Ten disinfectants were tested: seven pure and three diluted to 30% (v/v). The following tests were performed *in vitro*: i) Red Blood Cells test (RBC) according to the protocol INVITTOX 37, ii) chorionic allantoic membrane with trypan blue staining (CAM-TBS) assay, protocol INVITTOX 108, and iii) hen's egg test-chorionic allantoic membrane (HET-CAM) was performed according to the method described in the Journal Officiel de La Republique Française. The *in vivo* data were obtained from the INCQS database. The results were arranged in contingency tables to determine the accuracy, specificity and sensitivity of each method to *in vivo* the test. In *in vivo* test 1 product has been classified as non-irritant (NI), 5 weak irritant (WI) and 4 moderate irritant (MI). In the RBC test 1 product was classified MI, 4 severe irritant (SI) and 5 maximum irritant (Mx). In CAM-TBS 2 MI and 8 severe irritant and HET-CAM all products were classified as SI. The three *in vitro* methods showed five false positive results, implying low specificity. But the three methods showed good sensitivity.

I-8-324

Proposal of a mechanism-based selection of reference chemicals for development / evaluation of *in vitro* eye irritation methods

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The COLIPA (European Cosmetics Association) Task Force Eye Irritation is actively involved in the development of *in vitro* methods to replace the Draize rabbit eye test, now banned for evaluating ingredients used in the cosmetic industry. One of its key projects is focused on a validation study of optimized existing Reconstructed human Tissue (RhT) test methods (MatTek EpiOcular EIT® and SkinEthic® Human Corneal Epithelium (HCE)). After the evaluation of numerous chemicals and completion of pre-validation datasets, a prospective ECVAM validation study is now in progress to reliably discriminate non classified chemicals from all classes of eye irritants. In order to further develop *in vitro* assays that accurately identify the eye irritation potential, selection of appropriate test chemicals is of critical importance.

Here, we provide a set of 49 chemicals which: 1) are single chemical entities; 2) are supported by high quality *in vivo* data; 3) cover the whole range of irritant effects/potencies; 4) cover different chemical classes/physical states and 5) are readily available. All chemicals have been tested in at least one RhT assay and in the Bovine Corneal Opacity and Permeability test method. The use of such a reference list would facilitate early assessment of new method performance with respect to existing tests and its possible contribution to a tiered testing strategy. This poster provides a detailed analysis of a chemical dataset still under refinement, proposed by Colipa for use in the development and evaluation of *in vitro* methods for eye irritation testing.

I-8-345

A novel rapid assay useful for eye irritation testing using a human corneal epithelium model reconstructed in a collagen vitrigel membrane chamber

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A collagen vitrigel membrane (CVM) is composed of high density collagen fibrils equivalent to connective tissues *in vivo*. Also, it possesses excellent transparency and permeability of protein with high molecular weight. Recently, we made a novel CVM chamber adapted to three-dimensional culture. In this study we reconstructed a human corneal epithelium model in the chamber, investigated its histology and barrier function, and evaluated its utility in an eye irritation test.

HCE-T cells (a human corneal epithelium-derived cell line) were cultured in a CVM chamber for 2 days to form a confluent monolayer. Subsequently, a corneal epithelium model was reconstructed by culturing it in the air-liquid interface for 7 days. Histology and barrier function during the process of reconstructing the model were analyzed by fluorescent stain and TEER (transepithelial electrical resistance) measurement, respectively. More than 20 reference chemicals known as eye irritants were used to challenge the model and were evaluated regarding time-dependent changes of TEER.

Histological observations revealed that the reconstructed human corneal epithelium model was composed of around five cell layers and its outer 2-3 layers strongly expressed tight and gap junctions-related proteins. The decreasing ratio of TEER at 10 seconds after exposing the model to each chemical was well correlated with the eye irritancy previously reported as Draize score and/or GHS classifications. These results suggest that the human corneal epithelium model reconstructed in the CVM chamber provides a novel rapid assay useful for eye irritation testing.

I-8-388

In-house validation of the EpiOcular™ eye irritation test (EpiOcular-EIT) with 60 test substances and its implementation into the tiered testing strategy for assessment of ocular irritation according to the GHS

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The bovine corneal opacity and permeability (BCOP, OECD TG 437) test is regulatorily accepted for the identification of corrosive and severe ocular irritants (GHS category 1). However, there is currently no regulatorily accepted *in vitro* test available for the differentiation of ocular irritants and non-irritants (GHS category 2 or no category).

Human reconstructed tissue models have been suggested for incorporation in a tiered test strategy to ultimately replace the Draize eye irritation test (OECD TG 405). In this model, cell death induced by slight or moderate irritants is determined by the MTT assay. We established and evaluated the EpiOcular™ assay to discriminate ocular irritants from non-irritants. Test substances that decreased viability to ≤ 60% (compared to control) are considered eye irritants (GHS cat 1 or cat 2) and test substances with less effect on viability are considered non-irritants.

The tests were performed with 60 test substances including a broad variety of chemicals and formulations for which *in vivo* data (Draize eye irritation test) and BCOP data were available: 18 severe irritants/corrosives (GHS category 1), 21 irritants (GHS category 2), and 21 non irritants (no GHS category). For the assessed data set the EpiOcular™ assay provided sensitivity (cat1+cat 2) >90% and specificity (no cat) >70% resulting in overall accuracy of >80%. Applying an alternative viability threshold (50% instead of 60%) resulted in sensitivity, specificity, and accuracy >80%. The tiered testing strategy combining BCOP and EpiOcular into a "top-down" and "bottom-up" concept was evaluated and results will be presented.

I-8-394

COLIPA Eye Irritation Task Force strategy and programme for development of *in vitro* methods: continued developments and status

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The COLIPA (European Cosmetics Association) eye irritation programme for development of *in vitro* methods is focused on identification of new *in vitro* test systems/endpoints that use the understanding of mechanisms of eye injury/recovery to predict human ocular responses to chemical exposure. The core approach to achieve this is based on optimisation of existing *in vitro* assays through applied research and method refinement. Applied research focuses on models using Depth of Injury (DoI) as a mechanistic basis for eye irritation. An ongoing project builds on the standard BCOP assay to correlate DoI consistent with the degree of irritancy observed *in vivo*, using phalloidin staining to demonstrate loss of F-Actin in cells that have been killed. This approach could result in new/improved *in vitro* methods that would proceed to formal validation.

Key projects on method refinement focus on Reconstructed human Tissue (RhT) assays using MatTek EpiOcular® and SkinEthic® HCE human corneal models. We have, working with test method developers, completed pre-validation datasets to enable entry into a prospective ECVAM validation study for discrimination of non-classified chemicals from eye irritants (all classes). This ECVAM validation study is in its experimental phase (from mid-2010). A further project integrating use of HPLC/UPLC into RhT assays addresses a known limitation of possible interference with absorbance measurement of MTT by photometry for intrinsically coloured test materials. We continue to work in collaboration with external organisations such as ECVAM, academia and regulatory authorities to achieve validated alternatives.

This poster provides a detailed overview and update of the COLIPA Eye Irritation programme strategy and content.

I-8-408

Eye irritation of eye make-up removers assessed by *in vitro* methods and a clinical study

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Safety assessment of cosmetics intended for use in the eye area requires evaluation of ocular irritancy exclusively using *in vitro* tests due to recent legislative restrictions within the EU. Ten commercially available eye-make up removers were subjected to testing of eye irritation hazard using a battery of *in vitro* tests, including the bovine corneal opacity and permeability (BCOP) test, EpiOcular™ tissue construct assay, cytotoxicity tests using Balb/c 3T3 fibroblasts (Neutral Red Uptake and Neutral Red Release) and Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) assay. Results of the *in vitro* tests were compared to the outcome of a clinical in-use test under ophthalmological control after application of the products to the external eyelid. Individual alternative assays predictions for mild irritant formulations were not entirely consistent in terms of rank ordering relative to the human reactions respecting the foreseen conditions of use. The EpiOcular assay provided the most concordant results with human reactions. Negative HET-CAM results were in agreement with the mildest clinical symptoms recorded in the clinical study. However, the severity of clinical symptoms was not related to the irritation score obtained using the HET-CAM assay. The NRR assay seems to provide relevant additional results to EpiOcular and HET-CAM assays. The results confirm that a battery of *in vitro* tests with different endpoints might be required for reliable assessment of eye irritation; however, not all of the currently used assays seem to be correlated sufficiently with adverse clinical signs *in vivo*.

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I-8-428

Inter-laboratory validation of the alternative HET-CAM test

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The purpose of this study was to perform the HET-CAM test for inter-laboratory validation between TRIDSKIN and NATURA. Three independent assays were performed to evaluate the potential ocular irritation using the HET-CAM method. Besides the positive results (SDS) and negative ones (saline) three samples were tested, i.e. two bi-phasic oils and one sample powder. The average values of IS in the three assays for the powder sample was 1.0 according to the table of category of irritation, classified as slightly irritant. The only event observed was a discrete hyperemia and, in some cases, a discrete hemorrhage. As for the bi-phasic oils, a test for the inferior and superior phases was needed, once these samples obtained inconsistent results in the three essays. The superior phases of the bi-phasic oils showed IS equal to "0" being, therefore, classified as a non-irritant. However, the inferior phases of bi-phasic oils showed IS values between 3.0 and 3.91, being, therefore, classified as slightly irritant. The only event observed was a light hyperemia and, in some cases, a discrete hemorrhage. The positive control (SDS 1%) showed IS equal to 9.17 while the negative control (saline) showed IS equal to "0", therefore these samples are classified as severe and non-irritants, respectively.

I-8-433

Development of a new opacitometer for the Bovine Corneal Opacity and Permeability (BCOP) assay

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The BCOP assay is generally accepted as a valid alternative method to the Draize eye irritation test to detect corrosive and severe eye irritants. Routinely, opacity is measured by an OP-KIT opacitometer, which provides a centre-weighted reading of light transmission by measuring changes in voltage when the transmission of white light through the cornea alters. However, this may underestimate opacity that develops as spots or heterogeneous opaque areas on the periphery of isolated corneas. In addition, the BCOP test has not proven sensitive in distinguishing among mild and moderate eye irritants.

A prototype of a new laser light-based opacitometer allowing better measurement of opacities was developed by Van Goethem and colleagues. This new device showed improved sensitivity to detect subtle changes in corneal transparency. Furthermore, the new opacitometer allowed the analysis of the complete corneal surface and was able to detect more efficiently opaque spots located along the sides of the excised corneas. CARDAM, in co-operation with Peira Scientific Instruments (Beerse, Belgium), will construct a copy of this prototype and improve further the equipment for opacity measurements by 1) using the laser light-based opacitometer in combination with a camera and 2) modifying treatment conditions of the corneas in the cornea holders in order to better mimic the *in vivo* situation. A set of reference compounds with irritancy potencies, especially in the mild and moderate range, will be tested. These modifications of the classical BCOP assay should allow a more accurate definition of the eye irritating potential of compounds.

This research project is sponsored by the Stavros Niarchos Foundation (Greece).

I-8-438

Validation study on the Ocular Irritection® assay

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A validation study was undertaken to obtain additional prospective data and assess the relevance and reliability of the Ocular Irritection® assay according to the OECD principles for validation and the ECVAM modular approach. The primary goal of the study is to evaluate the ability of the Ocular Irritection® test to reliably discriminate non-classified substances from classified ocular irritants (categories 1 and 2) as defined by the UN Globally Harmonized System for classification.

The assay is based on a macromolecular reagent that, when rehydrated, forms an ordered matrix mimicking the highly ordered structure of the transparent cornea. Irritant substances produce a turbidity of the reagent by changes in protein conformation and degree of hydration that mimics the disruptive effects irritants may have on the corneal proteins and carbohydrates. Because of its nature, Ocular Irritection® presents the advantage of having long shelf-life (years) and is easily shipped around the world.

An international Validation Management Group was formed to manage and oversee the study comprising an independent chairman, co-chair, biostatistician and chair of the chemicals selection, in addition to the sponsor representatives (INT.E.G.RA and InVitro International) and the lead laboratory representative. A challenging set of 60 coded substances for which *in vivo* data are available were selected in collaboration with ECVAM and will be tested in 3 independent laboratories from Europe and the USA, including one naïve laboratory. The testing phase is planned from May to September 2011 after the training and transferability of the test method takes place.

I-8-532

Considerations for demonstrating the inter-laboratory reliability of Chorioallantoic Membrane Vascular Assay (CAMVA) and the Bovine Corneal Opacity and Permeability Assay (BCOP)

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In vitro assays evaluating ocular irritation potential are routinely used by personal care companies. Two of these *in vitro* assays include the Chorioallantoic Membrane Vascular Assay (CAMVA) and the Bovine Corneal Opacity and Permeability Assay (BCOP). These assays do not require the use of live animals, provide reliable predictive data and are rapid to conduct. The BCOP uses excised bovine corneas to predict ocular irritation. The CAMVA uses the vascular network of fertilized chicken eggs as a conjunctival model to predict eye irritation. Both BCOP and CAMVA have been used for over fifteen years for product development, worker safety, and safety claims substantiation.

This poster describes procedures and considerations for demonstrating the inter-laboratory reliability of the BCOP and CAMVA. It is important to have a valid assay that can be implemented consistently at several different laboratories. For Kao Brands Company, a large BCOP and CAMVA database exists that covers multiple consumer product categories such as hair shampoos, skin cleansers, and hair styling sprays (containing ethanol). Therefore, a proper review of candidate laboratories is important for seamlessly generating consistent results that can be used for assessing potential ocular irritation of new products. First, a candidate laboratory should be audited for proper facility operation and personnel training. Second, the laboratory's use of Good Laboratory Practices (GLPs) should be reviewed. Third, reference materials with known BCOP and CAMVA data (one irritant and two non-irritants for initial assessment) should be tested at each new laboratory for verification of proper assay performance.

I-8-541

Prospective validation study of reconstructed human tissue models for eye irritation testing

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A prospective validation study of two *in vitro* test methods using Reconstructed human Tissue (RhT) models (EpiOcular™ and SkinEthic™ Human Corneal Epithelium (HCE)) for the detection of eye irritation effects by chemicals is currently being conducted by ECVAM and COLIPA. These methods are being validated for their usefulness to identify chemicals as either not classified for eye irritation (NC) or irritant (Cat. 1 and Cat. 2) within the United Nations Globally Harmonised System (UN GHS), for inclusion into a Bottom-Up/Top-Down test strategy, with the ultimate goal of replacing the *in vivo* Draize eye irritation test. They are not intended to differentiate between mild/moderate (Cat. 2) and severe (Cat. 1) irritants, which would be left to another tier of the test strategy.

Pre-validation studies indicated that both methods predict eye irritant properties of chemicals with high accuracy (~80%). In the current validation study 104 chemicals are being tested with each method in three independent runs by three laboratories. Selected chemicals cover the full irritancy range, represent a wide range of chemical classes/functionalities, and are also balanced in physical state and chemical reactivity.

The Validation Management Group has organised Quality Assurance audits on each RhT production site to guarantee quality of supplied tissues and has developed guidance on study conduct for laboratories as well as performance criteria for assessing validity of the test methods. The participating laboratories have successfully completed transfer and transferability studies and are expected to finalise the testing phase in mid-2011. This poster presents all latest updates of the validation study.

I-8-576

Usefulness and limitations of the Cytosensor® Microphysiometer (CM) test method for ocular safety testing

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ICCVAM evaluated CM as a potential replacement for the rabbit eye test for identifying ocular hazards. CM is restricted to water-soluble substances. ICCVAM concluded that substances within a limited applicability domain (water-soluble surfactants, surfactant-containing formulations, non-surfactants) that are positive for severe effects in CM can be classified as ocular corrosives/severe irritants (EPA Category I, EU R41, GHS Category 1). False positive rates ranged from 0% (0/17, 0/18) to 10% (3/29) and false negative rates ranged from 9% (2/23) to 50% (6/12) depending on the hazard classification system used. ICCVAM also concluded that substances within an even more restricted applicability domain (water-soluble surfactant chemicals and certain types of surfactant-containing formulations, but not non-surfactants) would not require ocular hazard labeling (EPA Category IV, EU Not Labeled, FHSA Not Labeled) without any further testing if they are negative in CM. Although false positive rates were high (50% [3/6] to 69% [18/26]), false negative rates ranged from 0% (0/27, 0/28, or 0/40) to 2% (1/46 or 1/47) depending on the hazard classification system used. CM is not considered valid for identification of mild or moderate ocular irritants (EPA Categories II/III; EU R36; GHS Categories 2A/2B); these substances would require additional testing. ICCVAM also recommended a standardized CM protocol and future studies to expand the applicability domain. Some ICCVAM agencies have endorsed these recommendations, making CM the first *in vitro* test method available in the US for identifying a subset of substances that do not require ocular hazard labeling. A draft OECD TG for CM is currently being considered.

I-8-598

The impact of US adoption of the UN Globally Harmonized System on the use of *in vitro* methods for ocular and dermal irritation and corrosion

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Endorsed by the United Nations in 2003, the UN Globally Harmonized System for classification and labeling is intended to harmonize hazard classification and labeling criteria throughout the world for human health and ecotoxicity endpoints. While GHS was designed to correlate with existing classification systems and the European Union, Canada, and United States have committed in principle to adopting GHS in place of their own national classification systems, differences among classification systems have delayed adoption of GHS by various agencies. Harmonization with GHS impacts the replacement, reduction, and refinement of animals in testing, since *in vitro* methods for skin and eye irritation have been and are currently being validated according to GHS classification. This poster compares US EPA, US OSHA and GHS classifications for skin and eye irritation as they relate to validated *in vitro* methods for skin and eye irritation and discusses methods to harmonize these classification systems. The methods include: The Bovine Corneal Opacity and Permeability test method, the Isolated Chicken Eye test method, the Cytosensor Microphysiometer (CM) test method, and the Fluorescein Leakage test method for eye irritation, and Reconstructed Human Epidermis and barrier models for skin irritation. Widespread adoption of GHS will help speed harmonized adoption of existing and new *in vitro* methods for relevant endpoints.

I-8-629

The eyes have it: Calf versus adult eyes in the Bovine Corneal Opacity and Permeability (BCOP) assay

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The Bovine Corneal Opacity and Permeability (BCOP) assay uses excised bovine corneas obtained from cattle slaughtered for food use. Consequently, the age of the cattle is generally not provided with the corneas. This introduces a variable at the start of the assay, which can be reduced by using eyes from cattle at a defined age.

The OECD guidelines for the testing of Chemicals No. 437 recommends the use of eyes from cattle 6 to 12 months of age but encourages investigators to report the estimated age and/or weight of the animals providing the corneas. In a commercial operation capable of killing large numbers of cattle, it may not be possible to determine age and weight of animals as they are received from a number of suppliers. Additionally, random-source animals typically have not been raised in constant environment and are subject to numerous environmental variables. This study looked at the effect of the cattle's age on the BCOP assay. Five-month-old calf eyes used in this study were obtained from a well-managed barn-raised herd, which had weekly veterinary monitoring and controlled feed and medication.

In this study we compared the *In Vitro* Scores (IVS) from random source cattle corneas with those of corneas from a well-managed calf herd 5 months of age. Thirty over the counter cleaners, hair dyes, hair sprays, deodorants and moisturizers, which had IVS scores obtained from random-age animals were used in the evaluation. The use of five-month old calf eyes resulted in a highly successful correlation with data from adult eyes except with products with a high colorant content or with hydrogen peroxide containing materials. A second *in vitro* assay helped predict the potential ocular irritation.

I-8-630

PorCORA ocular reversibility assay testing with personal care products

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To ensure consumer safety, ocular irritation testing is routinely performed on personal care products. Two alternative ocular toxicity tests, the Chorioallantoic Membrane Vascular Assay (CAMVA) and Bovine Corneal Opacity and Permeability Assay (BCOP), are widely used in the cosmetic industry since they do not require the use of animals. These assays provide reliable data predicting ocular irritation and have high predictive value when compared to rabbit or human eye results. To complement the CAMVA/BCOP assays, the Porcine Corneal Opacity Reversibility Assay (PorCORA) was developed using an *ex vivo* model to predict reversibility of ocular damage caused by potential irritants. In the current study, three commercially a consumer products (a shampoo, a hair color glaze, and a 12% hydrogen peroxide product) were tested in the PorCORA for ocular damage and reversibility. The PorCORA indicates that under the exaggerated *in vitro* study conditions the surfactant-based shampoo may cause irreversible ocular damage: histological changes occurred in the squamous-cell layer of the corneas and mild to moderate changes in the basal-cell layer. However, scientific literature contradicts these results, and ocular damage reversibility does occur *in vivo* following exposure to shampoo. Furthermore, the PorCORA predicts that under the same study conditions used for the shampoo, ocular damage caused by a hair color glaze and a 12% hydrogen peroxide product are fully reversible with histology reporting only minimal or mild microscopic effects to the superficial squamous-cell layer. Like the shampoo, the scientific literature also indicates that ocular damage is reversible *in vivo* following exposure to hydrogen peroxide. In summary, the PorCORA assay, in conjunction with other alternative toxicology ocular irritation assays is a valuable and predictive method to determine the extent of ocular damage and reversibility that products may cause following consumer eye exposure.

I-8-631

Porcine Corneal Ocular Reversibility Assay (PorCORA) predicts EU R41 and GHS Category 1

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Currently, there is no alternative (non-"*in vivo*") ocular irritation assay that can measure corneal tissue damage and reversibility. With the support of two Colgate-Palmolive Grants for Alternative Research, we have developed an alternative assay: Porcine Corneal Opacity Reversibility Assay (PorCORA). PorCORA measures corneal damage and recovery over extended time periods using porcine corneas excised from by-product abattoir eyes. Test articles (liquid and solid) are dosed directly onto the corneal surface, and tissue damage and recovery are assessed by sodium fluorescein (NaFL) retention in the same corneas over time (up to 21 days). We have confirmed NaFL retention results and corneal recovery in the PorCORA system via several approaches. Both fluorescence and reflective confocal microscopy confirm damage repair indicated by fluorescein retention in the cultured corneas. In addition, we have shown histological evidence that also correlates well with NaFL staining in the PorCORA assay. Here we report the results of a 32-reference chemical validation including chemicals from the following classes: acetates, acids, alcohols, alkalis, esters, hydrocarbons, inorganics, ketones, surfactants, and several solid compounds. To determine if the PorCORA system can predict R41 or GHS Category 1 we considered corneas that retained NaFL at 21 days post-dose to be R41 and GHS Category 1. ECETOC historical rabbit eye data was used to classify EU and GHS eye irritation for the 32 compounds tested. PorCORA predicted 11/11 compounds classified as R41 and 12/13 compounds classified as GHS Category 1. Since PorCORA can predict these categories, compounds that cause damage that is reversible in the PorCORA system may be considered R36 or Category 2. Thus PorCORA is a highly predictive method to distinguish between ocular irritancy classifications R36 or R41 and Category 1 or 2 without the use of live animals.

I-8-632

PorFocal, for your eyes only!

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MB Research Laboratories has developed a procedure for the culturing of excised porcine corneas for a period of up to 21 days. Using these corneas, we have developed a method, PorFocal, to evaluate the effects of multiple doses of low-level irritants. Corneas are excised from enucleated porcine eyes from the abattoir, and can be cultured in a living state for up to 21 days. In order to detect and quantify low-level damage to corneal tissue, this assay was created to assess ocular irritation by measuring cell viability using a dead stain, Ethidium homodimer (EtH), and fluorescence confocal microscopy. For the PorFocal assay eight cultured corneas (4 per test material) were treated with either Dulbeccos's Phosphate Buffered Saline (dPBS), or 0.01% Benzalkonium Chloride (BAK) for a total of 10 doses (2x/day on days 1, 2, 6; 1x/day on days 0, 3, 7, 8) at 50 μl/treatment. On day 8, these corneas were incubated for 30 min with 2 μM EtH dead cell stain and imaged using confocal microscopy. The EtH stained dead cell nuclei were imaged in 6 random 450 μm x 450 μm x 56 µm-deep tissue fields via confocal z-stacks composed of eight 8 μm-thick optical slices. A maximum projection of image z-stacks was created so that no nucleus was counted twice. All dead nuclei (cells) were counted for each tissue field, the counts were summed, and statistical analysis was performed using ANOVA. PBS-treated corneas (n=4) exhibited 1659 dead cells and 0.01% BAK-treated corneas (n=4) exhibited 3591, a 216% increase in cell death, which was statistically significant ($p<0.001$). These data indicate that low-level damage can be detected by using confocal microscopy. Future directions for this project include increasing the amount of replicates to decrease variance. Also, the complimentary component of the staining kit is a live cell stain. This stain could be further developed, and a ratio of live to dead cells in each group could yield higher sensitivity corneal irritation measurements.

I-8-633

Historical data on personal care products over fourteen years using the Chorioallantoic Vascular Membrane Assay (CAMVA) and the Bovine Cornea Opacity/ Permeability Assay (BCOP)

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The Chorioallantoic Membrane Vascular Assay (CAMVA) and Bovine Corneal Opacity and Permeability Assay (BCOP) are two common assays used to determine ocular irritation for consumer-use products. These assays do not require the use of live animals, provide reliable predictive data, provide results similar to *in vivo* models and are rapid and inexpensive to conduct. Data from 321 studies performed from 1995 to 2009 (a total of 345 test materials assessed by CAMVA and/or BCOP) were compiled to determine the feasibility of predicting ocular irritation for various formulations. Review of the data from both assays found that hair shampoos, skin cleansers, and hair styling sprays (containing ethanol) were repeatedly predicted to be ocular irritants. In contrast skin lotions/moisturizers were repeatedly predicted not to be ocular irritants. Based on the findings for these product types, future ocular irritation testing (i.e., CAMVA/ BCOP) can be nearly eliminated as long as formulations are compared to those previously tested. For example, skin cleanser irritation appears to be solely dependent on surfactant species and level in these formulations.

For other product types (e.g., deodorants, make-up removers, hair styling, body sprays) it was concluded that these products should continue to be tested in CAMVA/BCOP for ocular irritation potential because either significant variability exists in the historical data (non-spray hair stylers) or the historical sample size is too small to permit definitive conclusions (deodorants, make-up removers, massage oils, facial masks, body sprays, and hair styling products).

I-8-634

Development of the Replacement Ocular Battery – tiered testing strategy of alternative toxicology tests to replace the need for rabbit eye tests

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Using a series of non-animal assays in a tiered approach, the Replacement Ocular Battery (ROBatt) accurately predicts the categories of acute ocular irritation corresponding to OECD, EPA and GHS guidelines. At present, no single alternative assay has been accepted by regulatory agencies to completely replace the use of live animals. The BCOP (Bovine Cornea Opacity/ Permeability) test has been accepted by OECD as a screen for severe and corrosive materials. EpiOcular™ and other ocular tissue models are in various stages of review or acceptance. The Cytosensor Microphysiometer has been accepted for sub-severe testing but it is only applicable to aqueous-based materials. The ROBatt approach uses a series of up to three non-animal assays to categorize both aqueous and non-aqueous materials.

An FDA-NIH Grant has been awarded to develop the ROBatt decision tree criteria. Initially, screening will use the Chorioallantoic Membrane Vascular Assay (CAMVA) to discriminate slight or non-irritants from moderate to severe irritants. Slight or non-irritating materials will be categorized using the Porcine Cornea Confocal Assay (PorFocal). 3D human reconstructed tissue models and/or the Bovine Cornea Opacity/Permeability test (BCOP) will be used for discriminating between moderate and severe to corrosive materials. Lastly, the Porcine Cornea Opacity Reversibility Assay (PorCORA) will be used to categorize severe irritants and corrosives.

Fifty validation chemicals from the ECETOC database of ocular irritation will be initially tested. Having performed over 6,700 CAMVA, 5,700 BCOPs, 3,000 MatTek EpiOcular™/ SkinEthic HCE™, and nearly 100 PorCORA assays, the researchers are confident of the ability of ROBatt to properly categorize any material to international standards.

Session I-9: Advances in Three Rs alternatives for reproductive and developmental toxicity

Session I-9: Oral presentations

I-9-657

Predicting reproductive and developmental toxicity: A tiered strategy

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The past decade has seen an enormous increase in the availability of high-information data streams (e.g., genomics) and computational power. These advances, coupled with a strong foundation of toxicological knowledge, have the potential to support remarkable advances in prediction of human risk. This talk will explore a tiered approach to the assessment of the toxicity of chemicals that combines cheminformatics, high-throughput and high-information content data streams, pharmacokinetic considerations, computational modeling and simulation, and relevant *in vitro* models. Cheminformatic databases are available that contain summaries of all toxicology studies conducted, including over 11,000 entries for reproductive and developmental toxicity. These data can be searched by chemical substructure using expert-based rules to identify analogs of new chemicals. The data can be used to develop a hypothesis about the toxicity of a new chemical, which can often be tested by targeted *in vitro* studies. High-throughput data streams such as ToxCast, along with toxicogenomics, can be used to refine and test hypotheses. A better appreciation of pharmacokinetics and its use in setting appropriate exposure levels in *in vitro* assays will move beyond simple predictors of hazard to predictors of quantitative risk. Computational simulations are still in relative infancy but have the potential to conduct thousands of virtual experiments, a feature that will be indispensable in identifying the biological pathways that are toxicologically relevant. Employing these tools in a tiered fashion will make it possible to evaluate the toxicity potential of many more chemicals than is currently possible, and in a manner that may be more human-relevant.

I-9-258

The embryonic stem cell test as tool to assess structure-dependent teratogenicity: the case of valproic acid

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Testing for reproductive and developmental toxicity of drugs and other chemical compounds *in vitro* is an attractive alternative procedure to time-consuming and expensive *in vivo* or *ex vivo* experiments. The embryonic stem cell test (EST) represents a scientifically validated method for the detection and classification of compounds according to their teratogenic potency. However, more work is required to assess its applicability domain and to improve its predictive capacity before gaining full regulatory acceptance. We chose valproic acid (VPA) as a model compound to evaluate the suitability of the EST for distinguishing between developmental toxicity potencies of substances with closely related structures. VPA is among the most frequently used anti-epileptic drugs worldwide. Further, it is used for migraine prophylaxis and in the treatment of bipolar psychotic disorders. Two severe side effects of VPA, hepatotoxicity and teratogenicity, have prompted research into derivatives of VPA. Here we investigate six closely related analogues of VPA whose teratogenic potential has been previously determined in the NMRI mouse model of encephalopathy. Distinct embryotoxicities *in vivo* of stereoisomers which differ only in their spatial configuration were reproduced by the EST. Similarly, an increased potency *in vivo* correlating with longer chain length of the congener was evident as higher toxicity in the EST. As toxicological endpoints, both differentiation and cytotoxicity *in vitro* have to be considered to assess teratogenicity comparable to *in vivo* results. In conclusion, our data demonstrate that the EST represents a valuable screening tool in potency ranking of structurally closely related substances of the same class.

I-9-300

BLTK1 murine Leydig tumor cells: a novel model for evaluating the steroidogenic effects of reproductive and developmental toxicants

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Leydig cells are the primary site of androgen steroid hormone biosynthesis in males, which is necessary for proper reproductive development and function. Several environmental toxicants target Leydig cell steroidogenesis, resulting in both developmental and reproductive effects including testicular dysgenesis syndrome. BLTK1 cells, a novel murine Leydig cell line (BLT-1 cells, clone K1), possess an intact steroidogenic pathway producing low basal levels of testosterone (T), and express all the necessary steroidogenic enzymes including Star, Cyp11a1, Cyp17a1, Hsd3b1 and Hsd17b3 as confirmed by RT-PCR and/or Western blot analysis. In addition, 3 ng/ml recombinant human chorionic gonadotropin (rhCG) induced cAMP (~100-fold), progesterone (P, ~10-fold) and testosterone (T, ~10-fold) compared to basal levels, as well as induced Cyp17a1 and Hsd17b3 mRNA levels. Dose-dependent and temporal studies of the effects of triazine herbicides, phthalates (di- and mono-esters), triclosan and glyphosate on steroidogenic activity in BLK1 cells suggest different modes of action underlying altered steroidogenesis, with varying potency and efficacy as reflected in treatment-specific gene expression profiles. These studies suggest BLTK1 cells are not only a suitable *in vitro* model to screen chemical libraries for effects on steroidogenesis, but can also be used to elucidate the mechanisms underlying their endocrine disrupting effects.

I-9-647

hESC-based *in vitro* toxicity testing – a test strategy for assessing prenatal toxicity

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Within the Health Programme of the European Commission's 7th RTD Framework Programme, a consortium of 32 partners addresses the need to develop alternative strategies in order to optimise the identification of prenatal toxicants during the process of early drug development. Given that prenatal toxicity assessment often suffers from inter-species variations, confounding predictability and based on the unique properties of human embryonic stem cells, the consortium considered it justified to choose hESCs lines as their major research tool.

Three years into the project, several hESC-based *in vitro* assays, covering different phases of the embryonic development, have been created to form building blocks relevant for the design of a testing battery on prenatal toxicity with an emphasis on the nervous system. The battery will assess different aspects of prenatal toxicity such as functional impairments and changes in the differentiation capacity after exposure to well selected reference compounds. Furthermore, newly developed *in vitro* tests will be employed to identify predictive toxicogenetic markers deriving from Affymetrix array data, which will then

be employed on a qPCR chip as candidates for biomarkers able to identify prenatal toxicants.

The consortium is currently preparing for a proof of concept study in which the relevance of new tests will be evaluated by challenging selected tests with blinded compounds. We will introduce the new hESC assays including the applied reference compounds that are employed to train the tests. Furthermore, toxicogenomic data will demonstrate the progress in the biomarker identification study.

Session I-9: Poster presentations

I-9-201

Testing REACH – responding to the testing proposals system to reduce animal testing

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The European Coalition to End Animal Experiments (ECEAE) is an umbrella organisation representing 17 animal protection organisations across 15 member states in Europe, which campaigns on animal testing issues. The ECEAE has a team of toxicologists that, from the start of REACH, have committed themselves to serving the public consultation on testing proposals. This is a mechanism built into REACH to try to prevent unnecessary animal testing by enabling third parties to submit existing data on each registered chemical or other scientific arguments for why the animal test does not need to be conducted.

The ECEAE team has commented, to date, on all 81 testing proposals for 49 chemical substances. Proposed testing includes two-generation reproductive toxicity, developmental toxicity, 90 day repeated dose, *in vivo* genotoxicity studies, carcinogenicity, long-term fish toxicity and fish bioaccumulation tests. We have estimated that under the 2010 deadline, over 1 million animals alone could be used if all proposals to tests are accepted. (This does not cover all testing under REACH, as acute tests are not subject to the public consultation process.)

This presentation summarises the types of comments we have submitted, the problems we have faced in this process and an update of ECHA acceptance of these comments. To date, ECHA decisions have been very slow, but already we see that animal tests are more likely to be not conducted if it can be shown that they are superfluous to requirements rather than based on the presence of existing data.

I-9-261

Effects of developmental toxicants on microRNA expression during neural differentiation of murine embryonic stem cells

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Studying chemical disturbances during neural differentiation of murine embryonic stem cells (mESC) has been established at ZEBET as an alternative *in vitro* testing approach for the identification and classification of developmental toxicants. Many studies have shown an essential role of microRNAs in posttranscriptional regulation of gene expression during development and ESC differentiation. Thus, neural differentiation of ESC *in vitro* allows investigation of the role of miRNAs in chemical-mediated developmental toxicity. The main goal of this project was to analyze the expression of neural-specific miRNAs during neural differentiation of mESC while being exposed to the developmental neurotoxicants. The substances we mainly focused on comprise valproic acid (VPA), arsenic and curcumin. All these substances have been shown *in vitro* and/or *in vivo* to exert effects on miRNA expression and to affect neural development. The developmental neurotoxicity of these substances as well as their effects on miRNA expression during neural differentiation of mESC will be discussed. We could demonstrate that neural-specific or enriched miRNAs show different expression patterns during neural differentiation of mESC when cells are being exposed to VPA. So, the expression of mir-128a and mir-124a decreased with increasing VPA concentrations, whereas let-7c was 2-fold upregulated. The downregulation of mir-128a and mir-124a in cells treated with VPA was stronger compared to the concurrent downregulation of the neuron-specific marker βIII-tubulin. The effects of VPA on neuron-specific mir-124a expression may point to possible compound-mediated mechanisms exerted through mir-124a-dependent regulation pathways.

I-9-330

The developmental neurotoxicity of lead in rat primary aggregating brain cell cultures using transcriptomics and metabolomics approaches

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Tox-21c proposed a paradigm shift in the field of toxicology. Instead of relying on traditional animal experiments, the report proposes the application of the latest advances in science and technology to develop more relevant test strategies. The concept is that pathways of toxicity (PoT) can be identified using *in vitro* cell systems, high throughput testing, "omics" approaches, systems biology and computational modeling. The so-called "pathways of toxicity" are defined as changes in normal biological processes, e.g. cell function, communication and adaptation to environmental changes, which are expected to result in adverse health effects. An area of toxicology where Tox-21c could have a significant impact is developmental neurotoxicity (DNT). There is concern that exposures to environmental chemicals contribute to the increasing incidence of neurodevelopmental disorders in children. However, due to lack of DNT studies only very few substances have been identified as developmental neurotoxicants.

This study aimed to develop an *in vitro* approach using metabolomics and transcriptomics for DNT assessment. A 3D rat primary neuronal organotypic model was exposed to lead chloride (0.1, 1, 10 μM) from day 7 up to 21. Quantitative measurement of genes expressed in different cell types (nestin in neural precursor cells, neurofilament-200 (NF-200) in neurons, S100β in astrocytes and myelin basic protein (MBP) in oligodendrocytes) and mass spectrometry based metabolomics measurements were performed.

Treatment with lead chloride significantly down-regulated the mRNA levels of NF-200, S100β and MBP. In contrast the mRNA levels of nestin were significantly increased. The obtained data indicates different effects by lead chloride exposure on all cell types present. Moreover, the mass spectrometry analysis showed differences in metabolite levels between control and treated cells in a concentration dependent manner. Further analysis of the altered metabolites should give mechanistic insight into the DNT of lead. This study demonstrates that gene expression and metabolomic analysis can be sensitive endpoints for DNT assessment.

I-9-376

Towards automation of the Embryonic Stem Cell Test

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The Embryonic Stem Cell Test (EST) is a cell-based assay to evaluate embryotoxicity of substances and belongs to the portfolio of ECVAM (corresponding to ICCVAM) validated *in vitro* assays. The EST requires the production of embryonic bodies (EBs) by the classic hanging drop (HD) method. The EST-process is a time consuming, labour-intensive process in particular due to manual transfer of EBs into an adhesive 96-well plate. In this study the HD-method for EB production was adapted to novel top-loadable HD-plates in a 96-well format for a more efficient EST-process enabling the implementation into an automated process.

Embryonic stem cells (ESCs) were grown for 5 days in the HD-plate to achieve EB formation and induce cardiomyocyte differentiation. At day 5, EBs were directly transferred into an adhesive 96-well plate by placing the HD-plate on top of a receiver plate and adding excessive medium into the HD-wells. Adhered EBs were monitored for cardiomyocyte differentiation at day 10. ESCs aggregated in the hanging drop and formed round shaped EBs of uniform size within 5 days. Size analysis of EBs resulted in diameters of 319 μm ±3.0% at day 3 and 466 μm ±5.2% at day 5, respectively. The assessment of EB transfer into a 96-well receiver plate showed an efficiency of 87% ±5.7%. Differentiation of EB culture in flat bottom plates showed cardiomyocyte contraction efficiency of 88% ±13% at day 10.

We adapted the traditional manual HD method to develop a more efficient process using a top-loadable hanging drop culture format leading to approximately 80% time saving per 96-well plate.

I-9-523

Zebrafish embryo: an alternative model system for embryo toxicity and developmental neurotoxicity

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Environmental chemical exposures are likely to contribute to the increasing incidence of neurodevelopmental diseases in children, though most chemicals have not yet been evaluated for developmental neurotoxicity (DNT). Relying solely on test guidelines (OECD, US EPA) to address current and anticipated future regulatory demands for DNT testing would incur unacceptably high costs and face ethical drawbacks. There is a strong need for alternative methods which identify potential developmental and neurotoxic compounds with speed, reliability and respect for animal welfare.

We explored zebrafish embryos and larvae as an alternative model to predict potential morphological and neural disorders during early development. A teratogenic assay (Selderslaghs et al., 2009) has been developed and includes the time-related evaluation of embryotoxicity next to morphological endpoints like heartbeat, tail detachment, eyes, spinal cord, etc. at embryonic and larval stages up to 144 hours post fertilization (hpf). Spontaneous tail coiling (24-26 hpf) and swimming activity (120 and 144 hpf), are evaluated as measures for neurobehavioral disorders (Selderslaghs et al., 2010). These methods have now been evaluated for an extended panel of known positive and negative chemicals for developmental toxicity and neurotoxicity, and comparison of zebrafish test results with available mammalian and/or human data demonstrated promising values for sensitivity and specificity.

In conclusion, through these data we will demonstrate that zebrafish might be a valuable alternative vertebrate model filling a gap for developmental and neurobehavioral endpoints which cannot be covered by cellular *in vitro* systems.

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I-9-538

Using molecular signatures for identification of teratogenic compounds in the zebrafish embryo assay

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The zebrafish embryo has been suggested as an alternative model for assessing teratogenicity in mammals. We propose that the predictive capacity of the zebrafish embryo can be improved by including molecular markers. As a proof of principal we analysed shh-(sonic hedgehog signalling pathway) interfering compounds known to cause holoprosencephaly and the cyclopia phenotype. First we screened about 16 potential shh-interfering compounds with a cellular reporter gene assay (shh-light cells) and identified a couple of compounds that were significantly more potent than the model shh inhibitor cyclopamine. Furthermore, zebrafish were exposed to selected candidate compounds and analysed for shh-specific gene expression patterns by microarrays. A very specific shh-related gene response (54% of differentially expressed genes were linked to the shh-pathway) was identified for the reference compound cyclopamine. One of the candidate compounds (SANT-2) shares the same target (smo) with cyclopamine but failed to induce any changes in gene expression. The other compound (GANT-61) – known to interfere with the gli transcription factor – provoked gene expression patterns not related to the shh pathway. We concluded that (1) the candidate compounds were unlikely to interfere with the shh pathway (at least in the zebrafish model) and (2) cellular reporter gene assays may be too reductionist and require (subsequent) testing in more complex assays. The data clearly supported the idea (NRC, 2000) that developmental toxicity might be linked to interference with a limited number of signalling pathways.

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I-9-550

Integrated testing strategy for reproductive toxicity

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The current system of risk assessment of chemicals is complex, very resource-intensive and time-consuming which will become even clearer upon implementation of the REACH regulations. Within these regulations, the requirements for reproductive and developmental toxicology are particularly important since these studies require the most resources. Therefore, there is a great need to modernize the process of hazard- and risk assessment, requiring registrants to consider alternative methods of filling the data gaps. Due to the complexity of the reproductive and developmental processes, the use of alternative methods for these endpoints may be problematic. At this moment, there are only a few alternative methods identifying potential reproductive toxic agents with sufficient accuracy, speed and reliability. Simple animal-free *in vitro* models cover only a restricted part of the reproductive cycle. Most models represent underlying processes and dynamics insufficiently and are therefore of limited use as a stand-alone.

The EU project ChemScreen aims to fill these gaps and place the tests in a more innovative, animal-free, integrated testing strategy for reproductive toxicity, which will use combinations of available *in silico* and *in vitro* technologies. A first step in the project is to establish methods for prescreening and prediction of chemicals having specific toxicological properties that do not need further testing for reproductive toxicity according to REACH, i.e. chemicals that need classification as either genotoxic carcinogen or germline mutagen. Also in this step, methods for prescreening and predicting potential reproductive toxicity using repeated dose and reproductive toxicity databases and *in silico* methods are envisaged. A minimal set of medium- and high-throughput *in vitro* test methods to study sensitive parameters will be established as a second step to identify reproductive toxicants. For the short run these methods will be applied and tested for use in a category approach to verify the read across to an *in vivo* tested member, while the long run objective is to develop them into a stand-alone battery. In the final step, all this information will be integrated to allow conclusions on classification, labeling and risk assessments to be made among others by applying quantitative *in vitro-in vivo* extrapolation, and herewith to decide on the need for and specifics of further *in vivo* testing for reproductive toxicity.

This work was carried out with financial support from the Commission of the European Communities, the collaborative project ChemScreen (GA244236).

I-9-588

Assessment of impaired neurite outgrowth in live human neurons as functional readout for potential developmental neurotoxicants

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Models for the outgrowth of neurites and methods to quantify modifications of this process are at the centre of interest in the field of developmental neurotoxicology (DNT). We developed an imaging-based procedure to quantify neurite growth in LUHMES human neuronal precursor cells that spontaneously extend projections after plating. Labelling methods were all based on live cell stains to avoid additional manipulations that might affect fragile neurite structures. Although cells were grown at high density to allow extensive networking, the observer-independent test allowed for a signal-noise ratio of >50 with regard to the quantification of overall neurite growth. General cytotoxicity was measured simultaneously with neurite evaluation for each individual cell by calcein uptake. Toxicity affecting the extension of processes occurred for compounds such as U0126 independent of general cytotoxic effects. Under such conditions, high numbers of viable cells without neurites were detected. A test set of known negative and positive controls, including also the extension-prolonging compound Y-27632 was identified correctly. This unique multi-parametric imaging approach was finally used to examine to which extent unspecific cytotoxicants such as menadione, cadmium and sodium dodecyl sulfate would affect neurites. In some cases, apparently specific disintegration of cellular extensions was observed as indirect activity of such compounds. These data suggested that only a concentration ratio of >4 between EC_{50} (neurites) and EC_{50} (cell death) defines a directly neuritotoxic compound. The characteristics of the described novel test system suggest its usefulness both for high throughput screens (HTS) and for mechanistic research.

I-9-590

Migration assays in human embryonic stem cell-derived neural crest cells to detect neurodevelopmental toxicants

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Proper neural development consists of several tightly regulated processes including e.g. differentiation, neurite outgrowth and migration. The main migratory cell population during neural development, the neural crest cells (NCC), emerges from the neural tube and migrates throughout the body to give rise to multiple cell types including neural and non-neural cells. To study potentially adverse effects of different compounds on the migratory potential of neural precursors, we used human embryonic stem cells (hESC) differentiated into NCC. We were able to keep the cells in a neural crest progenitor state, which allows the expansion and freezing of the cells. Furthermore, the NCC were differentiated into peripheral neurons and Schwann cells, which confirmed their functional potential, while marker expression indicated the expected phenotype. To study the migratory potential of these cells and the effects of compounds on the migration capacity, we used the classical scratch assay and video imaging. LIVE cell imaging experiments demonstrated that NCC repopulate the scratch via migration independent of cell division. As mechanistic proof-of-concept we found that compounds which inhibit actin polymerisation inhibit the migration of these cells at concentrations that do not affect general cell viability or cell division. Typical developmental toxicants such as mercury and lead blocked migration in NCC, but not other cell types. We therefore believe that this human-based *in vitro* test system is a powerful tool to detect potential neurodevelopmental toxicants.

I-9-627

Embryonic stem vs. embryonic carcinoma cells: an miRNA perspective on developmental toxicology

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In the last year's research and technology developments, ethical concerns and legislations have urged toxicity testing towards a new pathway-based strategic vision. In the context of pathway identification, molecular signatures play a key role, particularly when dealing with complex spatio-temporal interactions, as they occur in organism development.

MicroRNAs (miRNAs) are noncoding RNAs involved in the post-transcriptional regulation of gene expression. The miRNAs are key players in animal development that seem also involved in toxicity responses. Moreover, as a single miRNA regulates many proteins, a small number of miRNA can give information on complex gene expression patterns crucial for the different aspects of animal development. These features make miRNA expression profiling a most promising tool for developmental toxicity assessment. We thus aimed to evaluate the feasibility of microRNA expression profiling as a tool to assess developmental toxicity in two human cell models.

NTERA-2, a human embryonic carcinoma cell line, and H9, a human embryonic stem cell line, were compared for their potential toward neuronal differentiation in terms of gene and protein expression. We then assessed the miRNA profile of differentiated and undifferentiated cells, in order to identify relevant changes in miRNA patterning upon neuronal differentiation. We finally evaluated miRNA expression in cells exposed to repeated doses of methylmercuric chloride (MeHgCl) during differentiation. By comparing undifferentiated, differentiated and MeHgCl-exposed cells we could observe changes in miRNA expression, suggesting that miRNA analysis could be a useful tool in developmental toxicity.

This work is part of the ethically reviewed FP 7 Project ESNATS.

I-9-628

A human embryonic stem cell approach for toxicity assessment in human early neural development/neurulation

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Concordance between species is a main limitation when using animal models to predict human responses to toxic exposures. In particular for prenatal toxicity, disasters like the thalidomide tragedy have proven the need to develop human based *in vitro* tests. Therefore the development of more predictive and accurate tools is a priority, in order to optimize hazard identification in the phase of drug development. In this sense, human Embryonic Stem Cells (hESC), due to their differentiation potential, might be an interesting cell source of material for establishing toxicological tests of human toxicity as early as embryogenesis.

As part of the ESNATS consortium, we have developed a cell system allowing the detection of toxic effects during the genesis of the developing human nervous system. To do so, cells of the H9 hESC line were differentiated *in vitro* into early neural precursors, apparently mimicking the early steps of *in vivo* neurulation. Based on that differentiation system, effects of nine toxicologically well-defined compounds were analyzed. In an initial step, the cytotoxicity of 6-aminonicotinamide, 5-fluorouracile, lead acetate, methylmercury, methotrexate, toluene, retinoic acid, valproic acid and warfarin were defined. The resulting dose-response curves were used to determine critical dosage levels such as the IC_{50} and the lowest non-cytotoxic concentration. These concentrations have been further analysed to assess changes of the gene expression profile after chemical treatments. The chemicals were clustered according to their toxicological responses in the differentiating cultures. These initial gene expression profiles are now being further analysed by microarray technology (Affymetrix gene chips) in order to detect new toxicity biomarkers.

Session I-10: Safety testing for carcinogenicity and genetic toxicity: Recent 3Rs advances

Session I-10: Oral presentations

I-10-670

In vitro versions of the Muta™Mouse Transgenic Rodent (TGR) Mutation Assay for hazard identification of chemicals

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Transgenic rodent (TGR) mutation assays, such as the Muta™ Mouse assay, provide the ability to quantitatively assess mutagenicity in virtually any tissue of an exposed experimental animal. An OECD guideline for proper conduct of the assay (no. 488) is pending final approval. However, the costs associated with this *in vivo* assay, and the desire to reduce, refine and replace laboratory animals in toxicity testing, has promoted interest in the establishment of *in vitro* alternatives. In 2003 we introduced the Muta™ Mouse FE1 cell line. The cells, which retain several characteristics of alveolar epithelium, are cytogenetically stable, metabolically-competent, contain wild-type p53, and yield reproducible responses upon exposure to a variety of mutagenic substances. We investigated the utility of the FE1 assay in a regulatory context by examining 9 non-carcinogens that have been highlighted for their ability to induce "irrelevant positives" in a traditional mammalian cell assay (i.e., Mouse Lymphoma Assay). In addition, we are conducting simultaneous *in vitro* (i.e., FE1) and *in vivo* exposures to selected compounds in an effort to investigate the mechanistic and empirical relationships between the different endpoints. More recently, we have integrated other genotoxicity endpoints into the FE1 assay; including micronuclei in binucleate cells and stable DNA adducts. We also successfully demonstrated the ability to culture and expose primary hepatocytes from Muta™ Mouse. The *in vitro* hepatocyte assay has been employed to examine mutagenic aromatic amines that generally require metabolism and activation by cyp1A2, a P450 isozyme that is almost exclusively hepatic.

I-10-134

In vivo Comet Assay: update on the ongoing international validation study coordinated by JaCVAM

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The *in vivo* rodent alkaline Comet assay is used worldwide for detecting genotoxic chemicals. The assay, however, has not been formally evaluated for its reliability and relevance. Thus, the Japanese Center for the Validation of Alternative Methods (JaCVAM) has been coordinating an international validation study to evaluate the *in vivo* rodent alkaline Comet assay as a potential predictor of genotoxic carcinogens. Our goal is to establish an OECD guideline. The study protocol has been finalized as version 14 based on the results of the 1st to 3rd phase validation studies conducted in four or five lead laboratories. A daily administration regimen of three doses per day of test chemicals to the animals has been adopted in the study protocol to combine the Comet assay with the micronucleus assay, resulting in both reduction and refinement of animal use. The 4th phase of the (definitive) validation study began in 1Q/2009 in accordance with the study protocol. In the first step, data obtained from 13 laboratories indicated good reproducibility of the assay results among laboratories when the assay was conducted with four coded test chemicals: 2-acethylaminofluorene; ethyl methanesulfonate (EMS); D-mannitol; and N-methyl-N-nitrosourea. The 2nd step of the 4th phase validation study is now on-going in 14 laboratories with many more coded test chemicals and a positive control, EMS, in order to investigate the predictive capability of the assay against the known carcinogenicity of the test chemicals.

I-10-265

ECVAM-coordinated pre-validation study of three cell transformation assays for the carcinogenicity testing of chemicals

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The *in vitro* Cell Transformation Assay (CTA) has been proposed as a valuable alternative to the traditional rodent carcinogenicity bioassay since it recapitulates critical stages of *in vivo* carcinogenesis, generates transformed cells that can be tumourigenic *in vivo* in suitable hosts, and can detect both genotoxic and non-genotoxic carcinogens. Following past activities of the European Centre for the Validation of Alternative Methods (ECVAM) concerning the use of the CTA, and to complement the findings of the OECD detailed review paper (DRP) on CTAs for the detection of chemical carcinogens, ECVAM coordinated a formal pre-validation study on the Syrian hamster embryo (SHE) and BALB/c 3T3 CTAs. The study objective was to address issues of protocol standardization, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. Three protocol variants (SHE pH6.7, SHE pH7.0 and BALB/c 3T3 CTAs) were evaluated in a multi-laboratory trial with six chemicals per assay. In October 2010, the study results were submitted to the ECVAM Scientific Advisory Committee (ESAC) for scientific review, the conclusions of which will serve a basis for the ECVAM recommendation on CTA. In agreement with the study Validation Management Team (VMT), the ESAC concluded in February 2011 that the study succeeded in generating standardised protocols, which appear transferable and reproducible for the SHE CTAs. Although promising, further optimisation of the BALB/c 3T3 protocol was recommended and use of the refined protocol, including the modifications suggested by the VMT and the ESAC, was encouraged to expand the data on assay reproducibility. These results, together with the extensive database summarised in the OECD DRP, support the utility of the CTA for the assessment of carcinogenicity potential. Moreover, the ESAC made detailed suggestions regarding the next steps considered to be necessary for the possible routine use of the CTA.

Session I-10: Poster presentations

I-10-170

Using high content imaging to automate the *in vitro* micronucleus assay: analysis of CHO-K1 and Balb/3T3 in the presence and absence of cytochalasin B

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The Micronucleus (MN) assay is a commonly used tool to identify the genotoxic potential of various agents. The OECD guideline for this assay (#487) allows for several variations (length of compound exposure, blocking of cytokinesis or not, metabolic activation, etc.) of the MN assay, along with the potential to screen with multiple cell types, tissue models or mammalian blood samples. Each of these variations requires flexibility of the automated analysis of the assay and is perhaps why attempts to develop an automated assay have proven to be difficult. In this study, we used high content imaging analysis to evaluate the genotoxicity of six compounds in two cell lines (CHO-K1 and Balb/3T3-a31) with and without cytokinesis block to determine whether compounds had different effects on the cell lines and/or whether cytochalasin B (cyt B) had an effect on the results.

The compounds tested exhibited dose-dependent changes for MN frequency, but the cell lines and cyt B test methods varied in the cells' sensitivity to each compound. The automated analysis allowed six compounds in seven-point dose-response curves, six replicates per dose, in two cell lines and two treatment schemes to be evaluated in approximately 36 h without any hands-on time required. High content imaging analysis of the MN assay is a flexible tool that offers a significant improvement in speed and throughput over manual methods, eliminating subjectivity and hours of manual scoring time.

I-10-185

A validation study on a Bhas 42 cell transformation assay using 96-well micro-plates

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The Bhas 42 cell transformation assay (Bhas 42 CTA) is a short-term system for predicting chemical carcinogenicity. The Bhas 42 cells were derived from the BALB/c 3T3 cells transfected by v-Ha-ras gene. The assay protocol of Bhas 42 CTA consists of two components, the initiation assay and the promotion assay, to detect tumor-initiating activity and tumor-promoting activity, respectively, of chemical carcinogens. In the promotion assay the Bhas 42 cells do not require to be pre-treated by an initiator such as 3-methylcholanthrene, showing that these cells are initiated. The Bhas 42 CTA was applied to 98 chemicals in-house and its proficiency in predicting carcinogenic potential and its capability of detecting Ames-negative and Ames-discordant carcinogens were previously reported in *Mutation Research*. Validation studies have recently proven that the Bhas 42 CTA is reproducible between and within laboratories and applicable to the prediction of chemical carcinogenicity (manuscript in preparation). These studies were carried out using 6-well micro-plates (6-well method). We have since developed the Bhas 42 CTA using 96-well micro-plates (96-well method) that has the potential to be utilized for high throughput automated applications. The present study was performed to validate this 96-well method. Four laboratories participated and the study was forwarded stepwise (pre-validation phase, phase I and phase II). All the test chemicals were coded and a total of 25 chemicals were tested including duplicate chemicals between phases. The study results proved that the 96-well method is transferable between laboratories, reproducible both within and between laboratories, and applicable to the prediction of chemical carcinogenicity. The 96-well method and the 6-well method gave the same judgment for 15 out of the 17 chemicals duplicated between validation studies.

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

I-10-207

The use of human 3D epidermal models for genotoxicity testing: Results with the Comet assay

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EU policy with regards to the safety evaluation of cosmetic ingredients marks the need for a broad replacement of animal tests due to the Seventh Amendment to the Cosmetics Directive. For many chemicals and cosmetic ingredients, the skin is the route of exposure (first site of contact). The *in vitro* genotoxicity assays that are currently used result in many false positives and there is no follow-up assay available in the target tissue skin to further evaluate a positive response obtained from *in vitro* assays. Therefore, genotoxicity (comet and micronucleus) assays using human 3D epidermal models were developed, representing a relevant test system with regards to barrier function of the stratum corneum and the possibility to apply formulations (realistic exposure conditions).

For the assay discussed here, the comet assay, EpiDerm™ models (MatTek) were cultured in an air-liquid interface and topically exposed to test compounds for 3 h, followed by cell isolation, lysis, electrophoresis, and preparation of slides. Inter-laboratory reproducibility of the 3D skin comet assay was demonstrated for MMS and 4NQO and results showed good concordance with *in vivo* data. Phase 1 of the pre-validation of the 3D skin comet assay was performed with 5 coded compounds at 3 different laboratories. Results of the collaborative study will be presented.

Results thus far indicate that the comet assay in 3D skin models is a relevant model for safety evaluation of compounds that penetrate the skin.

This work was sponsored by the European cosmetics industry (COLIPA) and the European Center on Validation of Alternative Methods (ECVAM).

I-10-214

Novel *in vitro* genotoxicity assays using reconstructed human tissues

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The poor specificity (tendency to yield a high number of positive results whereas *in vivo* genotoxicity results are negative) of *in vitro* genotoxicity assays (especially the mammalian cell based ones) is of concern in the context of the European legislation (7th amendment to the European Cosmetics Directive, REACH).

An *in vitro* micronucleus assay using human reconstructed skin tissues and target cells grown beneath the skin was developed. The purpose was to improve the relevance of exposure conditions in *in vitro* genotoxicity assays for dermally applied compounds. Previous results have shown that this method was reproducible and could be transferred to other laboratories. In addition, clastogens as well as aneugens could be detected. The system has now evolved to combine both the comet assay and the micronucleus assay. A set of 12 chemicals has been tested with this system. The results obtained will be shown. So far, they look promising. Most of the "irrelevant positives" yielded negative *in vitro* results using this system.

I-10-223

Comparable metabolite patterns of benzo[a]pyrene and cinnamic aldehyde in human skin *ex vivo* and human skin models *in vitro* indicate comparable metabolic capacities

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In vitro skin models are widely recommended in toxicological test guidelines for addressing penetration, as well as corrosion and irritation. However, the suitability of these test systems for other toxicological endpoints, such as genotoxicity or skin sensitization depends on their metabolic competence to transform non-toxic parental compounds into relevant metabolites of toxicological concern. Initial studies on metabolizing enzymes in skin models have focused on expression patterns of cytochrome P450 (CYP) monooxygenases and selected detoxifying phase II enzymes. Besides gene expression, comparatively little is known about enzyme activities present in skin models. Both CYP1A1 and 1B1 are thought to initiate the biotransformation of benzo[a]pyrene (BP), thereby resulting in a complex pattern of metabolites due to various parallel or alternate pathways of BP conversion.

We have analyzed the induction of CYP1 enzymes, as well as CYP activities in full thickness models, showing that these enzymes are preferentially expressed within the epidermal layers. In order to address the metabolic capacity directly, we have compared the metabolite patterns in BP-exposed 3D skin models, excised human skin and cultured primary human keratinocytes. Importantly, the patterns of eight BP-metabolites detected, including BP-r-7,t-8,t-9,c-10-tetrahydrotetraol, trans-BP-7,8-dihydrodiol, trans-BP-9,10-dihydrodiol, BP-1,6-dione, BP-7,8-dione, 3-OH-BP, 7-OH-BP and 8-OH-BP were comparable between human skin and the MatTek epidermal and full thickness skin models. All analyzed BP metabolites were also detected in primary human keratinocytes. However, these primary cultures appeared to metabolize BP at lower rates than human skin. Further, we also compared the metabolic transformation of cinnamic aldehyde into cinnamic acid and cinnamic alcohol in the skin models and human skin. Our results confirm a sufficient metabolic competence of the selected skin models and support their applicability for replacing *in vivo* tests for genotoxicity and other endpoints that rely on metabolism. The BfR coordinates a German multi-center project (BMBF Funding ID 0315226 A-D) aimed at developing and prevalidating the Comet assay in skin models. Initial observations also confirm that pro-mutagens are converted into their active forms, which can be detected in this assay.

I-10-239

The application and use of cell transformation assays in hazard and risk assessment – experience at a CRO

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The prediction and assessment of carcinogenicity of chemical compounds is an essential step in their development. The 2-year bioassay is the standard method for carcinogen detection, which is time and resource intensive. *In vitro* cell transformation tests using SHE-, Balb/c3T3-, Bhas 42- cells simulate the process of animal two-stage carcinogenesis. These tests are suited for the *in vitro* detection of a carcinogenic potential of test compounds in safety and risk assessment. Results from cell transformation assays can provide information, which in combination with data from other testing methods, are useful for identifying the carcinogenic potential of chemical compounds. The cell transformation assays are used: in order to gain additional information when the biological significance of the bioassay results is uncertain; for clarification of the meaning of positive results from genotoxicity assays in the weight of evidence assessment; for compound classes where genotoxicity data have only limited predictive capacity; for investigation of compounds with structural alerts for carcinogenicity; and to demonstrate differences or similarities across a chemical category. New technologies will contribute to a better understanding of the mechanism of these assays and to a more objective evaluation of transformed foci and colonies. We have investigated several biomarkers, characteristic for the carcinogenic process in humans. Promising results were obtained for butylcholin esterase, acetylcholine esterase and alkaline phosphatase. It is time for the cell transformation assays to be accepted as a useful short-term *in vitro* tool for assessing the carcinogenic potential of chemical compounds.

II-10-242

Development and validation of mechanism-based *in vitro* transformation assays for carcinogen screening

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The use of Syrian hamster (SH) as a model for carcinogen screening carries the advantage (e.g. over mouse and rat) that its somatic cells are, in the absence of carcinogen treatment, extremely resistant to oncogene/stress induced senescence bypass (OSISB), with spontaneous immortalization frequencies well below 10-8/primary cell passaged. Telomerase does not need to be reactivated in SHD cells (a very rare event that is a prerequisite in human cells for immortalization, clonal evolution and malignant progression), making them potentially a sensitive target for use in cell transformation assays. In defining the precise molecular mechanism underlying OSISB in the SHD system, we have produced a near-complete molecular description of the various combinations of mutational and epigenetic alterations leading to carcinogen-induced OSISB (immortalization) in primary SHD cells. Our data indicate that different human carcinogens show a distinct preference for inactivating different elements of ARF-p53/p16-RB senescence effector (human tumor suppressor) pathways by mutational and/or epigenetic means, in some cases leaving a clear molecular fingerprint characteristic of the mechanism of action of the particular class of carcinogen. We are now further developing the *in vitro* SHD immortalization system in order to characterize the extensive panels of non genotoxic carcinogens-immortalized SHD clones. In parallel, the improved understanding we now have concerning the mechanisms of carcinogen-induced OSISB will be applied to a molecular analysis of transformants from the related SH embryo cell morphological transformation (SHE-MT) assay in order to obtain a mechanism-based validation of this promising, but thus far uncharacterized system.

I-10-252

Reconstructed Human Epidermis (RHE) use for genotoxicity testing with fewer false positive results

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According to the Seventh Amendment, Council Directive 2003/15/EC, the use of animals for genotoxicity testing has been forbidden since 2009; consequently, alternative *in vitro* methods have been developed for the safety assessment of cosmetic products. These tests, using cell culture, are now being questioned due to their tendency to give false positive results and because the possibility to replace cell culture by reconstructed human epidermis (RHEs) is being studied. RHEs are particularly adapted for cosmetics testing due to their similarity to the physiological condition of skin.

Here we describe experiments using EPI/001 RHEs for the evaluation of the genotoxicity effect of substances known to give misleading positive results in cell culture tests. All RHEs were pre-treated with 3 μg/ml of cytochalasin B, in order to get binucleated cells, an essential condition for the micronucleus test. Then, dose-ranges of Urea, Eugenol, Resorcinol, Curcumin, Propyl Gallate and Phthalic anhydride diluted in acetone were topically applied (10 μl). Non-treated, acetone-treated and Mitomycine C (MMC)-treated RHEs were used as controls. Keratinocytes were released from the RHE, placed on histology slides and stained by May-Grünwald/Giemsa (MGG) protocol. The binucleated cells and micronuclei rate were evaluated using NIS Element taxonomy software (Nikon).

Pure acetone treatment did not modify the micronuclei rate and viability compared to non-treated RHEs. MMC treatment resulted in a dose-dependent increase of micronuclei scoring, a decrease of tissue viability and a decrease of the binucleated cell rate, while the 6 known false positive genotoxic substances did not give any significant increase of micronuclei scoring.

I-10-256

Genotoxicity evaluation of molecules possessing antifertility potential: Testing in microorganisms as an alternative to animal testing

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A large number of molecules, synthetic as well as natural, have been discovered for their use as antifertility drugs. Newly discovered molecules of antifertility drugs have to undergo genotoxicity studies to evaluate their mutagenic and carcinogenic activity. To test the genotoxicity of one drug on five different genotypic strains, approximately 3,000 animals are used. Response of the drug is observed in F1 hybrids after five weeks, with an efficacy ratio of 7.2%. Animal testing in preliminary trials should be avoided in view of the principles of the 3Rs. Genotoxicity evaluation using the Ames test in the discovery of various antifertility drugs can reduce the sacrifice of a large number of rodents and other mammals used in laboratories in preclinical research procedures. Among the numerous genetic bioassays tested in our laboratory, the Ames test has been found to be the most effective as a preliminary screening bioassay. Two recombinant strains of *Salmonella* bacteria, *Salmonella typhimurium* TA 1535 and TA 1538 that carry both frame shift and point mutations in the genes required to synthesize histidine, have been used. The antifertility molecule possessing mutagenic potential may restore the gene function and allow the cell to synthesize histidine, resulting in growth of both of the strains. The specially designed F334 strain of mouse (rodent) and *Schizosaccharomyces prombe* ade 6 and ade 7 (yeast) give results similar to *Salmonella* TA 1535 and TA 1538. This study shows that recombinant strains of microorganisms can be used as alternatives to animal testing in genotoxicity studies on molecules to be used as antifertility drugs.

I-10-264

Comparison of *in vitro* versus *in vivo* transcriptomics data of hepatocarcinogens

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Currently, there are substantial efforts to develop "omics-based" *in vitro* models for identifying genotoxic and non-genotoxic carcinogenic substances as an alternative to the classical 2-year rodent carcinogenicity bioassay. An important issue, however, is the *in vivo* relevance of the *in vitro* obtained data. In the current study, we compare the gene expression profiles generated after oral administration of hepatocarcinogens to rats with those derived after *in vitro* exposure of either epigenetically stabilized or conventional primary rat hepatocyte cultures. Three genotoxic hepatocarcinogens (aflatoxin B1, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 2-nitrofluorene), three non-genotoxic hepatocarcinogens (methapyrilene.HCl, piperonylbutoxide and Wy-14643) and two non-carcinogens (nifedipine and clonidine) were tested. After applying comparable statistical tools for data analysis, *in vivo*- and *in vitro*-derived gene expression profiles per compound were extracted and functionally classified. The major descriptive cellular pathway found *in vivo* for genotoxic carcinogens was DNA damage response, whereas for non-genotoxic carcinogens it was cell cycle progression. Those characteristic *in vivo*-derived gene groups were further compared with the *in vitro* data. In the conventional hepatocyte cultures, two out of the three *in vitro* tested genotoxic carcinogens mimicked the *in vivo* relevant DNA damage response, whereas only one genotoxicant was responsive in the epigenetically stabilized system. Exposure to the non-genotoxic hepatocarcinogens triggered a relatively weak response in both *in vitro* systems, with no clear similarities to *in vivo*. This study might be indicative for the importance of the "*in vivo* relevance of *in vitro* data" when prediction of genotoxic/non-genotoxic potential of chemicals is based on toxicogenomics techniques.

I-10-271

Alternative approaches for the evaluation of carcinogenicity and its use for quantitative risk assessment of cosmetic ingredients

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In 2010 a panel of experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including carcinogenicity, in view of the full marketing ban anticipated in 2013 for cosmetic products and ingredients tested on animals in Europe.

The evaluation of cancer hazard and risk assessment is rarely done in the two-year cancer bioassay for cosmetic ingredients. Rather, a combination of shorter-term *in vitro* and *in vivo* studies have been used, including *in vitro* and *in vivo* genotoxicity assays to assess genotoxic potential and repeated dose toxicity studies to assess the risk of non-genotoxic chemicals. It is clear that the animal testing bans will have a profound impact on the ability to evaluate and conduct a quantitative risk assessment for potential carcinogenicity of new cosmetic ingredients, which is mainly due to the ban on *in vivo* genotoxicity testing, any repeated-dose toxicity testing, and other tests such as *in vivo* toxicokinetics studies and *in vivo* mechanistic assays.

Although several *in vitro* tests, which are at different stages of development and acceptance, are available to support conclusions on cancer hazard identification beyond the standard *in vitro* genotoxicity assays, the available *in vitro* tests are focused on hazard evaluation only and cannot currently be used to support a full safety assessment by adequate dose-response information. However, for some chemical classes the available non-animal methods might be sufficient to rule out carcinogenic potential in a weight of evidence approach.

Taking into consideration the present state of the art of the non-animal methods, the timeline for full replacement is expected to extend past 2013.

I-10-286

Genotoxicity testing using the micronucleus and Comet assays in normal human cell-based 3D epithelial models

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Safety assessment of new products for human use requires genotoxicity testing. Current *in vitro* assays have low specificity (high rate of false positives) and previously *in vivo* assays were conducted. However, the 7th amendment to the Cosmetics Directive banned *in vivo* genotoxicity testing in 2009. 3D human tissue models, which have *in vivo*-like barrier function and metabolism, and which allow for topical exposure, are predicted to have improved biological relevance. Toward this end, the Reconstructed Skin Micronucleus (RSMN) and Comet assays (CA) that utilize the MatTek™ EpiDerm™ model have been adapted for use with tracheal, vaginal, oral, and corneal tissues.

EpiDerm is a 3D normal human cell-based epidermal model that contains *in vivo*-like barrier and biotransformation capabilities. RSMN assay results show statistically significant, dose-dependent increases in cells containing micronuclei (MNC) for 9 direct genotoxins and 6 genotoxins that require metabolic activation, and no increases for 4 non-genotoxins. CA results show increases in %tail DNA after treatment with model genotoxins. Utilizing the RSMN protocol with tracheal, vaginal, oral, and corneal tissue models, increases in MNC (0.3 to 1.2%) were observed after treatment with genotoxins. Similarly, CA results with tracheal, vaginal, oral, and corneal tissue models showed increases in %tail DNA. Hence, the RSMN and CA can be applied to other *in vitro* tissue models using real life exposure conditions. Together, RSMN and CA for skin, tracheal, vaginal, oral, and corneal tissue models will identify a wide spectrum of genotoxic hazards, and will increase confidence in the veracity of *in vitro* tests.

I-10-315

Effects of the genotoxic compounds, benzo[a]pyrene and cyclophosphamide on phase 1 and 2 activities in EpiDerm™ models

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The micronucleus assay in the 3D human reconstructed EpiDerm™ skin model (RSMN) is a promising new assay for evaluating genotoxicity of dermally applied chemicals. In order to complement the testing of metabolically activated chemicals, we have measured basal phase 1 (ethoxyresorufin O-deethylation and testosterone metabolism) and 2 activities (UGTs and GSTs) in EpiDerm™ models in a study design which mimics the pre-validated RSMN. We have also investigated whether the known genotoxins, cyclophosphamide (CPA) and benzo[a] pyrene (BaP), alter these activities. These studies demonstrated the presence of basal phase 1 and 2 activities of EpiDerm™ models. With the exception of GST (which decreased between 24 h and 48 h), all of the basal activities measured did not change over time. It was possible to measure enzyme induction using this assay design. Of the enzymes tested, EROD activity was significantly induced by BaP but not by CPA. CPA, BaP and the reference chemical, beta-naphthoflavone, all caused a small increase in GST activities, the magnitude of induction being markedly lower than that for EROD, which is consistent with literature findings for hepatic models. Since metabolic enzyme activities have been shown to be present, the RSMN assay does not require an exogenous metabolic activation system and is therefore a good model to reflect the metabolic capacity of human skin.

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

I-10-316

COLIPA validation of the Reconstructed Human Skin Micronucleus Assay (RSMN): Further pre-validation studies and investigations into increasing time efficiency

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The European Cosmetic Toiletry and Perfumery Association (COLIPA) has initiated a multi-laboratory project to establish and evaluate more predictive *in vitro* genotoxicity assays using 3D human tissues as replacements for current mammalian cell *in vitro* genotoxicity assays, which induce high levels of false positives. The reconstructed skin (RS) model, EpiDerm™, was combined with the micronucleus (MN) and comet assays because the skin is the first site of contact of many different products, including cosmetics. The resulting RSMN model offers the potential for a more realistic application/metabolism of test compounds for evaluating genotoxicity (Curren et al., 2006; Mun et al., 2009). For the assay discussed here, the MN assay, there was a good intra- and inter-laboratory reproducibility with model genotoxins e.g. mitomycin C and vinblastine sulfate (Hu et al., 2009) and studies with coded chemicals also showed excellent prediction of positive and negative chemicals (Aardema et al., 2010). We have extended the number of coded chemicals to 30 as part of the pre-validation process. We have also optimised the viability determination by adopting an image-based analysis of differential staining with ethidium bromide (dead cells) and Acridine Orange (live cells) instead of manual counting using Trypan Blue. The two measurements were comparable and afforded a significant increase in time efficiency. Studies to automate MN scoring are on-going. In conclusion, the RSMN model is a promising alternative *in vitro* method for genotoxicity testing.

This work is funded by COLIPA.

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I-10-322

Characterization of molecular events underlying induced morphologically transformed (MT) phenotypes in the Syrian Hamster Embryo (SHE-MT) assay

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Current legislative and ethical pressure will require a substantial reduction in the number of animals used in carcinogenicity testing for safety purposes. The Syrian hamster embryo morphological transformation (SHE-MT) assay is one of the most promising systems used by industry to predict the carcinogenic activity of chemicals. A pre-validation study conducted on behalf of ECVAM has concluded SHE-MT to be a reliable assay for carcinogenicity. It has been shown that the rate-limiting immortalization step in SHE cell transformation requires only bypass of the oncogene/stress-induced senescence barrier (SHE cells have constitutive telomerase activity) and that spontaneous progression towards immortalization is a rare event. Therefore, SHE cells should be further exploitable as a useful *in vitro* cell transformation model for carcinogen screening. However, the underlying molecular events leading to immortalization remain unclear. Recent studies in our laboratory involving a SH dermal (SHD) mass culture system have produced a near-complete molecular description of carcinogen-induced OSIS bypass (immortalization). This has led us to initiate a mechanisms-based validation of the SHE-MT assay. Using quantitative gene expression (qRT-PCR) analysis, High Resolution Melting (HRM) and gene sequencing, we set out to characterize early-events leading to immortalisation in the SHE-MT system. The latest results of this study will be presented. Ultimately, transformation biomarkers will be identified for future use by industry in carcinogenicity risk assessment.

I-10-352

Applicability and robustness of the Hen's Egg Test for micronucleus induction (HET-MN): results from an interlaboratory trial

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In vivo genotoxicity assays are often performed to clarify the biological relevance of *in vitro* tests because the latter ones exhibit low predictivity. Due to the new EU chemical legislation REACH and the 7th Amendment to the EU-Cosmetics Directive this testing strategy has to be modified, leading to a high demand for improved *in vitro* tests.

The hen's egg test for micronucleus induction (HET-MN) was developed several years ago to provide an alternative test system to the *in vivo* micronucleus test. In order to assess its applicability and robustness, a study was carried out at the University of Osnabrueck (labA) and at the laboratories of the Henkel AG & Co. KGaA (labB). Following transfer of the method to labB, a range of test substances, which had been pre-tested at labA, were tested at labB: the genotoxins cyclophosphamide, dimethylbenz(a)anthracene, methotrexate, acrylamide, azorubin, N-nitroso-dimethylamine and non-genotoxins orange G and myristic isopropylic acid.

In a second phase, additional compounds were examined in both labs: the non-mutagen, ampicillin, the "irrelevant" positives, isophorone and 2,4-dichlorophenol ("irrelevant" means that they were positive in standard *in vitro* tests but were negative *in vivo*), the mutagen, p-chloroaniline, and the aneugens, carbendazim and vinorelbine. All substances were correctly predicted in both labs with respect to their *in vivo* genotoxic properties, indicating that the HET-MN might have an improved predictivity compared to current standard *in vitro* test systems. The results support the promising role of the HET-MN assay as a supplement to existing testing batteries in the future.

I-10-366

Electrophilic reaction chemistry to predict genotoxicity through mechanistically derived grouping and read-across: links to adverse outcome pathways

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The last few years have seen a vast increase in interest related to chemical grouping approaches to allow for read-across for toxicity prediction. For grouping to be successful, analogues of the target compound must be determined on a rational and mechanistic basis. For a number of endpoints, such as genotoxicity, reactive mechanisms involving covalent bond formation following electrophilic reactions are important for grouping chemicals. This study describes recent work to define the reaction mechanisms associated with individual endpoints and to assess the differences across the electrophilic spectrum of reactivity, as related to different endpoints. For instance, genotoxic chemicals are at the hard end of the electrophilic spectrum. These definitions of chemistry are formed into profilers and are being made freely available through the OECD QSAR Toolbox. The use of the profilers will be illustrated with regard to relevant human health endpoints. These descriptions of chemistry are not predictive tools in themselves; however the groupings they facilitate allow for read-across. Compounds that may be reactive from a structural perspective may not be toxic for a number of reasons. The mechanisms can be rationalised in terms of adverse outcome pathways that attempt to demonstrate the link between the molecular initiating event and the effect on the organism or population. There is a clear need for these approaches to be supported by relevant mechanistic assays to support the grouping and category approaches.

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I-10-384

Opportunities to minimise animal use in regulatory toxicology; a cross-company review

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Regulatory toxicology studies required for pharmaceutical development have well-defined scientific objectives. The UK Laboratory Animals Science Association and the National Centre for the Replacement, Refinement and Reduction of Animals in Research established a group of toxicologists from the UK's major pharmaceutical companies and contract research organisations in order to share good practice and identify areas for application of the 3Rs whilst ensuring that the scientific objectives and regulatory requirements of such studies are still met.

A cross company review of numbers of animals used in general toxicology and carcinogenicity studies was carried out. The results showed that there is some variation in the numbers of animals used. The reasons for this have been explored and the information used to develop a series of approaches where small changes in practice may reduce animal use. We recommend these approaches are used where possible but acknowledge that they may not be appropriate for all studies or programmes.

Practical considerations are given on:

– Reducing the number of animals to obtain toxicokinetic (TK) data;
– Incorporating male fertility assessment into the six-month rodent toxicology study;
– Including fewer recovery animals; and
– Using transgenic mice, single control groups and appropriate strains in carcinogenicity studies.

The data collected demonstrate that the largest influence on animal numbers in rodent toxicity studies is for TK profiling. Therefore, the most significant contribution to reducing the number of animals is likely to be the development of analytical techniques which would allow analysis using smaller sample volumes.

I-10-391

In vitro genotoxicity testing using a metabolic competent human 3D bronchial epithelial model

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EU policy, including REACH and the Cosmetics Directive, implies an urgent need for reliable *in vitro* strategies with regards to the safety evaluation of chemicals. *In vitro* assays currently used are not sufficiently suitable for the reliable evaluation of substances for which the airways are the primary route of exposure (first site of contact), such as gasses, volatiles and aerosols. The LUMC developed a robust human 3D bronchial epithelial model that is cultured in an air-liquid interface and has several important functional characteristics including mucus production, cilia beating and metabolic activity. Moreover, using this model exposure to gasses, volatiles and aerosols is possible via air, simulating a relevant exposure route.

Human primary bronchial epithelial cells (HPBEC) were isolated from the large bronchi of the unaffected outer layer of resected lung tissue from patients undergoing surgery for lung cancer. Upon culture in an air-liquid interface, the metabolic activity of the human 3D bronchial epithelial models and feasibility of *in vitro* genotoxicity tests was investigated. These human 3D bronchial epithelial models were exposed to chemicals via the medium, a droplet on the tissue surface, or via the air, followed by the measurement of genotoxicity and cytotoxicity parameters, including tissue and membrane integrity, cell viability, micronuclei formation and comet induction. A dose-related response was observed for known positive chemicals mitomycin C and methyl methane sulfonate in the micronucleus test and comet assay, respectively. Results with additional chemicals will be presented. Preliminary results indicate that the model is promising for the safety testing of chemicals, including genotoxicity and acute cytotoxicity.

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I-10-403

The COLIPA strategy for animal-free genotoxicity testing

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The 7th Amendment to the Cosmetics Directive bans the use of animals for genotoxicity testing. This ban has perhaps had less focus from an animal alternatives standpoint because sensitive *in vitro* genetic toxicology assays already exist. However, the regulatory-required battery of these *in vitro* genotoxicity tests has a low specificity (i.e. a high percentage of irrelevant positive results for non-carcinogens). Since it is no longer possible to conduct follow-up *in vivo* genotoxicity tests for cosmetic ingredients with positive *in vitro* tests to further assess the relevance of the *in vitro* findings, valuable ingredients will be rejected.

To address this problem, the EU Cosmetics Association (COLIPA) Genotoxicity Task Force has been funding, directing and conducting a major program to develop approaches for genotoxicity testing of cosmetic ingredients. The program consists of three main projects:

(1) A "False Positives" project performed at Covance Laboratories (UK) to optimize current mammalian cell assays in order to improve specificity, showing that the selection of more relevant cells and toxicity measures can prevent >60% irrelevant positive findings.
(2) A "3D skin model" project focusing on developing and validating new methods based on reconstructed human 3D skin models. Results so far indicate good reproducibility of the assay as well as improved specificity compared to standard *in vitro* tests.
(3) Research into the metabolic capacity of human skin and 3D models which demonstrated the 3D skin models' "human skin like" metabolic competency, confirming their usefulness for testing of compounds with dermal exposure.

The outcome of this program is expected to help enable a sound assessment of the genotoxic hazard of cosmetic ingredients in the absence of *in vivo* data, and should also help to substantially reduce animal use in other sectors, such as the chemical industry.

I-10-414

Spectrophotometric measurements of transformation frequency in Bhas 42 cells using hydrogen peroxide

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Transformation assays using the focus formation method have widely been applied to study mechanisms of carcinogenesis and predict chemical carcinogenicity. Four decades after development of this system, transformation frequency is still quantified by time-consuming and subjective scoring of transformed foci under a microscope. To assess transformation frequency quickly and objectively, we have developed a spectrophotometric assay to measure transformation frequency in Bhas 42 cells, a clone established by transfection with the v-Ha-ras gene into BALB/c 3T3 cells.

We found that when Bhas 42 cell cultures containing transformed foci were treated with hydrogen peroxide, normal cells were selectively killed. We plated Bhas 42 cells into 96-well plates and treated the cells with 3-methylcholanthrene (MCA) to form transformed foci. At the end of the culture, hydrogen peroxide and WST-8 (a dye monitoring metabolic activity of living cells) were added, and OD at 450 nm was measured with a microplate reader. The cells were then fixed and stained with Giemsa solution, and wells with transformed foci were counted. Wells with transformed foci showed high OD values, whereas OD values of wells without transformed foci showed a blank level. The total OD values and the number of wells containing transformed foci in each plate increased dose-dependently with MCA concentration. The hydrogen peroxide method is a novel approach to quantify transformation frequency and will provide a high-throughput assay by combining automated systems.

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I-10-452

Development of a new genotoxicity assay using proliferating and metabolically active upcyte® hepatocytes

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One of the disadvantages of the currently used genotoxicity micronucleus assays is their high "false positive" (FP) rate (i.e. low specificity). Two reasons for the high FP rate are (1) the use of cell lines which lack p53, e.g. the rodent cell lines; and (2) the use of metabolically incompetent cells. Medicyte has investigated a novel technique which causes primary hepatocytes to proliferate whilst maintaining differentiated functions. The resulting "upcyte®" hepatocytes provide a cell model which is both p53 competent and metabolically active during proliferation. Here, we investigated whether upcyte® hepatocytes could be used in the micronucleus assay to differentiate between diverse chemicals from 3 chemical groups, which represent true positives (cyclophosphamide, mitomycin C, etoposide, nitroquinoline), true negatives (ampicillin trihydrate, melamine, tris(2-ethylhexyl)phosphate) and false-positives (2,4-dichlorphenol, benzylalcohol, curcumin and urea). MN scoring was carried out using FACS analysis and the stains EMA and sytox green. Using upcyte® hepatocytes all tested chemicals could be correctly discriminated either as positive, resulting in a dose dependent increase in MN formation, or as negative, resulting in no significant increase in MN formation above the control-treated cells. upcyte® Hepatocytes were able to detect positive genotoxins that need metabolic activation without the help of external CYP enzymes (such as S9 mix) and to correctly identify false positives as being negative. This study supports the use of upcyte® hepatocytes in early ADME/Tox assays such as genotoxicity assays. This will eventually lead to an *in vitro* hepatocyte system in order to reduce or replace animal experiments in preclinical drug development and toxicity testing of chemical compounds.

I-10-464

An efficient approach to carcinogenicity prediction through *in vitro* mutagenicity and cell transformation assays

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The need for tools able to predict chemical carcinogens in less time and at a lower cost both in terms of animal lives and money is still a research priority, even after several decades of effort in that direction. Now, new regulatory requirements (e.g., the Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) recently implemented in Europe) have further increased the pressure to develop new tools in this field. Drawbacks of the present testing strategies have come into the limelight again recently, especially in view of new international regulatory requirements. Among others, there is: a) the lack of alternative assays able to identify non-genotoxic carcinogens; b) the exaggerated rate of misleading ("false") positive results of the *in vitro* mammalian cell-based mutagenicity short-term tests; c) the extremely low sensitivity of *in vivo* mutagenicity short-term tests. Within this perspective, we analyze the contribution of the Cell Transformation assays, and we show that they are a valid complement to tools able to detect DNA-reactive carcinogens. We show as well that a tiered strategy with inexpensive and fast tests in Tier 1 (like the Ames test and the Structural Alerts), and the Syrian Hamster Embryo cells Transformation assay in Tier 2, is able to identify up to 90% of carcinogens.

I-10-484

Quantitative assessment of the effects of low dose ionizing radiation using a human hybrid cell transformation assay

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The issue of radiation induced carcinogenesis has received increased interest over the past few years. This concern is due to environmental exposures resulting from nuclear clean-up, nuclear reactor failure, increasing usage of diagnostic scans that use radiation or radioactive isotopes, and concerns raised in the press by scientists who support the linear-no-threshold (LNT) model where "no dose is safe". The LNT model is based on extrapolating dose response data from high doses to zero and assumes that the dose response would remain linear at the low doses. Understanding radiation risks at low environmental or medically relevant doses is difficult due to: the inadequate numbers of exposed individuals to make statistical assessments, the complex environmental exposures, the cost and numbers of animals required for *in vivo* data acquisition, and the availability of quantitative *in vitro* human cell based assays for carcinogenicity. The HeLa x human skin fibroblast neoplastic transformation assay has been shown to be sensitive to ionizing radiation. We have used this assay to study the effects of very low dose ionizing radiation from various sources including: gamma rays, X-rays, protons, iron ion beams, and radioisotopes including ^{125}I. The data clearly demonstrate that at doses <10 cGy, the dose response curve is not linear but has a "J" shape. This is suggestive of an adaptive response that can protect cells against the normally occurring spontaneous events. The data suggest that the LNT model does not reflect reality and that a beneficial effect may occur at low radiation doses.

I-10-565

Application of the threshold of toxicological concern concept in safety assessment of chemically complex matrices

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Toxicological safety assessment is usually performed as a sequential process, which is often time consuming and expensive. To obtain a more efficient process new concepts would be needed that integrate knowledge and information from different disciplines (i.e. chemical analysis, toxicological research, exposure and risk assessment) right from the start throughout the whole research process to fine-tune the required level of detail. The Threshold of Toxicological Concern (TTC) is one potentially new such concept that was developed to assess substances in food, where the identity is known but where toxicological information is lacking. Application of TTC to chemically complex matrices (CCM) is limited due to a lack of chemical identity.

We drafted a framework to enable application of TTC to such CCM. The approach is based on exclusion of specific groups of compounds following the Kroes et al. (2004) TTC decision tree and modifications as proposed by Munro et al. (2008). We concluded that the highest threshold which can be applied for unknowns after exclusion of specific groups of hazardous chemicals is 540 μg/p/d. To determine the amount of substances above a certain threshold a conversion of response in a chromatogram (visualized as "forest of peaks") into concentrations and subsequently into intake is needed. Those substances which appear above the applicable threshold should be identified, characterized and toxicologically assessed.

The safety assessment framework for CCM and required analytical and test strategy innovations (such as genotoxicity screening methods) that are under development will be presented.

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I-10-597

Air/liquid interphase technique as an alternative *in vitro* testing strategy for detecting biological effects of volatile compounds. First results and future perspectives of an ongoing prevalidation study

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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. The aim of this ongoing pre-validation study is to perform a multi-laboratory evaluation of an air/liquid interface culturing and exposure technique for testing the acute cytotoxic and genotoxic effects of gases on a biological cell model. A549 cells, grown on microporous membranes at the air/liquid interface, were exposed to several test atmospheres (NO_2, SO_2, formaldehyde, or ozone). Gas-mediated cytotoxicity was assessed after a one hour exposure via electronic cell counting (CASY® technology). Analysis of dose-response relationships showed a good reproducibility within and between the laboratories for all four gases. Comparison of the derived EC_{50} values with published LC_{50} values revealed a tight quantitative relationship between *in vitro* cytotoxicity and *in vivo* lethality. To evaluate the performance and reliability of electronic cell counting, the cytotoxicity of SO_2 was additionally assessed using two well established viability assays, the Neutral Red Uptake assay and the CellTiter-Blue® assay (Promega). A high correlation was found between EC_{50} values obtained with all three viability assays. The release of IL-6, IL-8, and MCP1 was analysed in order to evaluate the inflammatory effect of SO_2. Genotoxicity assessment via Comet assay demonstrated reproducible dose-response relationships for SO_2 and formaldehyde. No such dose-dependent genotoxicity could be observed for NO_2 and ozone.

The results of the present pre-validation study are promising with respect to the reliability and relevance of the proposed *in vitro* method for inhalation toxicity testing. Extended prevalidation is underway to establish a tested training set of compounds sufficiently large to allow for optimization of the developed prediction model.

I-10-629

Comet assay atlas

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This Comet Assay Atlas was designed and edited under supervision of the Validation Management Team (VMT) of JaCVAM and is the initiative of an international validation study on the Comet assay. The object of the Atlas is to provide a standard to identify analyzable images for the image analysis. Although using an image analyzer, which gives data objectively, it is important to avoid bias for selection of nucleus to be analyzed. An operator should choose nuclei randomly from microscopic fields with high quality, taking into consideration conditions such as shape of nucleus, staining condition, cloudiness of nuclei, and existence of scattered debris. For example, peculiar shaped nuclei or much smaller nuclei compared with surrounding ones should not be selected for analysis. The other difficulty is to distinguish so called “hedgehogs” from nuclei with big tails. Hedgehogs may be derived from apoptotic or necrotic cells and not be derived from cells with damaged DNA. This Comet Assay Atlas gives guidance on how to distinguish between hedgehogs and true comet nuclei.

Session I-11: Safety testing for skin sensitization hazards: Recent Three Rs advances

Session I-11: Oral presentations

I-11-626

Alternative approaches for the evaluation of skin sensitisation and their use for quantitative risk assessment of cosmetic ingredients

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including skin sensitization, in view of the full marketing ban anticipated in 2013 for cosmetic products and ingredients tested on animals in Europe. In the absence of validated alternative methods, predictive testing for skin sensitisation still relies on the use of animals. The key mechanisms at the basis of the induction of skin sensitisation are rather complex but relatively well understood, and this knowledge is being exploited in the development of mechanistically-based non-animal test methods primarily designed for hazard identification. However, skin sensitization risk assessment decisions require not only hazard identification, but also sensitizer potency information to allow a safe level of human exposure to be predicted. It is proposed that a range of mechanistically based non-animal test methods would be necessary to yield an alternative measure of skin sensitiser potency. However, at present it is not possible to predict which combinations of non-animal information will be needed before risk assessment decisions could be exclusively based on non-animal testing data with sufficient confidence for the vast majority of cosmetic product exposure scenarios. The expert group concluded that, by 2013, full replacement of animal methods will not be available for skin sensitising potency assessment. The most positive view of timing for full replacement is another 7-9 years (2017-2019), although it is expected that the scientific ability to inform skin sensitization decisions without animal test data for some ingredients and exposure scenarios should be feasible ahead of 2017-2019.

I-11-051

The COLIPA research and method development program for identifying and characterizing skin sensitizers without animal testing

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At present, animal test methods (such as the mouse local lymph node assay) are required to characterize the potential for new chemicals to induce skin allergy. However there are currently several large research programs ongoing that aim to deliver new non-animal test methods for skin sensitization. COLIPA, the European Cosmetics Industry trade association, intensively participates in this international research effort through continuous funding of projects to explore the processes governing the induction of skin sensitization and the development of new methods incorporating the acquired knowledge. Our ongoing research portfolio (9 different research and method development projects investigating bioavailability, chemical reactivity, protein/peptide binding, skin metabolism, dendritic cell activation and migration mechanisms, T cell proliferation and multi-cell type interactions) continues to provide new insights into the biological processes driving skin sensitization and has already led to the successful development of three *in vitro* test methods for the detection of potential sensitizers: the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitization Test (MUSST). These tests are currently at the ECVAM pre-validation stage. In parallel, a focused evaluation of other available test methods as well as the use of all these methods for risk assessment purposes is being conducted. This comprehensive research and development program aims to define a toolbox of assays to be used in a risk assessment strategy capable of characterizing skin sensitizer potential and potency, the final goal being to perform this risk assessment without the need for animal testing.

I-11-651

Modeling for the molecular mechanisms of allergens on the innate immune synapse

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Because innate immune cells have been implicated in the immune mechanisms of allergic contact dermatitis, we hypothesized that ligands involved in the activation of natural killer or natural killer T-cells may be perturbed in keratinocytes (KC) as a part of their phenotypic changes associated with an allergen induced stress response. To test this hypothesis, we first studied human allergic contact dermatitis in positive patch tests to $NiS0_4$. ULBP-2 was up-regulated on KC *in situ* in ACD (gene expression and *in situ* staining). Studies of primary cultured human KC confirmed the *in vivo* observations regarding the effects of $NiCl_2$ on cultured human KC and ULBP-2 gene expression. Gene expression profiling of human KC revealed that $NiCl_2$ consistently up-regulated a small number of genes involved in innate immune synapse, including ULBP-2. Exposure of primary KC to $NiCl_2$ prior to incubation with highly enriched NK cells resulted in significantly enhanced cytotoxicity (^{51}Cr release), which could be blocked by ULBP-2 specific monoclonal antibody. The KC-derived cell line HaCat and the THP-1 cell line (monocyte lineage) also increased ULBP gene expression after exposure to nickel, indicating consistency of this phenomenon. Ongoing studies are examining other allergens in the training set of chemicals to determine how sensitive and specific the ULBP gene set will be in detected allergens and irritants *in vitro*.

I-11-125

Assessing the sensitization potential of compounds without animal testing

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The FP6 project Sens-it-iv (www.sens-it-iv.eu) has developed promising tests for the identification of skin and respiratory sensitizers. The human reconstituted skin test and the NCTC2544 test are currently tested as a tiered strategy, where the NCTC test identifies skin sensitizers (>99% accuracy) and the reconstituted skin test predicts potency in good correlation with the LLNA. The concordance of the tiered approach with the LLNA for skin sensitizers is 79% for potency ranking and 92% for classifying compounds into the correct potency groups (extreme, strong, moderate, and weak). A DC (dendritic cell) marker signature of 75 genes predicts skin sensitizers with 98% accuracy. In addition, information about the potency of chemicals is provided. Another DC-based test addresses the degree of maturation of DCs before and after exposure to chemicals using a chip coated with monoclonal antibodies against membrane markers. This test has the potential to become a medium through-put alternative to methods such as HCLAT and MUSST. Finally, a functional test assessing exposure driven DC migration has the potential of becoming a first post-screen test. To date, no misclassifications have been observed with this test. An *in vitro* T cell-priming test has been established. This test is the most complex assay of the tool box. Good predictivity has been demonstrated using proliferation as well as TNF-α and IFN-γ levels as read-outs. Thus, tools are available that (i) can identify skin sensitizers and (ii) are able to provide information about the potency of these sensitizers.

I-11-323

Towards the development of a BioMEMS-based microsystem to assess chemical sensitization: Allergy-on-a-Chip

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Skin sensitization remains a major environmental and occupational health hazard. Animal models have been used as the "gold standard" and method of choice for assessing chemical sensitization potential. However, a growing international drive and consensus for minimizing and/or eliminating animal usage have prompted the development of *in vitro* methods to assess chemical sensitization. We are in the process of developing a microfabricated platform that can capture the majority of the key steps involved in allergic contact sensitization. This "Allergy-on-a-Chip" microfluidics-based device integrates a reconstructed human skin compartment with a dendritic cell culture compartment. Intercellular communication is initiated through microfluidically-mediated signaling between the skin construct where the allergenic stimulus originates (via topical application of sensitizer) and an immune system compartment where the response to that stimulus occurs. The microfluidic device is designed to 1) activate dendritic cells following allergen diffusion and/or conversion by skin constructs, 2) allow sensitizer-activated dendritic cell migration via optimized chemokine gradients, and 3) ultimately induce sensitizer-mediated T cell activation. To date, we have fabricated a microdevice and quantified cell-responsive chemotaxis. Using this device, we have established an experimental system integrating a full thickness skin substitute and Mutz-3 cells that have been differentiated into immature Langerhans cells. In addition, we have successfully validated the efficacy of "on-chip" sensitization with a panel of sensitizers, and demonstrated quantitative dendritic cell activation metrics that can be used to distinguish irritants from sensitizers.

Session I-11: Poster presentations

I-11-083

Role of AU-rich element binding proteins in mRNA stability and potency of chemical allergens

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We previously demonstrated in the human promyelocytic cell line THP-1 that all allergens, including prohaptens, selectively induce a rapid p38α MAPK activation and IL-8 mRNA expression. While the prohapten isoeugenol fails to induce IL-8 release, all other allergens tested induced a dose-related release of this cytokine.

In the present study, we investigated whether this abnormal behavior of isoeugenol is regulated by AU-rich element (ARE) binding proteins, namely HuR and tristetraprolin (TTP). THP-1 cells were treated with isoeugenol and, for comparison, with the moderate contact allergen diethylmaleate (DEM), with the strong contact allergen DNCB, and with the irritant salicylic acid.

Data obtained provide evidence of a different regulation of IL-8 during contact allergen treatment. Distinct combination and regulation of the ARE binding proteins HuR and TTP following contact allergen exposure resulted in a different modulation of IL-8 mRNA half-life and release. Data shown demonstrated that increased expression of TTP results in destabilization of the IL-8 mRNA in THP-1 cells treated with isoeugenol, which can account for the lack of IL-8 release. In contrast, the strong allergen DNCB, failing to upregulate TTP while inducing HuR, resulted in longer IL-8 mRNA half-life and protein release. DEM induced TTP at a later time point; it did not induce HuR, resulting in increased IL-8 mRNA half-life compared to isoeugenol but shorter than DNCB. It is tempting to speculate that this different behavior of allergens may also occur *in vivo*, and may contribute to our understanding of allergen potency.

I-11-088

SenCeeTox®: a new *in vitro* method for predicting photosensitization

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The safety of over the counter (OTC) drugs, cosmetics and personal care products is an important part of product development. Standard tests for chemical sensitization have depended on animals. Amendment 7 of the European Cosmetics Directive requires the development of *in vitro* methods that replace animal usage. Although some *in vitro* approaches are under evaluation, SenCeeTox® is the only method designed to identify sensitization potential and provide a potency category. This assay relies on a concentration response of genes controlled by the antioxidant response element (ARE), cytotoxicity, direct reactivity, solubility, and dermal permeability to predict the sensitization potential. The aim of this study was to determine if SenCeeTox® can be used to identify photoallergens. Four known photo activated chemicals (oxybenzone, avobenzone, octisalate, and padimate-O) relevant to the OTC drug/cosmetic industry were selected. Glycerol, p-benzoquinone, and naproxen were used as negative controls, while ciprofloxacin and TSA were used as positive controls for photoactivation. Test compounds were prepared in DMSO and then diluted into PBS. The samples were divided into two groups, one exposed to 6 J/cm^2 UVA light, and one that remained in the dark. Following the light exposure, an aliquot was removed and evaluated for direct reactivity using glutathione (GSH) depletion. A second aliquot was mixed with culture medium and applied to a human keratinocyte (HaCaT) cell in 96-well plates. Following a 24 h exposure, cells were assessed for cytotoxicity (MTT) and ARE controlled gene expression (qRT-PCR). Analysis of cell viability, gene expression, and reactivity data indicates that SenCeeTox® can be used to identify photosensitization.

I-11-100

The Colipa research and method development program for identifying and characterizing skin sensitizers without animal testing

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At present, animal test methods (such as the mouse local lymph node assay) are required to characterize the potential for new chemicals to induce skin allergy. However there are currently several large research programs ongoing that aim to deliver new non-animal test methods for skin sensitization. Colipa, the European Cosmetics Industry trade association, intensively participates in this international research effort through continuous funding of projects to explore the processes governing the induction of skin sensitization and the development of new methods incorporating the acquired knowledge. Our ongoing research portfolio (9 different research and method development projects investigating bioavailability, chemical reactivity, protein/peptide binding, skin metabolism, dendritic cell activation and migration mechanisms, T cell proliferation and multi-cell type interactions) continues to provide new insights into the biological processes driving skin sensitization and has already led to the successful development of three *in vitro* test methods for the detection of potential sensitizers: the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitization Test (MUSST). These tests are currently at the ECVAM pre-validation stage. In parallel, a focused evaluation of other available test methods as well as the use of all these methods for risk assessment purposes is being conducted.

This comprehensive research and development program aims to define a toolbox of assays to be used in a risk assessment strategy capable of characterizing skin sensitizer potential and potency, the final goal being to perform this risk assessment without the need for animal testing.

I-11-111

Prediction of skin sensitization potential of preservatives using CD54 and/or CD86 on THP-1 cells

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Evaluation of skin sensitization potential is a major part of safety assessment of new ingredients in cosmetics and drugs to be applied topically. To evaluate the skin sensitization potential, animal test methods such as the Guinea Pig Maximization Test have been used. From the standpoint of animal welfare, the establishment of *in vitro* skin sensitization test methods is necessary. Evaluation of changes in cell surface marker expression induced in dendritic cells (DC) or DC-surrogate cell lines following exposure to contact allergens represents one approach for the development of non-animal test methods for skin sensitization. The aim of this study is to confirm the predictive potential of an *in vitro* test method for skin sensitization. The aim of this study was to optimize an *in vitro* skin sensitization test using THP-1 cells (monocytic leukemia cell line) with CD54 or CD86 expression markers. We evaluated 11 preservatives (e.g., 1,2 Hexanediol, Phenoxyethanol, MCI/MI) in various concentrations. By evaluating the expression patterns of these indicating markers, we could classify the chemicals as sensitizers or non-sensitizers. These data suggest that the THP-1 cells are a good model for screening for contact sensitizers.

I-11-112

Validation of a skin irritation study using a Japanese model; LabCyte EPI-MODEL24, additional study

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Based on the EpiSkin statement and its protocol, we created a program to validate the usefulness, relevance and reproducibility (including intra- and inter-laboratory variability and transferability) of a skin irritation study using a Japanese model (LabCyte EPI-MODEL24) from 2008-2009. From these data, a good transferability of the LabCyte EPI-MODEL24 and the original protocol was obtained. The skin irritation categories (Non-Irritant or Irritant) for 25 blinded chemicals using this model and protocol showed good inter-laboratory and good predictivity in each laboratory. However, OECD peer review panel indicated 1-bromohexane, category 2, was misclassified into the "No" category by five of the six laboratories, and recommended that the issue be solved. In accordance with this recommendation, J-TEC revised the protocol.

In this study, our goal was to re-evaluate the predictive capacity of the revised protocol using 20 blinded chemicals from the reference chemical list in the OECD Performance Standards.

Based on the reference list in the OECD Performance Standards, a catch-up validation of the LabCyte EPI-MODEL24 SIT by three labs was performed. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of accuracy (75-84.2% overall accuracy, 90-100% overall sensitivity, and 60-70% overall specificity) on the MTT assay excluding 60% of specificity at one laboratory. Two of three laboratories are sufficient with acceptance criteria according to the OECD Performance Standards and the VMT considered that this assay had acceptable reliability of accuracy for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

I-11-116

Application of the TTC and weight of evidence for the safety assessment of botanicals: Calendula and Juniper

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Botanical materials are increasingly being used in personal care products. The safety evaluation of these poorly characterized complex mixtures is a challenge due to the high degree of variability in composition. The first step is to identify each constituent chemical and its concentration range through literature search, supplier information, and/or analytical testing. For each chemical, skin penetration estimates can be derived from molecular weight and LogP values. The maximized, probable systemic exposure can then be established utilizing the concentration of the ingredient in a product and its application rate. If a NOEL or NOAEL does not exist for a chemical constituent, TTC is used as the first step in the safety evaluation. Based on Cramer Class the associated TTC value is compared to the systemic exposure. If the TTC value is exceeded, it is determined if there is a published Possible Average Daily Intake (PADI) for the chemical ("comparative approach"). If there is no known allowable daily intake or if systemic exposure exceeds this value, then a margin of safety must be estimated for the chemical. In order to estimate a margin of safety, a chemical of similar structure with a known NOEL or NOAEL can be used as "read across" surrogate (Chemical grouping approach). The MOS is then determined by dividing the NOEL or NOAEL by the systemic exposure and then applying the standard toxicological safety factors. A combined "weight of evidence" approach is very useful when evaluating such materials that tend to concentrate plant or oil components.

I-11-123

Acute dermal toxicity using the OECD TG 404 integrated testing strategy combining the use of the EpiSkin test methods

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Identification of corrosive and skin irritant chemicals was based, for regulatory purposes, on their ability to produce irreversible or reversible alterations of the skin at the site of contact. During the skin corrosion validation study of the EpiSkin test method, some *in vivo* corrosives were identified as non-corrosives *in vitro*. Since underclassification of chemicals may be due to non-specific reduction of MTT in solution, MTT interference corrections were performed on 5 chemicals detected as direct MTT reducers, indicating the need to adapt the EpiSkin skin corrosion test method by including specific controls for MTT reducers.

A stepwise testing strategy for the prediction of skin irritation and then skin corrosion was developed using the validated EpiSkin test methods to support the ongoing revision of the OECD test guidelines TG404 and TG431. When applying the testing strategy on about 50 reference substances (from the ECVAM validation studies), 20 *in vivo* irritant chemicals were identified *in vitro* as non-corrosive but correctly classified as irritants. In addition, 17 *in vivo* non irritants and non-corrosives were correctly predictive *in vitro* using both skin corrosion and irritation test methods. Finally 12 corrosive chemicals identified by NICEATM/ICCVAM as incorrectly predicted *in vitro* were evaluated. The results showed that corrosive chemicals misclassified in the *in vitro* corrosion test were identified *in vitro* as irritants.

This analysis of these new data should decrease the need for testing for both dermal skin corrosivity and skin irritation of substances for which sufficient evidence already exists.

I-11-137

Signature biomarker analysis for prediction of skin sensitizers using a cell-based *in vitro* alternative to animal experimentation

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Atopic contact dermatitis is a common inflammatory skin disease that affects a significant proportion of the population, and the incidence is increasing due to repeated exposure to sensitizing chemicals. The REACH regulation requires that all new and existing chemicals within the EU should be tested for hazardous effects. As the identification of potential sensitizers currently requires animal testing, this will have a huge impact on the number of animals needed for testing. Further, the 7th Amendment to the Cosmetics Directive (76/768/EEC) imposed a ban on using animals for testing cosmetic ingredients for all human health-related effects by 2013. Thus, development of reliable *in vitro* alternatives to animal experimentation for the assessment of the sensitizing capacity of chemicals is urgent.

We have developed a cell-based assay, based on the monocytic cell line MUTZ-3, for the purpose of testing the propensity of new chemicals to cause sensitization. We have stimulated the cell line with >40 skin sensitizers, irritants and controls for 24 h in optimal growing conditions (≥90% relative viability) and analyzed the activity with genome-wide transcriptional profiling. By employing advanced computational statistics, we have identified biomarker signatures which distinguish sensitizers from controls with 90% accuracy. Thus, we have identified a potent predictive biomarker signature for skin sensitization and demonstrated that the mRNA microarray is a powerful assay in itself. Being based on a human biological system, the assay is considered to be more relevant and more accurate for predicting sensitization in humans than the traditional animal-based tests. Further, the identified marker profiles are believed to describe biological pathways involved in sensitization.

I-11-142

Evaluating the sensitization potential of surfactants: Using *in vitro* methods in a weight of evidence approach

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An integral part of hazard and safety assessments is the estimation of a chemical's potential to cause skin sensitization. Non-animal test methods are in the process of being developed and formally validated. In order to gain more insight into the responses induced *in vitro*, representative surfactants were tested in methods that are in the prevalidation process. The battery of *in vitro* tests (animal data available) included peptide reactivity assays, the KeratinoSens assay, the hCLAT assay and EpiSkin irritation assays. Seven of eight surfactants were negative in the GPMT and in the KeratinoSens assay, seven of eight were positive in the LLNA, none formed covalent adducts with test peptides, and all were negative in the hCLAT assay. Six of eight surfactants would be rated as being irritants by the EpiSkin assay, seven of eight induced IL-1α, and all but one were positive in the LLNA based on ear swelling. A weight of evidence approach would classify seven of eight as being non-sensitizing skin irritants and would confirm that the LLNA tends to overestimate the sensitization potential of surfactants. As results obtained from LLNAs are considered as the gold standard for the development of new non-animal test methods, results such as these highlight the necessity to carefully evaluate the applicability domains of different test methods in order to develop reliable non-animal alternative testing strategies for sensitization testing. The results show how *in vitro* methods could possibly be used to interpret contradictory results from animal tests in a weight of evidence approach.

I-11-145

The Myeloid U937 Skin Sensitization Test (MUSST) for the prediction of skin sensitization potential

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Skin sensitization is a delayed type allergy consisting of a cellular immune reaction to small molecular weight chemicals, so far predicted using animal test methods such as the local lymph node assay (LLNA). In line with the 3Rs, *in vitro* alternatives are being developed based on early events of the skin sensitization process. One of these is the capacity of dendritic cells to recognize a chemical as a danger. The Myeloid U937 Skin Sensitization Test (MUSST) models this by measuring the up-regulation of CD86 expression on U937 cells. A chemical is classified as a sensitizer if it induces a dose-dependent up-regulation of CD86 expression at non-toxic doses in two concordant experiments. The MUSST prediction is exemplified with 2 sensitizers (phenyl benzoate, methylchloroisothiazolinone), 2 pre/prohaptens (ethylene diamine, eugenol) and 2 non-sensitizers (lactic acid, benzaldehyde) correctly classified by the assay. The predictive performances of the MUSST are evaluated with a panel of 50 reference chemicals (31 sensitizers and 19 non-sensitizers) against the LLNA data to support its submission to and its acceptance by ECVAM for a pre-validation. With the 40 classified chemicals (10 are inconclusive), the MUSST displays a concordance of 83% with 81% sensitivity and 84% specificity.

The performances, further evaluated with an extended set of 83 classified reference chemicals, show concordance, sensitivity and specificity above 75%.

The MUSST is an efficient assay for skin sensitization hazard characterization and is promising as a tool to be integrated within a battery of assays to perform a skin sensitization risk assessment.

I-11-152

Development of a skin sensitization test using a three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts on collagen vitrigel membrane for application to cosmetic products

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Although several *in vitro* skin sensitization tests using human cells have been developed, it is difficult to use these to examine cosmetic products such as milky lotion and cream owing to their water insolubility. We have already established a test method using a three-dimensional human skin model consisting of normal fibroblasts, normal keratinocytes and normal dendritic cells on a collagen vitrigel membrane (VG-KDF-Skin-method). In this study, we compared this method with an *in vivo* method, and investigated the possibility of evaluating the skin sensitization potential of cosmetic products using this model.

The VG-KDF-Skin was treated with test chemical or cosmetic products for 1 h. After removal of these, this skin model was further incubated for 23 h. The supernatant was collected, and IL-1α, IL-4 and IL-8 were measured by ELISA. Test chemicals that showed over 150% of cytokine release compared to control were deemed to have a positive response.

Nine sensitizers and five non-sensitizers were examined. The accuracy, sensitivity and specificity of the VG-KDF-Skin method using IL-4 as an indicator vs. LLNA were 93%, 89% and 100%, respectively. The accuracy, sensitivity and specificity of the VG-KDF-Skin method, using IL-1α± vs. LLNA, were 50%, 56% and 40%, respectively.

Significant IL-4 release was induced, by model cosmetic samples containing a skin sensitizer of 2,4-dinitrochlorobenzene. The VG-KDF-Skin-method using IL-4 as an indicator would be useful for evaluating the skin sensitization potential of chemicals and products showing the properties of emulsions, creams or solids owing to their water insolubility.

I-11-153

Statistical prediction model for skin sensitization potential using an integrated dataset from h-CLAT, DPRA, and DEREK

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The validation of alternatives to animal testing for skin sensitization is ongoing and a number of testing methods are currently being developed. For such methods, the use of only a single alternative test method may not provide sufficient predictive performance to make an assessment of skin sensitization potential. As a result, it may be necessary to develop an assessment strategy which combines multiple forms of testing. The objective of this study was to confirm the predictive performance obtained using a parametric regression analysis of multiple testing results. A parametric regression model presents the advantage of easy interpretation, compared to non-parametric regression models such as neural networks or support vector machines. Multiple linear regression analysis and logistic regression analysis were performed via the Weka software using an integrated dataset for 101 chemicals obtained via LLNA, h-CLAT, DPRA, and DEREK. LLNA results (positive or negative) and LLNA EC3 values were selected as outcome variables. A Box-Cox transformation was applied to the variable data to improve both normality and predictive performance. Use of the logistic regression analysis for the prediction of LLNA results yielded predictive performance with an accuracy rate of 89.1%, a sensitivity rate of 93.4%, and a specificity rate of 76.6%. Use of the multiple linear regression analysis for the prediction of log-transformed LLNA EC3 values yielded a predictive performance with a multiple correlation coefficient of 0.791. By applying a statistical regression model to the dataset from LLNA, h-CLAT, DPRA, and DEREK, the predictive performance was improved over the prediction of the single test method.

I-11-169

Papain characterization: an approach to the cytotoxicity profile

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Papain is a vegetal enzyme applied in several medical devices due to its characteristic properties, such as proteolytic activity and cicatrization induction, which allow distinct biomedical applications. However, when dealing with compounds for human use, toxicity and biocompatibility studies are mandatory in order to assure efficacy and safety. On this account, in this work we report a study based on different commercially available enzymes. Each papain formulation was characterized in terms of biochemical properties, through enzymatic activity determination and protein content, as well as according to their physicochemical properties, through UV spectra and IR techniques, in order to identify possible differences in the enzyme, since distinct purification and stabilization methods are used at industrial level nowadays. The samples were subjected to a cytotoxicity assay with equivalent protein content and enzymatic activity. Human keratinocytes (HK) in high density were used to test papain at several concentrations (from 0.25 to 2% (w/v)) for 24 h of contact at 37°C, 97% humidity and 5% CO_2. The viable cells were measured by MTS/PMS and formazan product was quantified at 490 nm. The results revealed that despite the specific characteristics of each enzyme produced, regarding activity and IV and UV spectra, the cytotoxicity profile was similar for all samples. The enzymatic activity does not seem to play a major role in the cytotoxic effects, indicating that the characteristic cytotoxicity is related to the enzyme concentration. It is relevant to note that, under the evaluated conditions, all formulations were cytotoxic even at low doses.

I-11-173

Towards the 21st century: Advances and refinements in the prediction of sensitisation potential using the TIMES platform

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The TImes MEtabolism Simulator platform for predicting Skin Sensitisation (TIMES-SS) is a hybrid expert system that was developed at Bourgas University using funding and data from a consortium comprising experts from industry and regulatory agencies and coordinated by IQF. The model was developed with the aim of minimising animal testing, to be scientifically valid for regulatory purposes, and to be mechanistically transparent. TIMES-SS encodes 2D structure-toxicity and structure-skin metabolism relationships through a number of transformations, some of which are underpinned by mechanistic 3D QSARs. An external evaluation exercise was completed in 2007 where LLNA data were generated for 40 new chemicals and compared with predictions made by TIMES-SS. The results were promising with an initial concordance of 75%. An extensive evaluation followed to assess the results in light of reaction chemistry principles. The number of chemicals underpinning a given reaction chemistry alert was reviewed and four validation substances were subsequently tested. Recently, a 3-year research proposal was initiated as part of a new industry consortium. The skin (a) biotic metabolism simulators and the skin sensitisation model will be refined in light of new data and chemical insights. The applicability domain of the underlying experimental data will be evaluated to reflect current recognised inconsistencies between different *in vivo* assays. The feasibility of developing a respiratory sensitisation model will also be investigated. This presentation will provide an overview of the current status of TIMES-SS, highlight current refinement activities, and outline the strategy for deriving a respiratory sensitisation model based on preliminary investigations.

I-11-176

Evaluation of KeraSkin™-VM, a new reconstructed human epidermis model as an alternative to the skin irritation test method according to the OECD TG439

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Several alternative *in vitro* methods for the evaluation of skin irritants have been developed recently. The most promising one is the reconstituted human epidermal model. In July 2010, the OECD officially endorsed the validated reference method (VRM) using three 3D reconstructed human epidermis models, EpiSkin™ , EpiDerm™ SIT (EPI-200) and the SkinEthic™ RHE, as replacements for the *in vivo* skin irritation test. In this study, KeraSkin™ -VM model (MCTT Co, Korea), a new human epidermis model reconstructed using Asian skin tissues, was evaluated as another *in vitro* skin irritation test method using 20 reference chemicals according the performance standards of OECD TG 439. The test protocol was performed using the *in vitro* RHE-based test method with a minor modification, in accordance with the requirements of the performance standards of OECD TG439. The results obtained with the modified irritation protocol were comparable to those of VRM with the EpiDerm™ SIT (EPI-200) models. When comparing the performance of the KeraSkin™ -VM with UN GHS categories, an overall accuracy of 80%, sensitivity of 90% and specificity of 70% were obtained, and the accuracy of the KeraSkin™ -VM was comparable to that of the VRM. In this study, we demonstrated the new reconstituted human epidermal model KeraSkin™ -VM showed a good performance in terms of intralaboratory variability and predictive capacity to screen skin irritants. Further studies are on-going to improve the protocol and predictability for the interlaboratory validation study of KeraSkin™ -VM.

I-11-177

Development of a Fluorescence intensity Increased Method (FIM) to evaluate the skin photosensitization potential of chemicals

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In order to develop *in vitro* assays for the detection of the photosensitizing potential of chemicals, we tried to apply published *in vitro* sensitization assays to detect photosensitization. Among them, we focused on the ARE (antioxidant response element) assay and the SH test. The ARE assay is a luciferase-based assay using AREc32 cells which have an eightfold repeat of the ARE sequence as an upstream promoter of the luciferase gene. The SH test detects changes of cell-surface thiols by haptens using flow cytometry. Both assays were reported to be useful as *in vitro* sensitization assays.

Using the SH test, we evaluated changes of cell-surface thiols on THP-1 cells upon treatment with known photosensitizers and UVA exposure. Furthermore, we evaluated cell-surface amines in addition to cell-surface thiols. Using the ARE assay, we determined the optical condition of UVA exposure necessary to detect photosensitizers. As a result, we confirmed that most photosensitizers induced changes of cell-surface thiols or amines on THP-1 cells upon 5 J UVA exposure. However, piroxicam and p-aminobenzoic acid (PABA) did not induce changes of cell-surface thiols and amines under this condition but did induce ARE expression in AREc32 cells upon 2.5 J UVA exposure. The results of both assays using photosensitizers and non-photosensitizers were in good concordance with those of literature information.

In conclusion, our data suggests that changes of cell-surface thiols and amines on THP-1 cells and the ARE luciferase assay are useful to detect the photosensitizing potential of chemicals.

I-11-182

Evaluation of a photosensitizer by non-radioactive local lymph node assay (LLNA)

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Concerns over photoallergy of cosmetics and drugs are escalating, but validated alternative test methods for the detection of photosensitivity are yet to be developed. We investigated the modification of the non-radioisotopic local lymph node assay (LLNA) using BrdU with flow cytometry as a new alternative test method for the identification of photoallergic potential. Before a full-scaled experiment, proper test concentrations of test material were decided through a preliminary photo-irritation test. Treatment method and scheme were the same with LLNA. Mice received topical application of chloropromazine (CPZ, 0.01, 0.025, 0.1, 0.5 w/v%) or vehicle on both ears and were irradiated with ultraviolet A (UVA) afterward for 3 consecutive days. Each dosed group consisted of two sets – irradiated and unirradiated. Mice were sacrificed 24 h after intraperitoneal injection of bromodeoxyuridine (BrdU). Weight of ears and lymph nodes were measured to evaluate photo-irritation and lymph nodes were isolated and underwent lymphocyte preparation. Potential for photoallergy was determined by BrdU incorporation into lymph node cells, B/T cell ratio and *ex vivo* cytokine production. We regarded the test article as photosensitizer when SI was above 3 in the irradiated group and SI was below 3 in the unirradiated group. Stimulation index (SI) and cytokine release like IL-2 and IL-6 were significantly increased by CPZ at concentrations above 0.1%. In contrast, the B/T cell ratio was significantly increased from the lowest concentration (0.01%). Phototoxicity could be identified using ear swelling and cytokine profile changes. These findings suggest that photoallergic potential could be determined using SI, B/T cell ratio and cytokine production. Further studies with more diverse chemicals are on-going for the validation of the method.

I-11-186

An *in vitro* test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8

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Several studies have suggested that IL-8 is a biomarker to discriminate haptens from irritants. To develop a high throughput method to identify possible haptens, we established a stable THP-1-derived IL-8 reporter cell line, THP-G8, which translates SLO and SLR luciferase genes under the control of IL-8 and G3PDH promoters, respectively. After 6 h of treatment with chemicals, normalized SLO-LA (nSLO-LA) was calculated by dividing SLO luciferase activity (SLO-LA) by SLR-LA, and fold induction of nSLO-LA (FInSLO-LA) was calculated by dividing nSLO-LA with chemical treatment by that without treatment. THP-G8 increased nSLO-LA in response to LPS or several haptens. FInSLO-LA was positively correlated with IL-8 mRNA induction in THP-1 stimulated with LPS or haptens. When we examined the effects of 15 haptens and 7 irritants on nSLO-LA, however, THP-G8 significantly increased their nSLO-LA (FInSLO-LA≥1.4) by 13 haptens as well as 5 irritants. Interestingly, pretreatment of N-acetyl cysteine (NAC) suppressed increase in FInSLO-LA induced by all haptens (suppression index (SI) ≤0.8), while NAC did not suppress increase in FInSLO-LA by most irritants. Then we evaluated the performance of this reporter assay with the criteria of haptens as FInSLO-LA≥1.4 and SI≤0.8 more than 2 out of 3 independent experiments, which resulted in test accuracies of 82% for these 23 chemicals and 88% for the chemicals proposed by Casati et al. This newly developed assay would be a candidate to replace animal tests for skin sensitization because of its accuracy, convenience, and high throughput

I-11-187

CEFIC-LRI workshop on skin sensitisation methods

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Skin sensitisation is an important toxicological endpoint that is assessed for all chemicals. Due to animal welfare benefits and the ease of potency quantification, the LLNA (OECD 429) is commonly the first choice test for sensitisation testing. Moreover, under REACH, the use of other tests (i.e. the traditional guinea pig test (OECD 406)) needs to be scientifically justified. Discrepancies between results obtained with the LLNA and guinea pig tests have been reported for some classes of chemicals (e.g. surfactants, unsaturated fatty acids, siloxanes). Such substances are not considered sensitising based on historic test data and the absence of human evidence of sensitisation. These results suggest a need for improved characterization of test results to enable a better understanding of potential confounding chemistries. To broaden awareness among stakeholders, a CEFIC Long-range Research Initiative (LRI) workshop reviewed these experiences with a panel of experts from regulatory, academic and industrial organizations. Focused discussions involved the definition of "gold standard", applicability domains and the use of LLNA, guinea-pig and human experiences for the development of non-animal tests. The workshop findings and recommendations will serve as a guide in a research strategy to understand critical aspects of these test results and the development of reliable alternative methods, while advancing a flexible and intelligent skin sensitisation testing strategy across different chemical classes.

I-11-189

A strategy for the hazard identification and potency categorization of skin sensitization using a combination of non-animal tests

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The combination of several *in vitro* methods is a crucial approach in identifying the skin sensitization hazard adequately for a wide variety of chemicals without the use of animals. We have been developing an *in vitro* sensitization assay, the human Cell Line Activation Test (h-CLAT), emulating dendritic cell activation. In this study, we investigated a battery system: the combination of h-CLAT, the direct peptide reactivity assay (DPRA), an *in vitro* assay, and the *in silico* system, DEREK. Currently ECVAM is pre-validating both h-CLAT and DPRA. As a first step, the integrated testing strategy (ITS) was investigated. Final score calculated from the scores of each robust data set from each ITS component was used for the evaluation. ITS demonstrated a higher accuracy (85%) compared to DPRA, h-CLAT or DEREK alone. Secondly, the tiered approach using h-CLAT and DPRA were investigated as a practical system. The optimized tiered approach indicated the possibility of not only detecting the hazard but also for classifying the potency of chemicals. The predictivity for the potency classification was 72.3%, while the "under prediction" rate was especially low. Our results brought the non-animal testing system one step closer to replacing animal testing. Finally, we have been developing a novel *in vitro* test, EpiSensA, using a reconstructed epidermis model, which is expected to solve some of the current problems (i.e. lipophilic chemical evaluation). By adding EpiSensA to the tiered approach, the non-animal testing system will be used as an alternate to animal testing and will be leveraged in risk assessments.

I-11-190

ECVAM prevalidation study on skin sensitisation alternatives: progress update

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In the field of *in vitro* alternatives in toxicology, several alternative methods for acute local health effects have already been validated. In contrast, sensitization and other repeated dose endpoints have remained a significant challenge. However, recent progress with *in vitro* assays in skin sensitization toxicology has resulted in the development of mechanistically based test methods which could make a valuable contribution to the replacement of the existing animal tests. These approaches comprise the Direct Peptide Reactivity Assay (DPRA), the Myeloid U937 Skin Sensitization Test (MUSST) and the human Cell Line Activation Test (h-CLAT). Each of these test methods has been the subject of substantial evaluation including inter-laboratory assessments, and their status of development has led to their acceptance by ECVAM for inclusion in a Prevalidation Study in which the three test methods are challenged with a set of coded chemicals in three laboratories each. 24 chemicals, well characterised with respect to their sensitisation potential (or lack of), have been selected and will be tested once for the assessment of the between- laboratory reproducibility. A subset of 15 chemicals will be assessed a further two times for the evaluation of the within laboratory reproducibility. It is anticipated that results from the DPRA will be available late in 2011, whereas results from the cell-based assays are expected to be delivered during 2012. Assuming a successful outcome, future activity will require consideration of how to deploy these assays in a structured assessment of skin sensitization potential. An overview on the study organisation and progress will be provided.

I-11-194

Implementation of non-animal approaches for cosmetic safety assessments for skin sensitisation

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Skin sensitisation is a key endpoint for the safety assessment of cosmetic ingredients, and the mouse Local Lymph Node Assay (LLNA) is currently the standard test method that predicts the skin sensitising potential of chemicals as well as estimates of their relative potency. A key question that still remains to be addressed is to what extent skin sensitisation safety assessments can be made in the absence of animal data. Therefore, COLIPA, the European Cosmetics Association, undertakes efforts to continuously evaluate emerging tools and approaches in the field, comprising predictions based on physico-chemical properties (including *in silico* tools), indications for the presence or absence of structural alerts ((Q)SAR), read-across based on similar chemicals with available experimental data, *in vitro* methods, historical data and exposure-based waiving approaches (e.g. Threshold of Sensitisation Concern). These tools and approaches are integrated into safety assessment strategies such as the quantitative risk assessment (QRA) approach for skin sensitization, for which the analysis of the weight of evidence is considered as a basic element. Based on the outcome of a COLIPA workshop held in 2010, guiding principles are suggested in or-

der to further develop individual tools and how to combine them in order to finally enable safety decisions without the need for animal tests. The generation of data from alternative tools suited for hazard characterisation and potency evaluation is considered to be the key research area. The design of safety assessment strategies integrating all relevant information is complementing the extensive COLIPA research program that aims at developing and evaluating such alternative test methods.

I-11-198

The molecular mechanisms of IL-8 production by hapten-stimulated monocytes: analysis using a stable THP-1-derived IL-8 reporter cell line, THP-G8

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We have established a stable THP-1-derived IL-8 reporter cell line, THP-G8, in which SLO and SLR luciferase genes are regulated by IL-8 and G3PDH promoters, respectively. Using THP-G8, we developed a high throughput-screening test for haptens. To elucidate the mechanism of IL-8 induction by haptens, we stimulated THP-G8 cells with a representative allergen, 4-nitrobenzylbromide (4-NBB), and a non-hapten, sodium lauryl sulfate (SLS), together with various signal transduction inhibitors. MRS2578, the P2Y6 receptor-selective antagonist, and the ERK inhibitors PD980059 and U0126 significantly suppressed SLO-LA induction by both 4-NBB and SLS, consistent with recent reports of UDP involvement in the production of IL-8 via ERK activation. To understand the mechanism by which UDP is released from THP-G8 stimulated with chemicals, we used 9 chemical inhibitors against reactive oxygen species (ROS) (NAC and DPI), mitochondria complex I (rotenone), calcium signaling (BAPTA-AM), phosphoinositide-3-kinase (LY-294002), Rho-dependent kinase (Y-27632), and various Cl^- channels (glybenclamide, arachidonic acid, and GdCl3). All these inhibitors except for GdCl3 significantly suppressed 4-NBB-induced SLO-LA, while only NAC and DPI but not the others suppressed UDP-induced SLO-LA. The results suggest that UDP release by 4-NBB-stimulated THP-G8 is at least partly mediated by 1) ROS production by mitochondria, 2) intracellular Ca^{2+} elevation, 3) PI3K, 4) Rho-dependent kinase, and 5) some Cl- channels. Only DPI significantly suppressed SLS-induced SLO-LA, SLS releases UDP by a mechanism different from that of 4-NBB. These data suggest that THP-G8 could be a useful tool to investigate the molecular mechanism of IL-8 production as well as to identify skin sensitizers.

I-11-200

Study on development of *in vitro* photosensitization test using human-derived monocytes

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The European Parliament amended for the seventh time the Council Directive 76/768/EEC, which includes the ban of testing on animals and of the marketing of products/ingredients tested on animals. According to the framework of the 7th Amendment to this Directive, a timetable was laid out for the phasing-out of animal testing. This timetable states that the cut-off date for the marketing ban of cosmetic products tested on animals for UV-induced toxic effects including photosensitization is 2013. However, there is no regulatory alternative method for the photosensitization test. Thus, we performed this study to develop *in vitro* photosensitization using human-derived monocytes. Photosensitization is a delayed-type hypersensitivity reaction with an essential requirement for ultraviolet (UV) radiation. Some chemicals including some drugs are known to cause photoallergic reactions. In this study, we evaluated the expression of CD40 and CD54 in THP-1 cells exposed to known photoallergens, such as 6-methylcumarin and chloropromazine, to develop the new alternative method for identifying photosensitizing potential chemicals.

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I-11-202

In vitro skin sensitization test; human Cell Line Activation Test (h-CLAT)

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We have developed an *in vitro* skin sensitization test using THP-1 cells (human monocytic leukemia cell line), named "human Cell Line Activation Test" (h-CLAT). This test is based on the augmentation of CD86 and/or CD54 expression in THP-1 cells following exposure to sensitizers. We have evaluated about 100 chemicals, which have a different potential for skin sensitization, by h-CLAT and compared the results with LLNA or human test data. The accuracy of the h-CLAT vs. LLNA was over 84% and for h-CLAT vs. human test data was about 80%. Most chemicals were evaluated correctly, but a few chemicals were failures. The chemicals that were evaluated as "false-negative" were e.g. benzoyl peroxide, isoeugenol, phthalic anhydride, and abietic acid, and the chemicals evaluated as "false-positive" were 1-bromobutane and diethylphthalate. The result of "false-negative" might be caused by lacking metabolic activity in THP-1 cells, low solubility to water, and weak sensitizers. Next, we calculated the estimated concentration to induce marker expression with an RFI=150 for CD86 (EC150) or 200 for CD54 (EC200) in the h-CLAT, and these values were compared with the LLNA EC3 values. We especially classified into 2 groups, "strong" and "weak", on EC150=10. As a result, the strong group in h-CLAT has a high correlation with the extreme and strong groups in LLNA, and the weak group in h-CLAT has a correlation with the moderate and weak groups in LLNA. This result suggests that h-CLAT could be useful not only for hazard identification, but also for estimating chemical allergic strength.

I-11-206

Evaluation of SenCeeTox®, an integrative model for identifying chemical sensitizers

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Allergic contact dermatitis is the result of an adaptive immune response of the skin to direct exposure to an allergen. Because it is the most common manifestation of immunotoxicity in humans, a strict screening of all ingredients in consumer products is required. Current methods rely on animal testing (e.g. local lymph node assay) for determining chemical sensitization. Ethical concerns and regulatory changes in the EU have stimulated the development of alternative tests for the assessment of potential sensitizers. There is a common view that a strategy for the integration of the currently available methods will be required. Ceetox Inc. has developed an integrative approach, namely SenCeeTox®, allowing the EC3 value to be estimated and differentiating the degree of response from non-sensitizer (NS), weak (W), moderate (M), and strong (S), up to extreme (E). The purpose of this study was to evaluate the predictive capacity of this approach in a blinded manner. L'Oréal provided a set of 40 compounds (20 positive and 20 negative), consisting of 24 proprietary and 16 public domain chemicals that were assessed by CeeTox. All 40 compounds could be classified; those placed into E, S, and M were considered positive, while compounds classified as W or N were considered negative. Results obtained for the prediction of positive and negative compounds were promising. However, the model failed to accurately predict each sensitization category. Refinements and automation of the algorithm and the incorporation of additional assays should improve the model's ability to predict potency.

I-11-217

A combined model of sebocytes with human epidermal-dermal equivalents for evaluating the effect of topical application of sebum inhibitors

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Agents with sebum inhibitory activity could be used to prevent or treat acne and seborrhea. Once formulated, such agents can no longer be tested in monolayer cell cultures, and traditionally they were tested in animals. Thus, we developed an *in vitro* model system to evaluate topical, formulated agents for their effect on sebaceous lipids. This system utilizes epidermal-dermal equivalents which are overlaying cultured primary sebocytes. Following topical treatment, lipid production is quantified by Nile red, a fluorescent dye that selectively binds to neutral lipids. The predictability of the new *in vitro* system was validated using flutamide, a known agent with sebum inhibitory effect. A melanocortin receptor 1, 5 (MC1R, MC5R) antagonist, JNJ-10229570, was shown previously to inhibit sebaceous lipid production in ChT-induced primary sebocyte culture and in human skins-SCID mice. When the same JNJ-10229570 was applied topically for 5 days onto the epidermal-dermal equivalents overlaying the cultured sebocytes, a similar inhibitory effect was observed, verifying the usefulness of this new co-culture system. In conclusion, we developed an *in vitro* combination system for evaluating topical agents for their effects on the sebaceous lipids that replaces the use of animal models.

I-11-226

Methods development through recognition in 3Rs: L'Oréal commitment

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Dissemination of advances on alternative methods represents a step to promote alternatives to animal testing in line with the EU Cosmetics Directive. L'Oréal has, based on these principles, developed test methods to screen and test potential effects on chemicals.

We have focused initially on approaches for skin irritation. A peer review on various aspects of alternative techniques was performed at all stages of the R&D with a focus on *in vitro* methods improvement of chemicals selection (screening) as well as quality testing. To ensure quality and objectivity, experts from international committees oversee the content of EpiSkin and SkinEthic RHE protocols. Details of the approach will be presented for both skin corrosion and irritation with a set of 50 reference chemicals. Using computational approaches, an automated workflow algorithm was developed to predict a molecule's potential for skin irritancy based on *in vivo* Draize data. The practical approaches developed by L'Oréal in the areas of eye irritation (SkinEthic HCE defined with 90 chemicals), skin sensitization (MUSST assay optimized with 50 chemicals), phototoxicity and genotoxicity will be described. For chronic and systemic toxicity testing, a realistic approach relied on the combination of data generated for multiple endpoints. Preliminary studies indicated that the method had good sensitivity and specificity (91% and 78%) while defining a LD_{50} threshold at 2000 mg/kg.

Combination of *in silico*, read across and *in vitro* strategies assure realistic scientific approaches suitable for the safety assessment process within industry.

I-11-230

Contact sensitizers modulate the arachidonic acid metabolism of PMA-differentiated U-937 monocytic cells activated by LPS

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Contact sensitizers are defined as reactive molecules that have the ability to modify skin proteins to form an antigen. In addition to the haptenation mechanism, inflammatory signals, leading to the activation of dendritic cells, are described to be crucial for the effective induction of an antigen-specific T cell immune response. However, the sensitization phase is often a silent process, without obvious clinical manifestations of inflammation/irritation. Even more, anti-inflammatory properties of some molecules do not prevent them from inducing skin sensitization. The aim of this study was to better understand how sensitizers modulate an inflammatory response. To address this purpose, we used the human monocytic-like U-937 cell line differentiated by phorbol myristate acetate (PMA) and investigated the effect of 6 contact sensitizers (DNCB, PPD, hydroquinone, propyl gallate, cinnamaldehyde and eugenol) and 3 non-sensitizers (lactic acid, glycerol and tween 20) on the production of pro-inflammatory cytokines (IL-1β and TNF-α) and on the arachidonic acid metabolic profile after bacterial lipopolysaccharide (LPS) stimulation. Our results showed that among the tested molecules, all sensitizers specifically prevent the production of PMA/LPS-induced COX-2 metabolites (PGE_2, TxB_2 and PGD_2). We further demonstrated that there is no unique PGE_2 inhibition mechanism: while the release of arachidonic acid (AA) from membrane phospholipids does not appear to be a target of modulation, COX-2 expression and/or COX-2 enzymatic activity are the major steps of prostaglandin synthesis that are inhibited by sensitizers. Altogether these results add a new insight into the multiple biochemical effects described for sensitizers.

I-11-234

Computational system for predicting chemical reactivity towards macromolecules and subsequent adverse effects

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The ultimate goal of this work is the development of a computational tool for predicting chemical reactivity towards macromolecules (proteins, DNA) and subsequent adverse effects such as skin sensitization, genotoxicity, etc. Firstly, we focused on contact dermatitis, which results from the interaction between a hapten (electrophile) and the side chain of nucleophilic amino acids of proteins. Therefore the reactivity of chemicals towards peptides was studied. The domains of reactivity categories are defined according to the types of interaction mechanisms such as Michael addition, epoxide ring opening, Schiff-base formation, acylation, etc. The chemicals acting by these mechanisms have specific structural functionalities with different hardness/softness, which can be assessed by quantum-chemical parameters and used to predict chemical reactivity towards macromolecules. Experimental data of reactivity of chemicals on synthetic cysteine/lysine peptides (Direct Peptide Reactivity Assay) and/or glutathione are used to calibrate the boundaries of defined reactivity categories as well as for defining new categories for protein binding potency. The ability of chemicals to interact by ionic or radical mechanisms were assessed by quantum-chemical parameters evaluating the stability of formed intermediates and hence, the energetic feasibility of the respective transformations. The classification of parent chemicals according to the reactivity categories will be used to provide a hazard assessment for subsequent adverse effects.

I-11-243

Use of melanocytes and keratinocytes in co-culture for the assessment of the potential of a Brazilian flora nut as a de-pigmentation agent

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Popular reports indicate the use of a nut from Brazilian flora as a skin depigmentation agent. This study proposes to use human melanocytes and keratinocytes in co-culture *in vitro* and to treat them with extracts obtained from the nut's shell. L-DOPA, a stimulator agent of melanogenesis, the melanin production pathway responsible for skin pigmentation, was standardized in co-culture of melanocytes and keratinocytes as standard control for the presence of melanocytes by pigmentation. To obtain the extraction samples the nut shells were crushed and heated in an incubator at 200°C to give two viscous extracts with different major components, called extracts (A) and (B) respectively. Based on topical use cream formulations developed in previous studies, tests of solubility of the extracts are being carried out, using the same concentrations of the formulations in the culture medium. Murine fibroblasts, human melanocytes and keratinocytes are being tested in co-culture and then treated with extracts (A), (B), negative and positive control, in order to assess the depigmentation property of the extracts as inhibitor of melanogenesis by DOPA reaction test. Hydroquinone, a known inhibitor of the melanogenic pathway, has been used as positive control of extracts (A) and (B), which subsequently will have their results compared to hydroquinone.

I-11-245

Use of fresh, functional human skin tissue in assessing anti-inflammatory effects of human pharmaceuticals

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It is estimated that inflammatory disorders involving the skin account for 15% of all visits to general practitioners in Europe. Experimental research in human skin disorders is generally performed using animal tissue or a synthetic human skin preparation. While advancements in treatments have been made using such models, these are not without their disadvantages. Herein, we present cytokine data obtained from fresh, full thickness human skin biopsies which were exposed to a UV light source or an inflammatory chemical.

Fresh human skin from cosmetic procedures was obtained with full consent. Full-thickness skin biopsies were placed into a transwell filter in a 24-well culture plate with 1 ml of culture medium with the epidermis facing upwards and the dermis suspended in the culture medium. Biopsies were either exposed to a UV light source (253 nm) or had lipopolysaccharide (LPS; 1, 10 & 100 μg/ml) or phytohemagglutinin (PHA; 10, 100 & 1000 μg/ml) applied to the culture media to artificially inflame the skin. Biopsies were cultured for up to 24 h at 37°C in 5% CO_2/air with the supernatant samples obtained at various time points and cytokine levels assessed by ELISA. Exposure of skin to UV light for 15 min or the addition of LPS or PHA to the culture media was generally shown to cause an increase in all cytokines and immunomodulators studied, with PHA shown to have the most marked effects. The results presented above demonstrate that full-thickness human skin can be used to model inflammatory skin conditions.

I-11-259

Peptide reactivity assay using spectrophotometric analysis for screening of skin sensitizers

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Evaluation of the skin sensitization potential is an important step for the safety assessment of new ingredients in cosmetics and topical drugs.

Haptenation is a determinant step in the induction of skin sensitization. Thus, tests to measure reactivity of chemicals with peptides or proteins using HPLC and or LC/MS have been developed as an *in vitro* skin sensitization testing method. In this study, we tried to examine the possibility of spectrophotometric analysis for evaluating the peptide reactivity using two kinds of synthetic peptides, Ac-RFAACAA and Ac-RFAAKAA, with 30 chemicals. Free thiol and amino groups of the non-reacted peptides were measured by UV-VIS spectrophotometer using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and by fluorometer using fluorescamine™ after reaction with chemicals.

Most sensitizers reacted with cysteine peptide and some chemicals reacted with both peptides or with only lysine peptide. Glutaric dialdehyde and benzocaine highly depleted lysine peptide but did not have much effect on cysteine peptide. Most of the non-sensitizers showed a low depletion rate for both peptides. Therefore, results from two model peptides should be integrated for the understanding of sensitization potential. These results suggested that the peptide reactivity test using spectrometric methods could be an easy, fast and high throughput screening tool for the prediction of skin sensitization potential.

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I-11-274

Human hair follicle equivalents *in vitro* for substance testing

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The human hair follicle plays an important role in skin biology. Being highly vascularized and surrounded by dendritic cells, it supports penetration of substances into the skin and further into the bloodstream. The continuous implementation of test procedures on human skin equivalents into OECD guidelines for substance testing was the first move toward animal free testing of cosmetics/chemicals.

Substance testing on human hair follicles *in vitro* can add significant value to the current test procedures. By carefully analyzing and recapitulating the growth and differentiation mechanisms of hair follicle formation, we recreated human hair follicles in tissue culture that were capable of producing hair shaft and revealed a striking similarity to their *in vivo* counterparts. Extensive molecular and electron microscopy analysis were used to track assembly of follicular keratinocytes, melanocytes and fibroblasts into the final hair shaft, producing microfollicle architecture. The hair follicle generation process was optimized in terms of efficiency, reproducibility and compliance with regulatory requirements for later transplantation. In addition, we developed a procedure to integrate the *de novo* created human micro-follicles into our existing human skin equivalents for substance testing. Tissue culture data, histo- and immunostaining of the organotypic cultures as well as marker analysis are presented. The later use of the established system for the evaluation of the role of hair follicles in dermal substance transport mechanisms for cosmetic and pharmaceutical products will be discussed.

I-11-276

A new perspective to evaluate sensitizing agents using microarray analyses

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The need for standard methods to assess undesired effects and the increasing public interest to avoid using animals for substance testing has led researchers to turn to cell-based methods. Cells obtained directly from donors are usually limited in quantity, therefore permanent cell lines have been tested as alternative assays. In this study, the U-937 permanent human monocytic cell line was used to analyze the gene expression response to four compounds showing different sensitizing activity in parallel to a test botanical extract. Global gene expression response was assessed using DNA micro-arrays. Salicylic acid and eugenol produced relatively weak responses, which could not be differentiated from the control treatment, whereas in the citronellal treatment, from 100 genes differentially expressed (fold change, FC > ±2) 90 genes were upregulated, including 37 genes involved in inflammatory response. In the propyl gallate and botanical extract treatments, 2955 and 414 genes, respectively, were identified as differentially expressed (FC > ±2) and are related to cell cycle and general cell maintenance. 87% of the genes modulated by the botanical extract were common to propyl gallate. 40 genes are modulated by both citronellal and propyl gallate. However, 35 of them show inverse regulation, i.e. the genes upregulated by citronellal are downregulated by propyl gallate and *vice versa*, indicating that these substances sensitize cells by different mechanisms. These results indicate that cells can show different gene expression responses to sensitizing substances and any set of diagnostic genes needs to consider genes involved in various pathways and biological functions.

I-11-280

Evaluation of SENS-IS®, an Episkin® based model for identifying chemical sensitizers

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In the context of the 2013 ban given by the EU Cosmetics Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance for the cosmetic industry. A range of different *in vitro* chemistry-based (DPRA, GSH reactivity) and cell-based methods (MUSST, hCLAT, Keratinosens) have been developed and we are currently evaluating some of them for their applicability to cosmetic ingredients and physicochemical diversity. Although these assays appear to be promising for hazard identification, potency assessment is still limited. Possible limitations may be linked to the metabolism that may differ between the models and native skin, to bioavailability, which is not considered in monolayer cultures, and to the danger signal that may be different in monolayers as compared to a natural tridimensional microenvironment.

ImmunoSearch developed SENS-IS, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®), thus providing a possible way to encompass these limitations and come closer to potency assessment. With the aim of evaluating the predictive capacity of this approach on a cosmetic ingredient constituted set, L'Oréal provided in a blinded manner a set of 40 proprietary as well as public domain chemicals that were assessed by ImmunoSearch. We present here the results of this study and will analyze the genomic signatures among chemical and ingredient classes.

I-11-281

Enterprise and university join efforts to develop *in vitro* alternative methods in Brazil

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Modifications in the regulation for cosmetics in Europe prohibiting animal tests for evaluating the sensitization potential of chemicals and new ethical standards of society compelled us to start developing *in vitro* alternative methods. In Brazil, regulatory boards, universities and society are also discussing alternative methods and their relevance for product development. To accelerate the Brazilian participation in that field of research, Natura Innovation and Products Technology Ltd and the Federal University of São Paulo joined efforts and established a partnership to study an *in vitro* model to replace an animal test. Our aim is to distinguish a sensitizer from a non-sensitizer substance using dendritic cell lines. In this way, 400 nM phorbol myristate acetate (PMA) was used to differentiate the human monocytic leukemia cell line (THP-1) and the human histiocytic lymphoma cell line (U937). After checking the pattern changes in the marker expression on THP-1 and U937 cells, such as CD14 and CD1a, differentiated cells were incubated with two strong sensitizers (dinitrochlorobenzene, p-phenylenediamine), three moderate sensitizers (methyl-chloro-isothiazolinone, methyl-isothiazolinone, cinnamaldehyde), two weak sensitizers (citronelal, citral) and two non-allergens (SDS, lactic acid). For each chemical, 5 concentrations were used in order to give a predicted cell viability range of 20-95%. Preliminary results showed that the expression pattern of CD86/CD54, as well as that of the secretion of IL-1β, IL-8, IL-18 differed depending on the chemical.

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I-11-293

Development of SENS-IS®, an Episkin® based model for measuring chemical sensitizer potency

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For the cosmetic industry, due to the ban starting in 2013 given by EU Cosmetics Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance. A number of assays has been developed and are currently under evaluation. However their ability to assess sensitization potency is limited. These limitations might be due to the use of monolayer culture and not native human skin. To overcome these limitations we developed SENS-IS, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®). The selection of biomarkers was done by analysis expression profiles of mouse ear skin treated with several sensitizers and irritants. These studies were completed by analysis of mRNA expression in suction blisters sampled from sensitized patients challenged with $NiSO_4$ or fragrance mix or SLS. The first selected panel of biomarkers was then further refined on monocytic cell lines challenged with chemical or on 3D reconstructed epidermitis. We will present here the results of these studies and will show the predictive capacity of this approach on a set of 50 chemicals selected from a panel of perfume ingredients.

I-11-301

Characterization of alcohol- and aldehyde-deshydrogenase activities in normal human skin compared with reconstructed human skin models

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Skin is considered the body's envelope and a physical barrier to its environment. However, it contains numerous metabolizing enzymes which give it a potential role in terms of metabolism and detoxification. The 7th amendment to the European Cosmetic Directive bans the use of animal testing to evaluate the efficacy and safety of new cosmetic ingredients. This policy has forced the cosmetic industry to develop reconstructed human skin models (skin models) as tools for alternative methods to animal experimentation. For this reason, the models need to be characterized and compared with normal human skin (NHS) in terms of metabolic capabilities. In this work, we characterized alcohol deshydrogenase (ADH) and aldehyde deshydrogenase (ALDH) activities. Previous studies showed that NHS and reconstructed human epidermis such as Episkin™, SkinEthic-RHE™ and the full thickness model of Episkin™ expressed several ADH and ALDH isoforms. Their global catalytic activities were quantified in NHS and skin models using cinnamyl alcohol and cinnamic aldehyde as substrates, respectively. Apparent V_{max}, K_m and ratio V_{max}/K_m (estimating metabolic clearances) were calculated for each tissue from metabolite measurements of dose effect studies. Results showed that in NHS and in skin models, ADH and ALDH enzymes are functional and that ALDH activity is more important than ADH activity (ratio V_{max}/K_m ALDH > ratio V_{max}/K_m ADH). To conclude, the skin models can be easily used to study the detoxification process of primary alcohols or aldehydes, considered as potential sensitizers, and define their levels of cytotoxicity at the skin level.

I-11-304

In vitro alternative for chemical allergenicity screening using plasmacytoid dendritic cells

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Human dendritic cells (DC) have been used as an alternative to existing animal models for contact sensitization. Such methods are necessary to comply with the ban on animal testing imposed by the Cosmetics Directive in the EU. We investigated whether normal human plasmacytoid DC (pDC) can be used to identify contact allergens. pDC were exposed to chemical allergens (n=49) or irritants (n=42) and the highest concentration that yielded >50% viability was used in the study. Allergens were identified based on the stimulation index (SI) calculated by the fold increase in CD86 expression levels. A material that had an SI ≥1.5 in at least 50% of the pDC donors (n=2-5 donors) was considered an allergen. Historical LLNA and human clinical data were available for 75 of the 91 materials. An SI of ≥1.5 fold was obtained for 41 of 43 allergens but not for 26 of 32 non-allergens. Based on the results, a prediction model was developed for chemical allergenicity. The pDC assay has sensitivity = 95%, specificity = 81%, and accuracy = 89%; these results were comparable to the standard LLNA assay. Transferability of the test method was evaluated using 7 test articles in 3 laboratories. The results showed all samples were correctly identified. In conclusion, the CD86 expression level in pDC appears to be a sensitive and specific predictor of allergenicity. These results will be submitted to ECVAM for inclusion in a formal validation study.

I-11-313

In vitro testing of contact allergens: Which cell types are suitable?

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Allergic contact dermatitis (ACD) is an adaptive inflammatory response of the skin triggered upon exposure to certain chemicals or metal ions. Since many ingredients in consumer products might exert allergenic potency, there is a need for appropriate screening and characterization of these chemicals. Still, up to now the identification of potential allergens completely relies on animal testing. Due to economical and ethical reasons, however, the development of *in vitro* test systems for identification of potential sensitizers is mandatory.

Since dendritic cells (DCs) play a pivotal role in the initiation of ACD, several attempts were made to use monocyte-derived DCs and, due to easier handling, cell lines with DC-like properties. However, only few investigations exist that focused on the suitability of different cell types for their use in *in vitro* test systems. In the present study we compared monocyte-derived DCs and monocyte-derived Langerhans cells with the widely applied cell lines Mutz-3 and THP-1, respectively.

Four known allergens were tested for their ability to alter the expression of several immunomodulating surface molecules. We used multicolor flow cytometry to detect differences in expression patterns of surface markers that were previously associated with cell maturation. In addition to the upregulation of CD86, we observed both a dose-dependent upregulation of programmed death ligand 1 (PD-L1) and a downregulation of the dendritic cell immunoreceptor (DCIR). While both monocyte-derived cell types displayed highly significant changes in the expression levels of these surface markers upon exposure to allergens, the corresponding changes observed in cell lines were much smaller.

I-11-329

Skin-sensitizing capacity and potency: pre-validation of an alternative two-tiered *in vitro* assay

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Due to new legislation, the use of *in vivo* models to assess the allergic potential of chemicals is under debate. Animal models are not capable of distinguishing skin sensitizers from non-skin sensitizers. Earlier studies show that production of IL-18 by NCTC2544 keratinocytes could provide an *in vitro* tool to discriminate between contact and respiratory allergens and irritants (Corsini et al., 2009). Spiekstra et al. (2009) developed an Epidermal Equivalent (EE) model that is capable of determining the irritating potency of a chemical by measuring the production of IL-1α. Since IL-18 cannot be a marker for irritating potency and IL-1α cannot be used to distinguish between skin sensitizers and non-skin sensitizers, a two-tiered approach was developed to predict whether a chemical is a potential skin sensitizer and how strong this chemical reacts.

Pre-validation of Tier 1: During the inter-laboratory transfer phase of the pre-validation, a total of 5 individual laboratories tested 4 different chemicals to distinguish the skin sensitizers from non-skin sensitizers. All 5 labs were able to recognize the non-skin sensitizer. False negative results showed that the readout of fold increase in IL-18 production, compared to the vehicle, might not be sufficient to recognize skin sensitizers. Therefore, using dose-response data might be a better option to use as readout.

Tier 2: Currently, 5 laboratories are working on the inter-laboratory transfer of the EE model. Two different skin sensitizers will be tested, i.e. 1 extreme and 1 moderate skin sensitizer, to determine whether the model can rank skin sensitizers according to their potency.

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I-11-332

VITOSENS: a mechanism-based *in vitro* assay for chemical-induced skin sensitization

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VITOSENS is an *in vitro* assay based on exposure-induced expression changes of gene transcripts in dendritic cells derived from $CD34^+$ progenitor cells in human cord blood. The assay was initially designed as a classifier and it is able to discriminate chemical skin sensitizers from non-sensitizers (Hooyberghs et al., 2008). Moreover, by combining different VITOSENS variables we were able to model an *in vitro* potency value that closely fits *in vivo*-derived data, and over the entire range from weak to extremely sensitizing chemicals (Lambrechts et al., 2010a). As such, the assay can provide valuable information in the context of chemical risk assessment. Finally, a reliable test system should be based on key elements of the *in vivo* disease process it is screening for. We demonstrated the functional relevance of the *in vitro* VITOSENS gene markers. In a first step we proved their differential protein expression (Lambrechts et al., 2010b) and in a second phase we evaluated changes in DC maturation after pharmacologically counteracting the sensitizer-induced activity of the markers (Lambrechts et al., in press). In conclusion, these results point to the feasibility of applying VITOSENS as a mechanism-based *in vitro* assay to classify chemicals according to their inherent sensitizing risk. The assay can represent the antigen-presenting aspect in an integrated approach towards an alternative for skin sensitization testing.

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I-11-334

Development of PEPT-IS®, a peptide-binding based assay for assessing chemical sensitization using lipocalin derived peptides

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For the cosmetic industry, due to the ban staring in 2013 imposed by EU Cosmetic Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance. A key step in the skin sensitization process is the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. A number of assays have been developed and are currently under evaluation, but their ability to predict weak sensitizers is poor. To augment sensitivity of the assay we selected a peptide from skin-expressed lipocalin proteins. The lipocalin protein family is a large group of small extracellular proteins with the ability to bind a range of small hydrophobic molecules that bind to specific cell-surface receptors and form complexes with soluble macromolecules. In the context of sensitization, lipocalins display a number of interesting characteristics. Members of the lipocalin family, odorant binding protein, bind a number of sensitizers, numerous animal-derived allergens are lipocalins and, finally, we observed that NGAL or lipocalin 2 is specifically expressed in skin challenged with sensitizers. We selected a peptide from a conserved region that contains homologies with the epitopes recognized in animal allergens and that contains cystein and lysine amino acids. Using this peptide we developed PEPT-IS®, a peptide binding assay using a single peptide with 4 h incubation. We will present here the results of these studies and will show the predictive capacity of this approach on a set of 30 chemicals.

I-11-338

Gene profiles of a bronchial epithelial cell line (BEAS-2B) induced by exposure to low-molecular weight chemicals

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Being the first cells that encounter xenobiotics that enter the body through inhalation, the major function of the airway epithelium was once thought to be that of a physical barrier. However, the epithelium also responds actively to changes in the external environment, e.g. the presence of low-molecular weight (LMW) chemicals, by secreting a large number of molecules and mediators that signal to cells of the immune system. To obtain more insight into the role of the respiratory epithelium on a molecular level, gene profiles of a bronchial epithelial cell line (BEAS-2B) were identified after exposure to a panel of LMW chemicals. BEAS-2B cells were exposed during 6, 10, and 24 h to a panel of 18 LMW chemicals (i.e. 9 respiratory sensitizers, 4 irritants, and 5 skin sensitizers). Overall changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide microarrays. Analysis of the identified gene profiles were performed by means of GOFFA (gene ontology for functional analysis) and pathway analysis tools. Furthermore, Fisher Linear Discriminant Analysis (LDA) was used to reveal gene signatures that can discriminate between different chemical classes related to challenge specific properties.

This work was partly funded by the EU FP6 Integrated Project Sens-it-iv (LSHB-CT-2005-018861) aiming at the development of novel strategies for *in vitro* assessment of allergens (www.sens-it-iv.eu).

I-11-386

Integrated adaptive testing strategy for skin sensitization assessment

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Bayesian Network Integrated Testing Strategy (ITS-1) pilot phase showed potential to be a useful tool for decision making with alternative test information and offered novel insights. In a next generation ITS, ITS-2, the aim was to confirm findings from the pilot phase using a significantly enlarged data set and to develop a practical, optimal testing strategy for skin sensitization hazard testing. To this end, we continue to combine *in silico*, *in chemico* and *in vitro* data related to skin penetration, peptide reactivity, activation of Nrf2-dependent gene activity and dendritic cell activation. However, we have introduced several changes. We have extended the number/modified input tests as they have evolved over time. For example, we evaluated the value of the newly developed peroxidase peptide reactivity assays that consider metabolic activation and report the results based on dose-response with one of the existing direct peptide reactivity assays. We have replaced the AREc32 assay with the more standardized KeratinoSens assay. In addition, we have included cytotoxicity as a cofactor that may be an indicator of effects related to danger signal formation / local trauma thought to affect sensitizer potency. We also include mechanistic evidence, such as reactivity domain characterization, which is not directly related to potency yet as an important co-factor in potency determination. In this way we have started the evolution from an integrated testing strategy towards an intelligent testing strategy that will rely on increasing mechanistic evidence generated with systems biology data.

I-11-397

Predicting sensitizing potential of cosmetic ingredients: enlargement of the applicability domain of the MUSST assay by using complementary U937-based assays

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Allergic contact dermatitis resulting from industrial, environmental or domestic exposure to sensitizers is the most common manifestation of immunotoxicity in humans. Skin sensitization risk assessment and, more precisely, the sensitizing potential of ingredients used in the cosmetic and pharmaceutical industries so far essentially relies on available animal test methods, such as the mouse local lymph node assay. In the context of the 7th amendment to the Cosmetic Directive as well as the recent EU-legislation on chemicals (REACH), the cosmetic industry is particularly concerned by the challenge of finding *in vitro* alternatives to assess the sensitizing potential of chemicals.

Contact sensitizers induce several phenotypic and functional changes on dendritic cells (DC) *in vivo* and *in vitro*. One of these changes, the induction of CD86, is the most frequently analyzed endpoint for the *in vitro* prediction of contact sensitizers using different cellular models based on DC or human myeloid cell lines. We developed the Myeloid U937 Skin Sensitization Test (MUSST) based on the induction of CD86 on U937 cells. Years of in-house experience with this assay led us to identify its limits, and to develop further methods and further models (including 3D-models) to overcome these limits. We will describe here how an adequate use of CD86-mRNA test, U937-apoptosis assay and the Episkin-U937 co-culture assay can complement the MUSST assay to enlarge its applicability domain and thus to cover a larger physicochemical diversity encountered in cosmetic ingredients.

I-11-439

In vitro skin irritation testing of greasy and sticky substances

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Skin irritation evaluation is an important consideration for safety assessment and is therefore required by various regulatory authorities for notification and import of test substances. The objective of the present study is to improve the skin irritation test validated by ECVAM for plant extract testing.

We suggest that the washing step must be revised since the plant extracts are more lipophilic and that a positive irritancy result could be due to a longer time of exposition than to an irritancy potential itself. For that purpose, different ways of washing were tested in addition to controls other than those validated by ECVAM. Reconstructed human epidermis (RHE) Skinethic® samples were used, and were topically exposed to substances for 42 min. Then the RHE were washed normally (removing the excess of substance with a cotton-tip and 25 ml PBS) or specially (removing the excess of substance with a cotton-tip soaked with mineral oil, DMSO, or SDS 0.1% solution, then with 25 ml PBS). The parameters evaluated were viability (MTT test), IL-1α secretion (ELISA) and histology.

Two lipophilic controls were selected: Vaseline, as negative control and N Alkyl "suif" as positive control. RHE exposed to Vaseline have a weaker cell viability (-20%) than those exposed to PBS, highlighting that lipophilic substances could have a higher cytotoxic potential than hydrophilic substances. We tested different washes on these controls and concluded that the best washing procedure was with SDS 0.1%: it permits the effective removal of the excess of product from the surface without altering viability.

I-11-446

Updated NICEATM evaluation and international acceptance of the reduced murine local lymph node assay

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To minimize allergic contact dermatitis (ACD) occurrence, regulatory authorities require testing to identify substances with ACD potential. Such substances must be labeled with the hazard description and precautions necessary to minimize exposure. The murine local lymph node assay (LLNA) is an alternative test method for determining the ACD hazard potential of most types of substances and, compared to guinea pig tests, requires fewer animals, less time, and eliminates pain and distress. The reduced LLNA (rLLNA), which uses only the high dose, reduces animal use by a further 40% compared to the multidose LLNA. LLNA results from 1071 published and unpublished studies, representing 664 unique substances, were obtained. Accuracy for the rLLNA was 98.4% (1054/1071), with false positive and false negative rates of 0% (0/319) and 2.3% (17/752), respectively. These results reinforce ICCVAM's 2009 recommendation (which was based on 471 LLNA studies) that the rLLNA be routinely considered before conducting the multidose LLNA, when dose-response information is not required. Based on the ICCVAM/NICEATM joint evaluation, the rLLNA was included in an updated version of the OECD Test Guideline for the LLNA (TG 429) that was adopted in 2010. The availability of this international TG will allow for global use of the rLLNA for regulatory testing, which is expected to significantly reduce animal use for ACD hazard testing while supporting the protection of human health.

The views above may not represent the official position of any government agency. ILS staff supported by NIEHS contract N01-ES-35504.

I-11-456

Effect of skin barrier function and metabolic ability on the concentration-distance profiles of chemical compounds in reconstituted cultured human skin models

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Reconstituted cultured human skin models (RMs) have been used for *in vitro* skin corrosion/irritation tests. However, false-negative and false-positive reactions were obtained for the tests with several chemical compounds. We have already reported that the skin viability (skin irritation) showed a fairly good relationship with skin concentration of chemical compounds. In the present study, therefore, we examined reasons for the false-positive or -negative reactions under the assumption that the main reasons would be differences in concentration-distance profiles of the compounds between RMs and human skin.

An *in vitro* skin permeation experiment was performed to obtain permeability coefficients through whole skin (P_s) and stratum corneum-removed skin (P_{ved}). Several hydrophilic compounds and ethyl nicotinate as a model ester compound were used. Obtained esterase activity parameters (K_m, V_{max}) were compared between RMs and human skin.

The permeability coefficient ratios (P_{ved}/P_s) of hydrophilic compounds in RMs were much lower than those in human skin, although Ps value in RMs was almost the same in human skin. This result suggests that viable epidermis has high barrier function, as in the stratum corneum, for the RM permeation of hydrophilic compounds, which is a reason for the false-positive reaction. Esterase activity (V_{max}/K_m) in RMs was much lower than in human skin. False-negative results in RMs may be obtained when the parent compounds have skin irritation properties. Thus, differences of concentration-distance profiles of compounds between RMs and human skin must be considered to explain false-positive and -negative reactions in skin irritation tests.

I-11-458

Establishment and characterization of *in vitro* skin models mimicking hallmarks of atopic skin

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Atopic dermatitis (AD) belongs to the major health problems in the industrialized world with currently 10-20% of the children and 1-3% of the adults being affected. Loss-of-function mutations in the filaggrin gene (FLG) are a strong predisposing factor for AD, although its relevance for the pathomechanism is not yet understood in full. The generation of an *in vitro* model which exhibits hallmarks of atopic skin would allow for further evaluation of underlying pathogenetic mechanisms, for testing of new treatment options, and for toxicological studies in a simple, fast and cheap way.

In this study we have knocked down FLG expression in normal, human keratinocytes and investigated its impact on epidermal maturation and on the response to skin irritation in 3D skin models.

Histopathological evaluation showed disturbed epidermal differentiation and maturation in the knock down model. In contrast, in healthy tissues all relevant dermal structures were developed nicely. Moreover, skin irritation induced by an application of sodium dodecyl sulphate resulted in significantly higher LDH-leakage and IL-6/-8 levels ($p<0.001$) in the knock down models. This is well in accordance with the *in vivo* situation where the skin of an atopic patient shows higher susceptibility to skin irritation compared to non-atopic individuals. This study clearly demonstrates that deficiencies in FLG expression considerably impair skin barrier development and trigger skin irritation based on inflammatory responses. This FLG knock down construct is a first step towards the development of atopic-like skin *in vitro* model.

I-11-535

Retrospective analysis of the EpiDerm 3-minute prediction model for assessment of GHS skin corrosion packing group sub-category 1A

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OECD has adopted several ECVAM-validated reconstructed human skin models (EpiDerm and EPISKIN/SkinEthic) for testing skin corrosion (OECD TG 431). However, TG 431 does not satisfy international (GHS) labeling guidelines for transport of dangerous goods. GHS package labeling guidelines utilize 3 corrosion sub-categories (1A: very dangerous, 1B: medium danger and 1C: minor danger). Labeling a chemical as sub-category 1A has important consequences, including very small volume package limits for air transport, prohibition from passenger aircraft, protective storage conditions, costly containers and low market acceptance. Animal tests are still utilized for assessing the 1A label requirement. An *in vitro* method that discriminates 1A from 1B/1C classes will therefore have a substantial impact on reducing animal tests for this purpose. The current poster evaluates data obtained with the EpiDerm model for ability to discriminate between GHS 1A and 1B/1C classes. Data obtained from 49 chemicals tested during the ECVAM Phase I validation study plus 17 additional previously tested chemicals were retrospectively analyzed based on the MTT viability assay (50% viability cutoff) and the 3 minute exposure period. The combined set includes 15 1A, 25 1B/1C, and 26 non-corrosive chemicals. The 3 min prediction model is shown to produce a sensitivity of 93% (14/15) and overall specificity of 76% (39/51) for predicting sub-category 1A. Testing of additional chemicals (ECVAM Phase III validation study) indicates that data correction for direct MTT-reducing chemicals is important. Adoption of the 3 min EpiDerm prediction model would lead to a significant reduction in animal use for corrosion sub-group package labeling.

I-11-542

Functionality and specificity of gene markers for skin sensitization in dendritic cells

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Dendritic cells (DC) are sentinel players in this immunological cascade of skin sensitization. Using transcriptomic analyses, we recently revealed a discriminating gene expression profile in human CD34$^+$ progenitor-derived DC after exposure to skin sensitizers versus non-sensitizers. Starting from the differential expression in a small set of genes, a preliminary classification model (VITOSENS®) has been developed to identify chemicals as (non-)sensitizing in an animal-sparing approach.

The objective of the current study is to gain knowledge on the intracellular mechanism of the VITOSENS® assay. To this end, we investigated the role of the markers in the DC maturation process, and compared their activation profile by a skin sensitizer *versus* a non-sensitizing danger molecule.

To evaluate the functional relevance of VITOSENS® biomarkers in DC maturation, their response induced by the sensitizer dinitrofluorobenzene (DNFB) was pharmacologically counteracted. Flow cytometry analyses revealed that CD86 was down-regulated after COX2 inhibition, whereas expression of HLA-DR was reduced by stimulating CCR2. When exposing DC to DNFB *versus* lipopolysaccharide S (LPS), expression of the most discriminating genes, CREM and CCR2, was not altered by LPS as opposed to DNFB.

To summarize, the observations in this research indicate that a selection of the VITOSENS® genes may be functionally involved in the intracellular pathway of sensitizer-induced DC activation. By comparing their responsiveness towards a non-sensitizing danger signal and a sensitizer, VITOSENS® gene markers CREM and CCR2 appear to display a specific response.

I-11-548

In vitro assessment of skin irritation potential of surfactant-based formulations using 3D skin-reconstructed tissues and cytokine expression analysis

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The skin compatibility and safety of surfactant-based cleansers is critical in personal care products. It is desirable to minimize the dermal irritation and disruption of the skin barrier that can be caused by surfactants. We find that reproducible *in vitro* systems can accurately assess the irritation potential of the products, thus avoiding the use of animal testing. The three-dimensional EpiDerm™ model (MatTek Corp.) provides a testing platform for skin irritation assessment.

The potential dermal irritation of over 150 amphoteric and/or anionic surfactant systems was evaluated by MTT viability and IL-1α release. Diluted to 10% in water, formulations were applied onto the surface of 3D tissues for 1 h, followed by 24 h post-exposure analysis for cytokine expression. Transepidermal water loss (TEWL), the flux of water through the skin, is used clinically to assess skin barrier impairment following topical application; increased TEWL is indicative of an impaired skin barrier. An exaggerated patching model on subjects with impaired barrier due to atopic dermatitis was used to clinically assess formulation impact on skin barrier function. A correlation is found between the *in vitro* assay and *in vivo* clinical results. Also, the structure of the hydrophobic tail group of surfactant is observed to be important to surfactant mildness. The IL-1α release from coco betaine was significantly greater than that of cocamidopropyl betaine, despite both surfactants having the same hydrophilic head group. Likewise, sodium lauroyl methyl isethionate was significantly more irritating than sodium cocoyl isethionate, a mild surfactant.

I-11-567

Evaluation of the murine local lymph node assay (LLNA) for potency categorization of chemicals causing allergic contact dermatitis in humans

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ICCVAM and NICEATM jointly evaluated the usefulness and limitations of the LLNA to determine potency categorization of chemicals that may cause allergic contact dermatitis. The dose per unit skin area that induces a 5% positive response rate in the human maximization test or human repeat-insult patch test was used as the human induction threshold. Substances with induction thresholds ≤500 $\mu g/cm^2$ were classified as "strong" human sensitizers. The extent to which the LLNA EC3 (estimated concentration expected to produce a stimulation index of 3) correctly categorizes strong human sensitizers was evaluated using 136 substances with both LLNA and human data. Using EC3 ≤2%, the criterion adopted by the GHS, correctly categorized 52% (14/27) of the strong human sensitizers. However, nearly half (48% [13/27]) of the strong human sensitizers had an EC3 >2% (11/27) or were negative in the LLNA (2/27). ICCVAM concludes that the LLNA can be used to categorize substances as strong sensitizers when EC3 ≤2% but cannot be used as a stand-alone assay to determine sensitization potency categories. Additional information is required to categorize substances as other than strong sensitizers when EC3 >2%. To improve the accuracy of the LLNA for identifying strong sensitizers, ICCVAM encourages the development and evaluation of integrated decision strategies that consider other relevant information such as quantitative structure-activity relationships, structural alerts, peptide reactivity, *in vitro* data, human data or experience, and existing data from similar chemicals.

The views above may not represent the official position of any government agency. ILS staff supported by NIEHS contract N01-ES-35504.

I-11-568

Evaluation of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis hazard potential of pesticide formulations

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ICCVAM and NICEATM jointly evaluated the usefulness and limitations of the LLNA for assessing the allergic contact dermatitis hazard potential of pesticide formulations. Most of the 104 formulations evaluated are water-soluble and were tested in an aqueous vehicle (1% Pluronic L92). Of the formulations for which LLNA and guinea pig (GP) data were available for the complete formulation (n = 23), the LLNA classified 52% (12/23) as sensitizers, while GP tests classified only 13% (3/23) as sensitizers, indicating a greater sensitivity for classifying sensitizers in the LLNA. All three formulations identified as sensitizers in GP tests were also LLNA sensitizers. The LLNA identified, as sensitizer, an additional seven substances that the GP tests classified as nonsensitizer, an overprediction rate of 50% (10/20). Based on these data, ICCVAM and an international independent peer review panel recommended that the LLNA could be used for testing pesticide formulations. This recommendation was forwarded to ICCVAM member agencies, which agreed on this expanded use of the LLNA. Several agencies also indicated that they would communicate the ICCVAM recommendations to stakeholders, and encourage appropriate use. OECD TG 429, updated in 2010, reflects the results of this evaluation, which should expand the use of the LLNA for allergic contact dermatitis hazard testing, as well as reducing and refining animal use.

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I-11-574

International acceptance of the nonradioactive LLNA: BrdU-ELISA for evaluating allergic contact dermatitis hazards

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ICCVAM and NICEATM jointly evaluated the nonradioactive LLNA: BrdU-ELISA, which measures the amount of BrdU incorporation into DNA of proliferating lymphocytes as an indicator of potential allergic contact dermatitis (ACD) hazards. Accuracy was calculated by comparing results to the traditional radioactive LLNA for 43 substances using different stimulation indices (SI) as decision criteria. SI ≥1.6 generated optimal performance; the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives) and 9/11 LLNA non-sensitizers (18% [2/11] false positives). The maximum SI for the two false positives ranged from 1.6-1.9. Eighteen substances had multiple LLNA: BrdU-ELISA tests: 85% (11/13) of the LLNA sensitizers and 60% (3/5) of the LLNA non-sensitizers were concordant. Based on these results, ICCVAM concluded that the accuracy and reliability of the LLNA: BrdU-ELISA supported its use for identifying potential ACD hazards and recommended SI ≥1.6, since there were no false negatives when compared to the traditional radioactive LLNA. Additionally, when dose-response information is not required or negative results are anticipated, ICCVAM recommended using a single-dose reduced LLNA: BrdU-ELISA, thereby reducing animal use by 40%. In July 2010, the LLNA: BrdU-ELISA was adopted by OECD as Test Guideline 442B. Availability of this international test guideline will allow more institutions to take advantage of the reduction and refinement benefits afforded by the LLNA since there is no requirement for radioactive reagents, obviating the hazards associated with their use and disposal.

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I-11-575

International acceptance of the nonradioactive LLNA: DA for evaluating allergic contact dermatitis hazards

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ICCVAM and NICEATM jointly evaluated the nonradioactive LLNA: DA, which measures ATP content as an indicator of lymphocyte proliferation, for identifying potential allergic contact dermatitis (ACD) hazards. Accuracy was calculated by comparing results to the traditional radioactive LLNA for 44 substances using different stimulation indices (SI) as decision criteria. SI ≥1.8 generated optimal performance; the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives) and 9/12 LLNA non-sensitizers (25% [3/12] false positives). The maximum SI for the three false positives ranged from 1.8-2.5. Fourteen substances had multiple LLNA: DA tests: 80% (8/10) of the LLNA sensitizers and 75% (3/4) of the LLNA non-sensitizers were concordant. Based on these results, ICCVAM concluded that the accuracy and reliability of the LLNA: DA supported its use for identifying potential ACD hazards and recommended SI ≥1.8, since there were no false negatives when compared to the traditional radioactive LLNA. Additionally, when dose-response information is not required or negative results are anticipated, ICCVAM recommended using a single-dose reduced LLNA: DA, thereby reducing animal use by 40%. In July 2010, the LLNA: DA was adopted by OECD as Test Guideline 442A. Availability of this international test guideline will allow more institutions to take advantage of the reduction and refinement benefits afforded by the LLNA since there is no requirement for radioactive reagents, obviating the hazards associated with their use and disposal.

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I-11-579

Towards animal-free testing for skin sensitization: in-house validation of four methods: MUSST, h-CLAT, KeratinoSens and DPRA

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Allergic contact dermatitis is induced by repeated skin contact with an allergen. Assessment of the skin sensitizing potential of chemicals, agrochemicals, and cosmetic ingredients is crucial to define their safe handling and use. Up to now, animal tests have been used to identify skin sensitizing potential. Animal welfare as well as the 7th Amendment to the Cosmetics Directive and REACH demands animal-free alternatives. The mechanisms of induction and elicitation of skin sensitization are complex. To account for the multitude of events in the induction of skin sensitization an *in vitro* test system will consist of a battery of various tests.

Currently, we perform in-house validations of four *in vitro* assays addressing three different events during induction of skin sensitization: 1) The peptide reactivity assay (DPRA, 1) using synthetic peptides and HPLC analysis; 2) Two dendritic cell based assays on the cell lines U937 (MUSST) and THP-1 (h-CLAT) and flow cytometric detection of the maturation markers CD54 and/or CD86 (2, 3); 3) ARE-dependent gene activity in the reporter gene cell line KeratinoSens™.

We present the results of these assays with more than 40 substances of known sensitizing potential including the performance standards defined for the LLNA. The sensitivity, specificity and accuracy of individual tests were obtained by comparison to human epidemiological data as well as to data from the local lymph node assay.

I-11-580

Determination of dimethylfumarate and analogues sensitization potential in *in vivo* and *in vitro* models

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Dimethyl fumarate (DMFu) was used as a preservative in imported house furniture such as sofas, chairs but also shoes or socks. Since 2006, this product has been implicated in many cases of skin sensitization in humans. Several thousands of consumers throughout Europe were affected, presenting weak to severe symptoms. The French health authorities have planned epidemiological and biological investigations. The aim of biological assays was to evaluate DMFu and analogues' sensitizing potential using *in vivo* and *in vitro* models. Several *in vivo* reference methods are available: the guinea pig maximization test, mouse ear swelling test and the local lymph node assay (Thy-H^3-LLNA, OECD guideline 429). In accordance with the 3R rules, the latter one was chosen to evaluate sensitizing potential of DMFu and 5 analogues: 2 fumarates: diethyl fumarate (DEFu), monomethyl fumarate (MMFu) and 3 maleates: diethyl maleate (DEMa), dimethyl maleate (DMMa), dibutyl maleate (DBMa). The efficient concentration (EC_3) was determined for each product and allowed classification. Our results confirm the strong sensitizing potential of DMFu (EC_3=0.2%). In addition, they show that all tested analogues are either moderate (DBMa, DEMa) or strong (DEFu, DMMa, MMFu) sensitizers. These molecules were also tested using the h-CLAT assay (method under pre-validation at ECVAM). H-CLAT data are in agreement with LLNA; all the products are sensitizers, DMFu being the strongest. In conclusion, this study allows the precise determination of the sensitization potential of DMFu and analogues. Also, it favours the direct comparison of *in vivo* and *in vitro* approaches.

Session I-12: Epigenetics and its increasing relevance in toxicology and risk assessment

Session I-12: Oral presentations

I-12-718

An introduction to epigenetics

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The term "epigenetics" was coined in the 1940s and denotes the heritable changes in the phenotype or gene expression patterns caused by mechanisms other than changes in the underlying DNA sequence. Since its introduction, the epigenetics research field has experienced a true boost, particularly in the last two decades, with the in-depth characterization of the determinants of the epigenome and the establishment of cutting-edge technology to study these features as milestones. The major drivers of the epigenetic machinery, including DNA methylation, histone modifications and regulation by non-coding RNA species, will be briefly discussed in the current presentation. Furthermore, the increasing relevance of epigenetics for both the study and practice of toxicology will be illustrated.

Session I-12: Poster presentations

I-12-129

Computational modelling and the reduction of animal testing in toxicology studies

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Cytochrome P450 enzymes (CYP450) are central to drug metabolism, carrying out the metabolism of 75% of known drugs in current clinical use. Important effects such as adverse drug reactions and genetically determined differences in drug toxicity and efficacy depend on CYP450 activity, yet this cannot be easily predicted from protein structures alone and is often found through the use of animal models.

Computational methods allow for a better understanding of the molecular determinants of reactivity and specificity, potentially contributing significantly to both drug development, assisting in the prevention of the growing and expensive problem of late stage drug failures (which often occur due to CYP450 mediated ADME-Tox properties), and in the reduction (and eventual replacement) of animal testing at this stage of drug development.

Our aim is to provide validated computer models of the CYP450 enzymes that can be used in *in silico* screening of new drug molecules, thereby greatly reducing the need for animal testing in toxicity studies. By using 3D homology modelling and

molecular docking, we are investigating the species difference observed for rat and human on the binding of the two stereoisomers of well-known inhibitors of CYP450 2D6, quinidine and quinine. By investigating the structural and electronic features that determine selectivity in these reactions, we hope to properly evaluate the reliability of animal models in predicting human ADME-Tox properties while also creating a series of simple descriptors for the accurate prediction of species selectivity and human ADME-Tox properties, reducing animal testing in the pharmaceuticals industry.

I-12-566

Stabilisation of primary hepatocyte cultures via interfering with epigenetic control mechanisms

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Hepatocyte-based models have become standard *in vitro* tools to evaluate pharmaco-toxicological characteristics of compounds. However, primary hepatocytes in culture cope with the progressive deterioration of their specific *in vivo* phenotype, including xenobiotic biotransformation capacity, which largely restricts their application to short-term *in vitro* studies. To stabilize hepatocyte cultures, scientists have tried to mimic the natural hepatocyte micro-environment in culture through the use of extracellular matrix components, adding soluble medium components or co-culturing with other cell types. However, this has only led to a slight improvement of the viability and the preservation of the differentiated phenotype. Therefore, new strategies need to be explored. Since epigenetic mechanisms such as histone acetylation and/or DNA methylation play a predominant role in the regulation of hepatic gene expression, interfering with these pre-transcriptional processes could aid in developing a long-term hepatocyte model for *in vitro* testing and screening purposes. Indeed, we were the first to show that inhibitors of these processes, including Trichostatine A (TSA) and 5'-aza-2'-deoxycytidine, respectively, (synergistically) cause proliferative blocks, counteract spontaneous apoptotic cell death, and promote functional and morphological differentiation of primary hepatocytes in culture. Moreover, it was recently also found that TSA up- and down regulates microRNA (miR)-379 and miR-122, and miR-143, respectively, which all could probably be related to the inhibitory effects of TSA on hepatocellular proliferation. In conclusion, our data indicate that classical epigenetic regulators either alone or in combination with modulators of miRNA species, represent innovative tools to develop more stable and functional primary hepatocyte cultures.

I-12-589

A test strategy to detect developmental toxicants that affect neural development using human embryonic stem cells

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Developmental neurotoxicity (DNT) is caused by exposure to toxicants during sensitive periods of neurodevelopment and can lead to cognitive, sensory or behavioural deficits, persisting long after removal of the original stimulus. How the "memory" of the early developmental exposure is stored is unclear yet, and information on developmental neurotoxic effects of most chemicals is still very sparse. To address these issues, we established a test system based on human pluripotent cells with the potential to differentiate towards neural cells. We profiled not only the changes of mRNAs, but also of miRNAs and obtained a profile of a large set of chromatin modifiers. The large changes in the expression of chromatin modifiers were corroborated by altered nuclear staining patterns of histone modifications. To test the sensitivity of the earliest phase of neurodevelopment to epigenetic modifying chemicals, we used human embryonic stem cells, which were differentiated to Pax6-positive neural precursor cells. In this system we investigated the role of various signalling pathways for neural differentiation and how toxicants might interfere with those pathways. The cells were also exposed to epigenetic modulators. As read-out, we used mRNA expression levels of markers specific for certain neurodevelopmental stages and flow cytometry of a reporter gene, and identified pronounced developmental toxicity in the absence of cytotoxicity.

Session I-13: Toxicity testing in the 21st century

Session I-13: Oral presentations

I-13-679

Tox21 Special Session at the 8th World Congress

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The Toxicology in the 21st Century (Tox21) program is an ongoing collaborative effort among four U.S. Government agencies: the National Toxicology Program (NTP) of the National Institute of Environmental Health Sciences, the Environmental Protection Agency (EPA), and Food and Drug Administration (FDA), and the National Institutes of Health Chemical Genomics Center (NCGC). Tox21 is developing and deploying a wide range of high-throughput *in vitro* biological testing and computational technologies to identify the activities and mechanisms of action of thousands of chemicals, with the goal of providing a science- and data-driven basis for *in vivo* chemical testing prioritization and risk assessment. After a pilot phase that began in 2005 and Tox21 Phase I that began in 2008, the Tox21 program entered Phase II in March 2011 with the completion of a testing library of 11,000 environmental and pharmaceutical chemicals, a dedicated robotics system capable of testing the entire Tox21 library in triplicate 15-concentration quantitative high-throughput screening (qHTS) format across a different *in vitro* assay every week, informatics databases and algorithms to analyze, visualize, and model the data, and targeted testing paradigms to examine the predictive and *in vivo* relevance of the models created. Tox21 also has a robust technology development component focused on the enumeration of all potential toxicity pathways, incorporation of metabolism and cell-cell interactions into *in vitro* assays, and the incorporation of exposure information into the models developed. Strategies and progress in all of these areas will be presented.

I-13-680

Tox21: Activities of the U.S. National Toxicology Program (NTP)

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In 2008, the National Institute of Environmental Health Sciences/NTP entered into a Memorandum of Understanding with the NIH Chemical Genomics Center and the Environmental Protection Agency's National Center for Computational Toxicology on the research, development, validation, and translation of new and innovative *in vitro* and lower organism test methods that characterize key steps in toxicity pathways. This collaborative effort, known informally as Tox21, was expanded in 2010 with the addition of the U.S. Food and Drug Administration. In support of Tox21, the NTP has (1) produced a large library of environmentally relevant compounds for screening across toxicity pathways; (2) identified and/or supported the development of assays suitable for use in quantitative high throughput and high content screens (qHTS, qHCS); (3) established a WormTox Screening Facility with the goal of developing toxicological assays using the nematode *Caenorhabditis elegans*; (4) developed statistically-based approaches for distinguishing between active, inactive, and inconclusive responses in these screens and informatic tools for identifying predictive toxicity patterns; (5) expanded the NTP's publicly accessible Chemical Effects in Biological Systems (CEBS) database to contain all Tox21-related data as well as the NTP historical data; (6) conducted qHTS studies to probe mechanisms of inter-individual susceptibility to toxicants; (7) evaluated next generation molecular tools for mining the formalin fixed, paraffin embedded animal tissues in the NTP Tissue Archives for predictive gene signatures; and (8) supported assay and informatic developments through the NIEHS Small Business Innovative Research contract award process. Advantages and limitations of these activities will be presented.

I-13-681

Development of an integrative approach for the prediction of systemic toxicity: Combination of cell toxicity, pharmacological and physical chemical properties

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Ethical, scientific and economic constraints have motivated the scientific community to develop alternatives to animal testing. Developing alternatives for acute/chronic systemic toxicity testing represents a challenge because of the complex biological processes implied. A realistic approach could rely on the combination of data generated for multiple endpoints. The Ctox panel®, which is a multiparameter cell-based *in vitro* system for predicting rat acute systemic toxicity, is a typical example. Preliminary studies conducted in a blinded manner showed a good sensitivity and specificity (91% and 78%) while defining a LD_{50} threshold at 2000 mg/kg. However, the model failed to accurately predict very toxic chemicals displaying (LD_{50} below 300 mg/kg). Further to an in-depth analysis of the misclassified chemicals, we concluded that both pharmacological data (for the reduction of false negatives) and physical-chemical properties (for the reduction of false positives) had to be considered. The modified approach was applied to 76 non-proprietary compounds previously tested with the standard method. A significant improvement in the prediction of the GHS categories was observed. Indeed, 75% of the chemicals pertaining to GHS 1, 2 and 3 were correctly classified, compared to 50% with the standard model. In addition, at an arbitrarily defined LD_{50} threshold of 500 mg/kg, the sensitivity and specificity were 85% and 89% with the new model against 71% and 83% with the standard model. Future directions will consist of challenging the newly built model with a new set of chemicals and foreseeing the application of such a strategy for repeated dose toxicity.

I-13-682

AXLR8 strategic directions for development of alternatives in the EU

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Conventional approaches to toxicity testing and risk assessment are often decades old, costly and low-throughput, and of dubious relevance to humans. The call for a transition to a 21st century toxicity testing paradigm will require a robust understanding of the cellular response/toxicity pathways which that can lead to adverse effects when perturbed; appropriate *in vitro* systems to study chemical interactions at key targets along a pathway; and computational systems biology models to describe the underlying pathways as a basis for creating biologically realistic dose-response models.

The EU FP7 coordination support action project AXLR8 (=accelerate) aims to support the transition to a toxicity pathway-based paradigm for quantitative risk assessment and will: 1) organize a series of annual workshops to map research progress, gaps and needs in the FP6/FP7 program on alternative testing strategies. 2) Provide a range of tools and opportunities for enhanced interdisciplinary and international communication, coordination and collaboration in order to maximise the impact of available resources. 3) Work to streamline regulatory acceptance procedures to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems as they become available. 4) Produce annual progress reports on the state of the science, including recommendations on priority research and funding targets, in order to ensure a prominent role for European science in this rapidly developing global research area.

In 2010 and 2011 the first AXLR8 workshops (AXLR8-1 & AXLR8-2) have focused on progress made in the EU FP6/FP7 projects funded by the health theme of the DG RTD "*Alternative Testing Strategies: Replacing, reducing and refining use of animals in research*". The results of the discussions and recommendations of the AXLR8 Scientific Panel at the AXLR8-1 2010 workshop have been published in the AXLR8 Progress Report 2010. These results and the recommendations of the AXLR8-2 2011 workshop on a "Roadmap to innovative toxicity testing (ITT)" will be presented.

I-13-683

The OECD QSAR toolbox

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The OECD QSAR Toolbox is a software application intended to be used by governments, industry and other stakeholders to fill gaps in (eco)toxicity data needed for assessing the hazards of chemicals. The two main pillars of the system are (i) the knowledge-base for grouping chemicals into toxicologically meaningful categories and (ii) databases with measured physchem, fate, and toxicity data. The grouping engines allow selection of analogues accounting for underlying interaction mechanisms and metabolism. Read-across and trend analysis are used to predict the endpoint values for a target chemical. The data gap filling methods include also a library of QSAR models to estimate missing experimental values. Each estimated value can be individually justified based on category hypothesis, quality of measured data and computation method used for categorization and data prediction. As the rationales for analogues selection are often based on common mechanisms of action, good regulatory acceptance is expected for predictions provided by the Toolbox. Since October 2010 the OECD QSAR Toolbox version 2.0 is available for free and can be downloaded from the OECD website. Version 2 is available both as a distributed version and as a stand-alone version. This release is part of a four-year collaborative project between OECD, ECHA, LMC and other partners. The aim of this presentation is to elucidate the improvements of the main functionalities as well as the new features introduced in version 2 of the Toolbox.

I-13-684

ToxCast Update – predictive signatures and phase II

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Chemical toxicity testing is being transformed by advances in biology and computer modeling, and driven by the thousands of environmental chemicals lacking toxicity data and concern over animal use. The U.S. Environmental Protection Agency's ToxCast program aims to address these concerns by screening and prioritizing chemicals for potential toxicity using *in vitro* assays and *in silico* approaches. This project has evaluated the use of *in vitro* assays for understanding the types of molecular and pathway perturbations caused by environmental chemicals and to build predictive and systems models of *in vivo* toxicity. To date we have tested close to 1000 chemicals in over 500 high throughput screening (HTS) assays across multiple technologies utilizing human and other species genes, proteins, primary and cell lines. Chemicals displayed a broad spectrum of activity at the molecular and pathway levels. We saw many expected interactions, including endocrine and xenobiotic metabolism enzyme activity. Chemical bioactivity ranged across pathways, from no activity to affecting dozens of pathways. We found statistically significant associations between numerous pathways perturbed by chemicals at measured *in vitro* concentrations, and with *in vivo* doses resulting in chemical toxicity. Useful predictive and systems models for reproductive, developmental, and cancer pathways and endpoints have been developed. ToxCast and the Tox21 programs are providing HTS screening and prioritization based on predictive and systems models of toxicity, and meaningful data on thousands of environmental chemicals for guiding targeted testing of chemicals.

This abstract does not necessarily reflect Agency policy.

I-13-685

Taking a mode-of-action approach to designing a hepatotoxicity screening strategy using the HepaRG cell model and high content imaging

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The liver is central to the metabolism of xenobiotics and faced with harmful effects of toxic substances. Evaluating the risk of liver toxicity is a major issue and there is still no established *in vitro* screening strategy to reliably identify potentially hepatotoxic chemicals. In the approach described here, a mode-of-action targeted analysis of the literature has been used to identify toxicity pathways and the key biological events associated with them. This knowledge has then been used to design a multiparametric HTS experiment to classify chemicals based on their likely association with a specific mode-of-action.

We used a metabolically competent cellular model, HepaRG, and high content imaging implemented on a HTS platform. The HepaRG cell line expresses the major liver functions, including P450s, phase II enzymes, transporters and nuclear receptors at levels comparable to those found in primary hepatocytes.

The high content screening approach we adopted is based on automatic analysis of image-sets acquired with an epifluorescent microscope for the quantification of immuno-fluorescently stained biomarkers expressed by treated HepaRG cells. A quantitative high throughput screening format was employed using a 96-well plate format, which facilitated the testing of a set of 92 reference chemicals and drugs with known hepatotoxic activity. Multiple cellular phenotypic changes were analysed by staining with fluorescent dyes for identification and quantification of response parameters. A biostatistical model was then developed to associate the test chemicals with different mode-of-action based categories. A systematic comparison of the classification results with literature findings allowed a preliminary validation of the approach.

I-13-686

Virtual Embryo: Systems modeling in developmental toxicity

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High-throughput screening (HTS) studies are providing a rich source of data that can be applied to chemical profiling to address sensitivity and specificity of molecular targets, biological pathways, cellular and developmental processes. EPA's ToxCast project is testing 960 unique chemicals (drugs, pesticides, etc.) in over 500 distinct assays, testing for diverse biochemical activities, receptor binding activities, reporter gene activation and gene expression profiles, stress-response indicators, and perturbation in cell state and cellular function. Also included are assays to monitor effects in zebrafish embryos and pathways of differentiation in mouse embryonic stem cells. *In vitro* profiles (AC_{50} in μM) are compared using machine-learning algorithms to identify patterns of biological activity and optimal feature selection for predictive modeling. Early findings suggest that developmental toxicity does not emerge from a simple molecular stream. Because many cells in a system interact to generate emergent properties (growth, patterning, homeostasis, robustness), computer models are needed to capture the complexity of multicellular networks and the key events leading to dysmorphogenesis. A predictive Virtual Embryo framework utilizes detailed knowledge to build computational models that run a morphogenetic series of events and can analyze the complexity of developmental processes. Potential regulatory applications are to inform and guide application QSAR models for predicting developmental effects; extract and organize literature for information relevant to developmental processes and defects; standardize *in vitro* and HTS data for predictive modeling of the disturbances to developmental processes; prioritize environmental chemicals for targeted testing; and systems modeling to analyze key pathways and mechanisms.

This abstract does not reflect EPA policy.

I-13-687

Integrated approaches to testing and assessment: The expert panel on the integrated testing of pesticides

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Canada relies on well-established approaches to assess the safety and efficacy of chemicals and pharmaceuticals, including pesticides. Alternative testing strategies represent an exciting opportunity for the development of tests that can evaluate a larger number of compounds, in systems that may more reliably predict potential adverse effects in humans. This approach also has the potential to identify the cellular mechanisms that may be the root cause of adverse effects. These new approaches may reduce the reliance on animal-based test systems and increase the reliability and efficiency of testing, while maintaining the highest levels of scientific rigor.

The Pest Management Regulatory Agency of Health Canada requested that the Council of Canadian Academies convene an expert panel to evaluate the use of integrated approaches to testing and assessment for the regulatory risk assessment of pesticides. Specifically, the 15-member panel was asked to address the following questions:

- What is the current status of the use of integrated testing strategies by regulatory agencies around the world?
- What is the state of the science of integrated testing strategies?
- What are the potential impacts on the public's perception and confidence of IATA for pesticides?

Pesticide formulations represent, on the one hand, one of the most data-rich chemical groups in the field of regulatory toxicology while, on the other hand, one of the most data-poor. To this end, they make an excellent model, both for the development and evaluation of new testing protocols and as a validation tool against which alternative testing strategies can be assessed.

Session I-15: Shellfish toxin testing: How are the Three Rs being progressed in this field?

Session I-15: Oral presentations

I-15-240

Evolving from the mouse to the optoelectronic mouse for phycotoxin analysis in shellfish

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Regardless of ethical and technical concerns, for phycotoxin analysis in shellfish the mouse bioassay remains the reference method of analysis for the global monitoring of diarrheic and paralytic shellfish poisoning toxins. Alternative methods of detection have been described but to date each analytical method is specific for a particular toxin and its chemical analogues, with each group of toxins requiring separate analysis. An ideal scenario for the monitoring of phycotoxins would be to evolve multiple toxin detection onto a single, easy to use *in vitro* platform.

Surface plasmon resonance biosensor technology has been demonstrated as a highly promising bioanalytical tool. This technology offers rapid real time detection requiring minimal amounts of toxin standards which is crucial because of their limited availability. A micro-fluidic immobilization device and prototype multiplex SPR biosensor designed for the detection of up to 16 molecular binding interactions in a 4 line by 4 channel array on a single chip has been utilised. This dual system was evaluated in its ability to be fit-for-purpose for the simultaneous detection of three important phycotoxin groups. Domoic acid, okadaic acid and saxitoxin calibration curves in shellfish were achieved in separate flow channels with detection limits of 4000, 36 and 144 μg/kg of mussel, respectively. The assay was designed to achieve detection below recognised regulatory action levels. This "optoelectronic mouse" detection system exhibits enormous potential for multiple phycotoxin screening as an alternative to the mouse bioassay with the additional benefit of being able to distinguish between toxin families in a single analysis.

I-15-431

Regulatory and methodical shortcomings in assessment of marine biotoxins in fish and shellfish

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As aquatic food already represents over 40% of the globally distributed animal food products, the toxicological risk assessment of these products becomes more and more a matter of special importance.

The gold standard to assess toxins in aquatic food has traditionally been and remains to date the mouse bioassay. Besides ethical issues of the *in vivo* bioassays there are specific associated problems with these *in vivo* assays, e.g. inter-species

comparability and intra-species variability. Thus there is an exigent need for more robust, sensitive, and reliable tools for toxins detection that have adequate predictivity in human risk evaluation. Responding to the growing alertness regarding this situation the European Food Safety Authority (EFSA) recommends LC-MS (Liquid Chromatography coupled Mass Spectroscopy) as a substitute for the *in vivo* bioassays for almost all classes of marine toxins.

LC-MS is a quantitative analytical method which is dependent on the availability of standardized reference toxins. However there are neither sufficient standards covering the known toxin structures nor is it foreseeable when there will be enough information on the wide range of analogues and released intermediates. Notably, for this reason the reference laboratories in this field will carry on performing *in vivo* bioassays.

This presentation will discuss the existing alternative methods as tools for risk assessment this field.

I-15-559

Removing the mouse from shellfish toxin testing – Fifteen years of the Three Rs

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Until recently, the only method acceptable in EU law for detection of the marine biotoxins paralytic (PSP) and diarrhetic shellfish (DSP) poisoning in shellfish harvested for human consumption have been rat or mouse bioassays (MBAs). MBAs cause substantial suffering to animals and have a number of potentially serious limitations including variability and false negative and positive results. It is estimated that around 400,000 mice are used annually for marine biotoxin monitoring in Europe.

Over the last 15 years, UK laboratories have used a number of strategies for refinement, reduction and finally replacement of MBAs. Using a combination of approaches, a steady reduction in the use of mice has been achieved.

From 1996 to 2005, a thirty percent reduction in numbers of animals was achieved by the use of 2 rather than 3 animals per sample. In addition, the time each animal remained on procedure was appreciably decreased. The duration of the PSP assay was reduced by one third (from 30 to 20 minutes) and that of the DSP assay by 75% (from 24 to 5 hours). A defined clinical endpoint had also been introduced for the DSP MBA. The effect of these changes significantly reduced the suffering in all animals used in the test (i.e. for both positive and negative samples).

From 2005 to 2010 further sizeable reductions in the number of MBAs have been achieved by a move to *in vitro* methods. Initially use was made of qualitative pre-screens in the PSP assay and subsequently a fully quantitative analytical method (HPLC) was introduced for the common shellfish species, with plans to extend the method into other species. In 2011, a replacement method (using LCMS) has been validated and introduced for 97% of DSP testing giving further reduction of mouse use.

Had strategies such as those above been adopted more widely in Europe, 100,000s of animals need not have been used in tests causing substantial suffering.

Session I-16: Alternatives for potency testing of rabies vaccines

Session I-16: Oral presentations

I-16-381

Drivers and barriers to acceptance and use of 3R models for the quality control of veterinary rabies vaccines

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Vaccines are subject to requirements that regulate their quality and safety. Due to their biological origin, vaccines are complex products which can be influenced by various factors. As a result, each batch of the finished product is tested on potency and safety. The use of animals in batch release testing is a regulatory obligation and represents around 80% of the total number of animals used in the vaccine industry. Over the last decades this heavy reliance on animal experimentation has met serious ethical, scientific and economic objections. However, despite the increase of 3R models to ensure vaccine quality and European legislation requiring the adoption of 3Rs alternatives where possible, the acceptance and use of 3Rs methods falls behind. This raises the question which factors influence the acceptance and use of 3R models for regulatory purposes and how should this process be optimised. The author aims at clarifying the mechanism of regulatory acceptance and implementation by defining the main obstacles and drivers influencing this process. For this purpose a case study on the rabies vaccine has been conducted that examines the acceptance of 3R models, such as a serology test developed by the PEI in Germany, to replace the regulatory required NIH potency test for veterinary rabies vaccines. The case study consists of literature research and interviews with regulatory authorities and vaccine manufacturers. In order to fully understand the mechanism of regulatory acceptance, the findings are put in the context of technology acceptance in the area of risk regulation. This study serves as input to the discussion between regulatory authorities and industry on how to optimise the process of acceptance of 3R models for quality control of vaccines in general and veterinary-rabies vaccines in particular.

I-16-073

Potency testing of rabies vaccine: on the way to a new era

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According to current requirements the potency of rabies vaccine batches is determined in a vaccination/challenge experiment in mice. The test uses a large number of mice, causes severe animal distress and is poorly reproducible. Several attempts have been undertaken to replace the test taking into account the 3Rs without success to date. In 2009 a promising method testing antibodies induced after vaccination of mice was established and validated by Kraemer et al. for veterinary vaccines. The serological test distinguishes between potent batches and vaccine batches which do not fulfill the minimum potency requirement. It uses a single dose and thus saves many mice. It provides a qualitative result in contrast to the quantitative potency value of the classical mouse challenge potency test. Based on this and successful experience with the assay in German batch release, a collaborative study was organized in the framework of the EDQM Biological Standardisation Programme. The standard operating procedure to be followed, crucial reagents (e.g. reference sera, fluorescent anti-rabies-nucleoprotein conjugate), the reference vaccine and four rabies vaccine batches were supplied to the participants. Three independent repetitions of the assay were performed by 13 laboratories from Europe and North America, including manufacturers and official control laboratories. The study clearly demonstrated the suitability of the alternative test method to identify potent and insufficient rabies vaccine batches. In addition, in depth statistical analysis provided data to recommend the most suitable number of animals to be used for routine batch testing. The study results are published in Kraemer et al. (2010).

Based on the results of the collaborative study, the monograph Rabies Vaccine (inactivated) for Veterinary Use of the European Pharmacopoeia has been adapted by the respective expert group. The revised monograph is currently published in PHARMEUROPA 23.13 for public consultation. Finally, potency testing of inactivated rabies vaccines may begin a new era involving lower animal usage and considerably less animal distress.

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I-16-255

Rabies vaccines for human use: Potency testing without mouse challenge?

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The conventional batch potency test for cell culture-based inactivated rabies vaccines involves vaccination and viral challenge of mice. The test is highly variable, very time-consuming, needs huge numbers of mice and causes significant suffering to the animals. It was established in the 1950s in the absence of GMP at the National Institutes of Health (NIH). It has been broadly used until now despite its multiple drawbacks. In line with the modern concept of the consistency approach for vaccine quality, future batch potency control will combine data of a panel of tests throughout manufacturing to have improved knowledge on product characteristics. Immunogenicity testing is a key element, providing valuable information that complements protein quantification.

Consequently, we propose the development of an immunogenicity assay based on vaccination of mice and determination of neutralising antibodies. The assessment of the serum response is done by quantification with the WHO standard anti-rabies immunoglobulin based on the Ph. Eur. monograph on potency testing of human rabies immunoglobulin. Points for consideration of a potential impact on the antibody response are the vaccination scheme and the age of mice. The design for the development of a serological alternative assay should be harmonized worldwide to the greatest possible extent. Global acceptance is a pre-requisite for any alternative assay. The World Congress on Alternatives and Animal Use in the Life Sciences provides an opportunity to present this serological alternative assay for human rabies vaccine potency and to discuss its potential use in the global context.

Session I-18: Report on the ICCVAM International Workshop on Vaccines

Session I-18: Oral presentations

I-18-569

International workshop on alternative methods to reduce, refine, and replace the use of animals in human vaccine potency and safety testing

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Few medical interventions have had a greater impact on human health than vaccines. Immunization efforts have resulted in the global eradication of smallpox, and the elimination of polio, measles, and rubella in the Americas. Prior to the release of post-licensing production lots of vaccine, regulatory authorities require testing to ensure potency and safety, which can involve large numbers of animals that experience unrelieved pain and distress. NICEATM-ICCVAM organized an international workshop with ECVAM, JaCVAM and Health Canada to review the state of the science and identify priority activities to advance scientifically sound alternative methods that can reduce, refine and replace animal use in vaccine potency and safety testing. Nearly 200 scientists from 13 countries identified relevant knowledge and data gaps, and identified necessary priority research, development, and validation activities. Diphtheria and tetanus toxoids, pertussis, rabies, anthrax, inactivated polio, and combination vaccines were identified as the highest priority vaccines because they use large numbers of animals and induce significant pain and distress during testing. Research into specific mechanisms of vaccine protection and identifying clinically relevant immunological markers was considered necessary to successfully implement *in vitro* alternatives. Participants agreed that broader acceptance and use of alternative methods would require broader access to information, increased global communication among regulatory authorities, research institutions, and vaccine manufacturers, and harmonization of testing requirements. Implementation of the workshop recommendations is expected to advance alternative methods for vaccine potency and safety testing that will benefit animal welfare while ensuring continued protection of human and animal health.

I-18-570

International workshop on alternative methods to reduce, refine, and replace the use of animals in veterinary vaccine potency and safety testing

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Veterinary vaccines represent an important tool for improving animal and human health by preventing a wide range of infectious diseases in animals and reducing serious zoonotic diseases in people. However, regulatory testing to meet vaccine lot release requirements can require large numbers of animals that may experience unrelieved pain and distress. NICEATM-ICCVAM organized an international workshop in partnership with ECVAM, JaCVAM and Health Canada to review the state of the science of human and veterinary vaccine potency and safety testing, and to identify priority activities to advance scientifically sound alternative methods that can further reduce, refine and replace animal use. Nearly 200 scientists from 13 countries participated in the workshop during which they identified relevant knowledge and data gaps and priority research, development and validation activities to address these gaps. This included identifying opportunities to apply new science and technology to develop improved methods. The highest priority vaccines were Rabies, *Clostridium sp*., and *Leptospira sp*. vaccines because they require large numbers of animals and involve significant pain and distress. Vaccine challenge testing, which often requires live viruses and bacteria hazardous to laboratory workers, livestock, pets, and wildlife, were also considered high priorities. Collaborations between human and veterinary researchers working on vaccines for the same or similar organisms were recommended to leverage scientific resources and expedite progress. Implementation of the workshop recommendations will likely advance alternative methods for vaccine potency and safety testing to benefit animal welfare while ensuring continued protection of animal and human health.

Session I-19: Toxicity testing strategies – progress in skin sensitization testing: A COLIPA supported session

Session I-19: Oral presentations

I-19-704

Considerations for the development of an integrated testing strategy for skin sensitization

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Skin sensitization is a crucial endpoint for the safety assessment of cosmetic ingredients with significant social and economic impact. Currently, the mouse local lymph node assay (LLNA) is the standard stand-alone test method which allows the acquisition of potency data needed for risk assessment. A major challenge for the cosmetic industry is the development of skin sensitization safety assessment strategies for ingredients with non-animal data due to the 2013 ban. Therefore Beiersdorf undertakes considerable efforts to evaluate available tools, which include *in silico* methods, chemical reactivity predictions based on physico-chemical properties, indications for structural alerts (structure-activity relationship: SAR) and read-across of historical data. All of these should be integrated into quantitative risk assessment (QRA) concepts based on weight of evidence as well as *in vitro* methods. Moreover threshold concepts and metabolism studies have to be incorporated. Besides the QSAR and read across approaches a defined battery of *in vitro* assays has to be applied in order to replace the LLNA as a stand-alone method in skin sensitization risk assessment based on established exposure scenarios. This battery should include *in vitro* assays representing biophysical (e.g. direct peptide reactivity assay) and physiological approaches such as cellular stress (e.g. KeratinoSens), dendritic cell activation (e.g. peripheral blood mononuclear dendritic cell-assay) and T-cell activation (e.g. T-cell assay). The combination of the above mentioned QRA concepts and the *in vitro* assay battery will enable an integrated testing strategy aimed at replacing animal testing for skin sensitization risk assessment.

I-19-705

Development of *in vitro* skin sensitization assay system at Shiseido

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Shiseido has developed several *in vitro* skin sensitization methods since the 1990's. The h-CLAT is a method for detection of augmentation of CD86 and CD54 expression in THP-1 cells by test chemicals, while the SH test detects changes of cell-surface thiols on THP-1 cells exposed to test chemicals. In this study, we attempted to predict the published EC3 values of chemicals in LLNA from h-CLAT, SH test and cytotoxicity data by means of nonlinear analysis using a combinatorial approach. We used *in vitro* biomarkers (CV75, h-CLAT and SH test) as input layers and the LLNA thresholds, including EC3 values for LLNA-

positive chemicals and set maximum concentrations for LLNA-negative chemicals, as the output layer in artificial neural network analysis. Model evaluation was implemented using the leave-some-out cross-validation method. In brief, we divided the dataset used in input layers and the output layer into 6 disjointed subsets (about 10% of all datasets). In the leave-some-out cross-validation method, we assessed whether the model derived from nine datasets predicted the remaining dataset.

We found a good correlation between *in vitro* model predictions and reported LLNA EC3 values. We confirmed that h-CLAT and SH test results were correlated with reported LLNA threshold values, and found that these *in vitro* data can be used in combination with artificial neural network analysis to build an *in vitro* prediction model for risk assessment of skin sensitization. Shiseido will continue research aiming at the practical use of this system.

I-19-706

Non-animal test battery optimized for detecting skin sensitizing potential

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As the mechanism of skin sensitization is complex and hard to reproduce in one *in vitro* system, the combination of several *in vitro* methods is useful in identifying the skin sensitization hazard adequately for a wide variety of chemicals. We have been developing an *in vitro* assay, the human Cell Line Activation Test (h-CLAT), which emulates dendritic cell activation. In this study, we investigated a battery system: the combination of h-CLAT, the direct peptide reactivity assay (DPRA), an *in vitro* assay, and the *in silico* system, DEREK. As a first step, the integrated testing strategy (ITS) was investigated. Final score calculated from the scores of each robust data set from each ITS component was used for the evaluation. ITS demonstrated a higher accuracy (85%) compared to DPRA, h-CLAT or DEREK alone. Secondly, the tiered approach using h-CLAT and DPRA were investigated as a practical system. The optimized tiered approach indicated the possibility of not only detecting the hazard but also of classifying the potency of chemicals. The predictivity for the potency classification was 72.3% while the "under prediction" rate was relatively low. Our results brought the non-animal testing system one step closer to replacing animal testing. Finally, we have been developing a novel *in vitro* test, EpiSensA, using a reconstructed epidermis model, which is expected to solve some current problems (e.g. lipophilic chemical evaluation). By adding EpiSensA to the tiered approach, the non-animal testing system will be used as an alternate to animal testing and be leveraged in risk assessments.

I-19-707

Towards an integrated testing strategy for skin sensitization: Development, refinement and combination of non-animal methods

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Contact sensitizers are reactive molecules that have the ability to modify skin proteins to form an antigen which will be recognized by specific T cells activated during the sensitization process. In addition to the haptenation mechanism, contact sensitizers induce several phenotypic and functional changes of dendritic cells (DC) either directly or indirectly through intercellular signaling pathways implicating keratinocytes, fibroblasts and other skin cells. This rather complex and still not fully unraveled maturation process of DC induced by contact sensitizers allows them to migrate to the lymph node, present antigen and efficiently prime hapten-specific T cells.

Due to the complexity of the sensitization process it is now commonly agreed that alternative hazard identification and risk assessment could only be addressed by combining a battery of methods. We present here our current approach based on a set of >150 chemicals and raw materials aiming to combine *in*

silico and *in vitro* tools from chemical reactivity assay to DC-based assay into an integrated testing strategy for the evaluation of skin sensitization. Through this exercise, we will share the limits and gaps of such an approach, in terms of applicability domains, heterogeneity of *in vivo* reference data and of requirements for statistical significance. Moreover we will give a broad overview of ongoing prospective initiatives in assay development and method evaluation in order to fill these gaps and to adapt our testing strategy in the most appropriate manner to face physicochemical diversity of cosmetic ingredients and to meet the needs for risk assessment of such ingredients.

Session I-19: Poster presentations

I-19-213

Skin sensitisation: modelling the human adverse response

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Assuring consumer safety without animal testing is a considerable challenge, however we remain confident it is ultimately achievable. 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 (Fentem et al., 2008). Here we demonstrate significant progress in developing a non-animal risk assessment approach for skin sensitisation.

In collaboration with Entelos Inc. we previously developed a computational model of skin sensitisation using the published literature (Maxwell et al., 2008). Insights from this modelling exercise have allowed us to focus our subsequent non-animal test method development activities upon the identified toxicity pathways, namely skin bioavailability (Davies et al., 2011), protein binding (Aleksic et al., 2009), skin inflammation/dendritic cell (DC) maturation and T cell proliferation. Guided by our previous work (Maxwell et al., 2008), we are now developing a pragmatic, mechanistic model of skin sensitisation capable of integrating these non-animal datasets (e.g. peptide reactivity) to allow risk assessment decision-making without animal test data. The aim is for the model to predict the dynamics of the emerging sensitiser-specific T cell response. Therefore, we are also further characterising the induction and maintenance of the human immune response to skin sensitisers.

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Theme II
Policy/Law on Animal Use, Public Engagement and Ethics Review

Session II-1: Public accountability

Session II-1: Oral presentations

II-1-674

Strategies and tools for effective public participation

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The presentation introduces the principles and best practices of public participation, tools that can be used in the different stages of a participatory process, and strategies to assess the effectiveness of the process. Public participation is widely recognized as a critical aspect in a variety of public accountability, policy, regulatory, and environmental processes. It is sometimes a regulatory requirement. Despite this, few policy and decision makers, managers, and scientists involved in these processes have formal training or professional development opportunities to build their capacity in planning for and implementing participatory processes. Many of these professionals "do" participation every day, but many do not have the opportunity to reflect on their practice or to contemplate ways to do it better. The presentation is designed to offer this opportunity and to introduce useful knowledge and tools that could help professionals and scientists engage the public to make sound policy and management decisions. Through effective public participation, the processes and outcomes of planning, policy, and decision-making are expected to be more efficient, equitable, and sustainable. The presentation draws on a curriculum on "participation basics" developed by FORREX in 2009 to address the needs of professionals whose job requires them to engage the public but who have not had any formal training in public participation.

II-1-196

Openness and public accountability – the why, who, what and how of it

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In recent years, a number of expert reviews[1,2] have concluded that it is desirable for the public to have access to good information about the type of research undertaken using animals and the intended benefits, along with the number and species of animals used and the implications in terms of pain, suffering and distress for the animals involved.

Openness about animal research is also promoted both by animal protection organisations and representatives of those using animals[3] – albeit from different perspectives. However, there is no single "public", and "openness" can mean different things to different people.

This presentation will consider what is meant by "openness," who has responsibilities and interests in this regard, and, using examples, it will discuss how it may be better achieved. It will cover:

- Bodies regulating the use of animals in research and testing
- Regulators whose requirements generate a demand for animal tests
- Local or institutional ethics (or animal care and use) committees
- Pharmaceutical and chemicals companies
- Academic institutions
- Research funders
- Professional scientific bodies and journals
- The media
- Animal protection groups

Simply increasing the amount of information provided in the public domain will not lead to better understanding or a more nuanced debate. Information has to be meaningful, and it has to be honest.

1 Nuffield Council on Bioethics – The ethics of research involving animals (2005). www.nuffieldbioethics.org/animal-research

2 House of Lords – Select Committee on Animals in Scientific Procedures (2002). www.publications.parliament.uk/pa/ld/ldanimal.htm

3 Understanding Animal Research – Openness and accountability (accessed 6 April 2011). www.understandinganimalresearch.org.uk/policy_issues/freedom_of_information/openness_and_accountability

II-1-087

The use of genetically-engineered animals in research: An exploration of stakeholder opinions

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Regulation has been developed in response to public concerns about animal-based science. However, as new developments in biological research involving animals occur, public attitudes may be shifting. Understanding public attitudes towards the shifting use of animals in science is important: public monies fund animal-based studies, and the public are often beneficiaries. Our previous studies have shown that public acceptance of animal-based science increases when regulation is in place, but to a lesser extent for experiments involving genetically-engineered animals. To better understand these earlier findings, an interview-based study was conducted to explore the in-depth views of different stakeholders (n = 20 researchers, animal care staff and members of the public) regarding the creation and use of genetically-engineered animals in research. Responses indicated that interviewees were more willing to accept genetic engineering of animals if done for biomedical research, and were less willing to accept this use if the aim was to develop food for human consumption. There was universal agreement among participants that limits to genetic engineering should be established, any resulting pain and distress for animals should be minimized, and that better communication between the scientific community and the public is needed. Together, our studies provide examples of stakeholder engagement strategies that can be employed to understand the conditions under which people consider the use of animals in research to be acceptable.

II-1-168

Freedom of information and animal experiments

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There is widespread agreement that the provision of accurate, unbiased information is essential to an informed debate about the scientific validity and ethical justifications for animal experiments. As a practice usually regulated by public institutions, often financed by government and practiced within public institutions, animal experimentation is also a legitimate subject of concern for citizens and taxpayers. While mechanisms exist to increase transparency of public institutions, direct access to primary sources of information remains the "gold standard" and the objective of Freedom of Information (FoI) provisions. Nations and political cultures have different attitudes towards openness regarding the conduct of public bodies in general and animal experimentation in particular. Comparisons are made between the practices of different nations on this issue, with a particular focus on the US and UK. Consequences of greater disclosure and greater withholding of primary information regarding both conduct and regulation of animal experiments are examined, and perspectives on the suitability of animal experimentation as a subject of FoI specifically are explored. The value of alternative or complementary sources of information such as published scientific papers and project summaries are examined as well as the limitations of this information. Disclosure of primary information, ideally by a FoI mechanism, is of particular and unique benefit to the public, the scientific community and animal welfare and governments which do not currently facilitate this kind of transparency should review their practice and laws.

II-1-405

Who is concerned about animal care and use in developing countries?

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In spite of the advances made today to develop and disseminate information and guidelines for the care and use of laboratory animals in many parts of the world to improve the health, welfare, and psychological well-being of the research animals, using the Three Rs as a foundation in order to increase the accuracy, reproducibility and ensure quality control in the validity of animal based results, the trend is lacking in most developing countries. A comprehensive and systematic review of published reports revealed that adoption of the 3Rs, oversight by IACUC or an ethical committee on animal based research, and the forward looking towards alternatives to animal use in research are not visible in developing countries. These deficiencies may account for the lack of recognition of data from developing countries and be responsible for the absence of searchable reports comparable to those from the Western world and Far East. It is suggested that for developing countries to catch up on this matter, particular issues need to be addressed such as advocacy for involvement of governmental regulations, academic responsible conduct of research by investigators, institutional commitment to animal welfare in research as well as exposure to training opportunities by international resource agencies on animal care and use such as AAALAC and ILAR, before raised public awareness further worsens the already slow pace of scientific advancement in these countries.

II-1-573

Young people's perceptions of the use of animals in scientific and medical research in the United Kingdom

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Surveys of the UK adult population have shown that 70% agree with the use of animals in medical research. However, there are no comparable studies of young people. In 2006, the ethics of animal experimentation became part of all high school biology curricula. The aim of this study was to determine young people's opinions on animal experimentation. A 20 minute presentation followed by the opportunity for students to ask questions on the use of animals in research was delivered in high schools within West Yorkshire, UK. Electronic voting handsets were utilised to gather student opinions before, during and after the session.

The seminar was delivered to 466 science students, aged 11-17, from 11 schools. The majority of students (78%) had never or only occasionally thought about the use of animals in research before the seminar, with only 37% either agreeing or strongly agreeing with their use. After the session, the level of acceptance had increased to 66%. When asked how new medicines should be tested, 31% thought that non-animal experimental preparations should be used, 24% would utilise animals, whilst 22% would use prisoners. Students also had serious misconceptions about practices in research laboratories, for example, 53% thought that animals were kept in small confined cages.

This study demonstrates the need for scientists to engage in outreach activities in order to provide young people with necessary information to enable them to make an informed decision for themselves as to whether the use of animals in research can be justified.

Session II-1: Poster presentations

II-1-241

Meeting the 2013 deadline for cosmetic testing: an opinion on the status of alternative methods

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In 2003 the European Parliament voted to end testing on animals for cosmetic purposes. The final installment of the ban is 2013 when it will become unlawful to sell cosmetics that have been tested for toxicokinetics, repeated dose or reproductive toxicity endpoints using animals outside of Europe. There is the risk however that this deadline can, and will, be extended due to a perception that these endpoints are not yet replaceable.

In an attempt to raise debate about the genuine status of alternative methods, the BUAV have produced a scientific review of the alternatives for the three remaining endpoints plus skin sensitisation and carcinogenicity which have been mistakenly included in the 2013 deadline. The report has been disseminated to European politicians and Commission experts. The report concludes that, should prevalidation studies be successful, full replacement of skin sensitisation and carcinogenicity should be possible by 2013. Toxicokinetics and reproductive toxicity studies are actually not always required for cosmetics due to low exposure and can already be replaced in part by *in vitro* methods. Repeated dose can be replaced by the implementation of strategy that combines the results from tests on key target organs *in vitro*, and in many cases can be waived through the use of the Threshold of Toxicological Concern (TTC) approach. The review concludes that the 2013 marketing deadline can be met with minimal impact to the cosmetic industry and that to extend it would undermine the excellent work done by industry to meet the deadline.

II-1-333

Veterinarians play an important role in the promotion and education of the 3Rs

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The veterinary profession has continued to develop since 2500 BC when the first evidence of the awareness of diseases in animals was found in Chinese writings. Today, veterinarians are highly trained in multispecies biology and comparative medicine and enjoy broad public support, are highly respected, and are consistently ranked among the most trusted members of society. As the veterinary medical profession continues to adapt to the needs of a changing society, the veterinarian has the ability to become a 3Rs advocate and educate the public on the importance of animal research alternatives. Veterinarians may be in the best position to educate and engage the public in the 3Rs because of their understanding of comparative animal health, their knowledge of animal welfare and behavior, and their ability to form a positive doctor-patient-client relationship. Veterinarians have the necessary experience to suggest valid options for replacement and reduction techniques in research. Their education in animal behavior, husbandry, disease control and welfare allows them to assess the need, importance, and effect of refinement in experiments. Finally, their ability to cultivate a doctor-patient-client relationship allows the veterinarian's professional opinion to be taken seriously by the public. This support, led by veterinarians, could result in additional publicity, funding, and advocacy for the continued development and implementation of 3Rs protocols. A partnership-based cooperation among veterinary schools in the US and Canada to develop and implement alternative methods, and inclusion of study courses promoting 3R principles at undergraduate levels may help disseminate this knowledge further.

II-1-367

Activities of Ethics Committee on Animal Use (CEUA) of Oswaldo Cruz Foundation, Brazil

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The Oswaldo Cruz Foundation's Ethics Committee on Animal Use (CEUA/FIOCRUZ) was established in 1999 to evaluate, from an ethical point of view, institutional activities involving animal use. These activities are related to animal breeding, research, drug and vaccine production, quality control and teaching. Nowadays CEUA/FIOCRUZ is composed of professionals from different areas such as veterinary, biology, statistics, medicine, pharmacy and a representative of animal rights. CEUA/FIOCRUZ works by receiving online protocols that contain an introduction, relevance of the projects, aims, crew and a detailed description of procedures. CEUA/FIOCRUZ receives around 100 projects per year, but, 3 to 5% of these protocols are not approved due to different reasons, for example, they do not comply with ethical principles or the coordinator of the project does not respond to required alterations. Despite all efforts to reduce the running time for evaluation of a project, CEUA/FIOCRUZ takes from 4 to 7 months to analyze a protocol. In order to guide researchers, a basic Guideline of Procedures is available at the website with information about anesthetics, routes of bleeding and injection, euthanasia, etc. CEUA/FIOCRUZ is working toward ensuring that researchers are conscious about using alternative methods when available, reducing animal number to a minimum required and improving the welfare of the animals when animals must be used.

II-1-427

Effective and relative reduction of the use of animals at Sanofi Pasteur

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In the vaccine industry, the main target population is healthy children and adults. Safety and efficacy of vaccines should be ensured to protect people against infectious diseases. For the development of novel vaccines (R&D) and the quality control of commercialized vaccines (industrial operations) (10% and 90% of the use, respectively), although major alternative methods have been and are being developed, the use of laboratory animals is still necessary and mandatory. However, in the last decade, the Reduction efforts achieved by Sanofi Pasteur appeared to be clear and important: absolute decrease of number of animals. The progresses are even better if the increase of the business and the R&D investments are considered in the meantime.

The presentation aims at explaining the Reduction initiatives and achievements, and the associated indicators.

– Effective reduction was gained by replacement methods, optimization of study design, waiving approach (removal or replacement by *in vitro* assays), and consistency.
– Relative reduction is highlighted by the internal indicators. For instance, the improvements of vaccine production and testing induced a drop of number of animals required to release vaccine batches. Also, the integrated research strategy provides more data to document the safety and the efficacy of novel vaccines with the same number of animals.

The commitment to reduce the use of animals is a long-term development. In addition to the animal care and use program, an institutional 3Rs program has been set up to focus on the ultimate goal, Replacement.

II-1-471

Americans' attitudes toward animal testing: 2001-2011

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Public opinion surveys about animal experimentation that have been conducted by independent polling organizations, as well as those commissioned by groups for and against the practice, uniformly indicate that approval for it has declined significantly in the United States. The first national survey on the subject conducted in the late 1940s showed 84 percent of the public supported the use of animals in experiments. Today, depending on demographic factors, multiple sources suggest that support rests somewhere between 40 and 60 percent.

Since 2001, the Gallup Organization has annually conducted a national "Values and Beliefs" survey of approximately 1000 adults ages 18 and above to collect opinion data on 16 different controversial social issues, including "medical testing on animals." An analysis of the data from the past ten years reveals a significant increase in moral opposition to medical testing on animals, as well as marked differences in attitudes toward the practice based on gender, age, political affiliation and level of education completed.

This paper discusses the 2001-10 Gallup data and offers possible explanations for these demographic differences and the general changes in the public's attitude about this contentious issue.

Session II-2: Ethics review

Session II-2: Oral presentations

II-2-649

Ethical review of animal experiments: current practice and future challenges

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Over the last 20 years, ethical review of animal use in research, testing and teaching, and its practical implementation, has developed significantly across the world. However, the expectations and outcomes of the process can vary widely. This is not surprising given that:

– there are different concepts of what ethics and ethical review actually means in practice;
– ethical values and judgements inevitably differ between individuals, roles, and establishments, between societies, cultures, and legislative contexts, with differing historical precedent, and over time; and
– ethical review *processes* vary between, and sometimes within, different countries, e.g. in their level of authority, scope of interest, remit and organisation.

Some countries have long experience with ethical review processes and some are just beginning to embrace the principles. It would therefore seem beneficial and timely to start to work towards harmonised, worldwide "guiding principles". There are already many good statements of principle from eminent organisations. The difficulty is translating these into workable and worthwhile practical systems. As a first step, it is important to develop a common understanding of what we want to achieve through ethical review. In this context, our presentation will:

– explore the various beneficial outcomes that can come from "doing ethics" in general, and specifically from ethical review of animal use in the life sciences;
– discuss examples of the different ways in which the ethical review process is, or could be, designed to achieve specific outcomes, drawing on available guidance from around the world;
– raise questions for discussion, which might form the basis for further dialogue leading to development of international guidelines.

II-2-553

Ethical review of the use of animals in science – A reflection on the journey and future directions

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In the 1970s, Canada, Sweden and Australia became the first countries to establish committees to review the ethical acceptability of research using animals. Modelled on comparable committees concerned with human subjects, drivers for the establishment of animal ethical review committees (AERC) recognised that such activities presented ethical challenges and that, given the level of controversy in the wider community, scientists needed to demonstrate responsibility and accountability for their actions. Notably, the scientific community was involved in the development of these committees. In Canada and Australia, AERCs were established under an institutional self-regulatory framework whereas in Sweden the committees were under national legislation and external to institutions. Nevertheless, in each country the involvement of "lay" members was seen as important in achieving the expected outcomes.

Since then, AERCs have been established in many countries albeit with differences in their charter and standing, reflecting historical experiences and cultural differences. However, the role of these committees and expected outcomes has become politicised; government policy is most often the driver so that, in many instances, the role of the AERC is mandated in legislation or through contractual agreements with funding agencies. Despite almost forty years' experience, there have been few reviews into the operation of AERCs, but available evidence raises questions as to the effectiveness of the process and engagement with the wider community. The AERC process would be strengthened by reflection upon and reaffirmation of expected goals, and a better understanding and recognition of the broader social context within which an AERC operates.

II-2-555

The costs and benefits of animal experiments

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Most regulations governing animal experimentation require that the harms expected to be incurred by animal subjects should be balanced against the likely benefits of the project. Too often, however, expected human benefits are based on unrealistic assumptions. To critically assess the human clinical, toxicological and educational utility of animal experimentation, the published literature was comprehensively surveyed to locate relevant systematic reviews. In only two of 20 reviews located did the authors conclude that animal models were either significantly useful in contributing to the development of human clinical interventions or substantially consistent with clinical outcomes. Furthermore, one of these conclusions was contentious. Included were reviews examining the clinical utility of invasive chimpanzee experiments, of highly cited animal experiments published in leading scientific journals, and of experiments approved by ethics committees at least partly on the basis of specific claims that these animal experiments were likely to lead to concrete advances in human healthcare. Seven additional reviews also failed to demonstrate reliable predictivity of human toxicities such as carcinogenicity and teratogenicity. Results in animal models were frequently equivocal or inconsistent with human outcomes. When considering costs and benefits overall, one cannot reasonably conclude that the human benefits exceed the costs incurred by animals subjected to scientific procedures. On the contrary, the evidence indicates that actual human benefit is rarely – if ever – sufficient to justify such costs. Despite this, deficiencies in the implementation of regulatory and policy requirements to replace, reduce and refine animal use remain marked and widespread. A range of policy initiatives are warranted to address these deficiencies, and are reviewed.

II-2-084

A New Zealand commitment to continuous improvement in AEC decision-making: giving operational effect to key principles

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The role of New Zealand's National Animal Ethics Advisory Committee (NAEAC), as well as provision of advice to the Minister of Agriculture and the Director-General of Agriculture and oversight of the regulatory system governing use of animals in research, testing and teaching, is to support the work of the 33 animal ethics committees in the country. NAEAC clearly has a significant interest in ensuring that AECs have the information they need to make good decisions. To this end the committee:

1. responds to requests for guidance from individual AECs when they require clarification on legislation or ethical principles;
2. holds workshops for AEC members every two years, ensuring inclusion;
3. sends out two to three newsletters a year highlighting issues that have arisen both for AECs and for NAEAC;
4. holds one meeting a year in a regional area where visits to local AECs are made.

This paper looks at the support given by NAEAC to AECs, with a focus on the workshops held over the last 8 years and highlighting topics that participants have found most useful and those where difficulties have arisen.

Session II-2: Poster presentations

II-2-135

The use of activity maps in project authorization

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The use of animals in research raises ethical questions. In order to get a project authorized, persons planning animal experiments must apply to an authorizing body, often providing written information in a standardized form. The information is commonly evaluated on compliance with the 3Rs or accumulated harm to the animal versus benefit of the experiment. Activity maps are commonly used in project planning. Activity planning means to make a detailed plan for necessary activities. Persons and their responsibility and necessary manpower are identified. The activity map gives a chronological overview of activities over time and the dependency between activities is clarified. It is helpful to identify critical activities to make sure that competent and experienced personnel are responsible and available for the project. A project description might consist of a substantial document and important information is drowned in information overload; an activity map shall describe all activities in a maximum of 1 page (A4, A3). This gives a good overview of all activities. In 2010 a pilot experiment was performed at the Animal Facility at the University of Bergen, Norway. An activity map had to be prepared as an attachment to all project authorization applications. The pilot project identified the following:

– activities, both frequency and total number
– accumulated harm of all activities/procedures on the animal
– different activities' potential for the 3Rs, especially refinement
– specific activities' needs for improvement (refinement)
– quantification of harmful procedures
– severity categorization to be implemented

II-2-164

Advancing ethics review in IACUC oversight of animal research

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Institutional Animal Care and Use Committees (IACUCs) oversee U.S. research institutions' animal programs and have played an important role in reducing some of the worst abuses of animals in laboratories. After 25 years, however, IACUCs struggle with adequate consideration of alternatives, and are criticized for failing to tackle a central issue – the justification and necessity of using animals for research in the first place. Drawing from published studies, we examine how IACUC functioning compares to public expectation, particularly with respect to how IACUCs handle the ethical dimensions of animal research. Critiques include a tendency to focus on technical aspects of refinement and a limited role for the community representative. In addition, harm-benefit analyses are rarely performed, in contrast with public expectation that broader ethical issues are considered during the research proposal review process. Other ethical committee models demonstrate that such deliberations need not be out of the scope of IACUC responsibility. Recommendations are provided for how US IACUCs could improve consideration of ethical issues and better represent their communities, achieving harmonization with practices in other countries and international standards for ethics review. We also highlight new research pointing to persistent challenges even with optimization of the ethical committee framework.

II-2-335

Incorporating ethics in the alternatives to animal use in scientific research

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Conducting any scientific experiment involves certain methodological aspects, such as the identification of a problem situation, the establishment of an objective, the accuracy in the procedures, the techniques to obtain data, and the management of the information and the final results. These elements of methodology interact with the scientific, professional and social criteria that define and provide evidence on the context in which the research is carried out.

When addressing the so-called "alternative" proposals regarding animal use in research, it can be observed that these are consistent with methodological criteria, research strategies and techniques based on scientific knowledge and procedures implemented in areas such as biology, ethology and veterinary medicine. Ethical guidelines are not explicitly included in the alternative proposals. Directions and requirements are, however, considered as ethical positions in some academic, normative and committee-related documents.

Considering that ethics, within research and science, places more emphasis on the justification of the experiments rather than on the way they are carried out, some questions arise regarding the interaction and support that ethics may contribute to the reflection and development of the alternative proposals that address animal use in scientific experiments. Thus, some ethical choices regarding these alternative proposals are built from the approaches developed by contemporary philosophical trends that have included animal issues in their reflections.

II-2-348

Using language to find if Australian Animal Ethics Committees use emotion or ethics to assess animal experiments

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In Australia, the ethics of the use of animals for scientific purposes are assessed by Animal Ethics Committees (AECs) that are comprised of the four major parties involved in the animal experimentation debate: veterinarians, scientists using animals, animal welfare representatives and members of the public. AECs are required to assess animal experiments as ethical based on a cost/benefit analysis, suggesting the use of consequentialist ethics. However, people are more likely to use a mixture of frameworks when making ethical decisions. Therefore, we hypothesised that AEC members will make their decisions using argumentation relying on multiple frameworks, including ethical relativism, deontology and emotional ethics; frameworks commonly used in the public debate about animal experimentation. The language used by AEC members, examined using discourse analysis techniques, can indicate which ethical frameworks they rely upon. Using a role playing method, representatives from each of the four AEC categories discussed the ethical value of eight fictional protocols involving animal experimentation. The discussions were recorded and analysed using Nvivo for instances of emotional and ethical language. Data were analysed using ANOVAs and Tukey tests. Emotional language was more common than ethical language ($p < 0.0001$). Categorical differences found scientists used the least emotional language ($p = 0.012$) but the most utilitarian language ($p = 0.023$). As hypothesised, Australian AEC members did not base their decision exclusively on a cost/benefit analysis. Contrary to the guidelines in use, an ethical decision making process that takes into account emotion should be used to accommodate the AEC members' views.

II-2-485

Chimpanzees in US laboratories

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The use of chimpanzees for biomedical research and testing has sharply declined over the last decade, largely due to ethical concerns, public opinion, economics, as well as scientific failures of the chimpanzee model. The United States is the only developed country in the world to continue invasive research on chimpanzees, with 1000 chimpanzees remaining in six US laboratories. In 2006, The Humane Society of the United States launched its Chimps Deserve Better campaign, which seeks to end invasive research on chimpanzees in the United States and retire chimpanzees in laboratories to appropriate sanctuary. This presentation will provide information on the current trends related to chimpanzees in laboratories including new laws, demographics of chimpanzees in labs and sanctuaries, costs associated with keeping chimpanzees in labs vs. sanctuary, and scientific evidence regarding failure of their use. An update of the Chimps Deserve Better campaign will also be provided, including an undercover investigation into the largest chimpanzee laboratory in the world, legal and policy actions taken on behalf of chimpanzees in laboratories, efforts to secure permanent retirement for chimpanzees, as well as outreach to corporations, scientists and the public.

II-2-527

Facilitating the role of lay members in ethics and animal care and use committees

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Incorporating independent "lay" members within ethics committees allows a measure of "public" accountability and widens the range of expertise and perspectives on animal care and use. Without doubt, a confident and constructive lay person can help in making significant improvements to animal welfare and the implementation of the 3Rs. They can challenge existing assumptions and practices, which helps to develop and facilitate broader ethical discussions. However, lay members may lack experience in discussing the harms and benefits of animal use and the potential for humane alternatives, so it can be difficult to make a real contribution especially when faced with professional scientific, technical and animal care staff who are experts in the topics under discussion. "External" lay members who are also unfamiliar with local personnel and management practices can find it even more difficult.

If lay membership is to achieve real benefits, it is important to understand and try to alleviate any problems lay members may face. The RSPCA has run an annual Lay Members' Forum for over 10 years, which has provided "training" for lay members and yielded valuable insight into their information needs. Interestingly, delegates are not only lay members – they come from a range of roles and positions within research establishments, suggesting that some of the issues facing lay members are common to other committee members. Experience from the Forums and elsewhere has enabled the RSPCA to produce a range of resources to help lay members fulfill their roles. These will be described in the poster.

II-2-557

Guidance on the severity classification of procedures involving fish

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Severity classification is an important tool for implementation of the 3Rs and for ethical evaluation of research procedures. The revised EU Directive that comes into force in 2013 requires signatories to ensure that all procedures are classified as "non-recovery", "mild", "moderate" or "severe", using assignment criteria set out by the European Commission. A working group appointed by the Commission produced a report in 2009 that gives examples of procedures within these categories. These examples are, however, most relevant to research using terrestrial laboratory animal species. A working group set up by the Norwegian Consensus-Platform for the 3Rs (Norecopa) has published a complementary document that gives guidance on severity classification in fish research, including examples of "subthreshold", "mild", "moderate", "severe" and "upper threshold" procedures. This document will make it easier for fish researchers to implement the requirements of the new Directive. Norecopa has established a website (www.norecopa.no/categories) with links to these guidelines and more information on severity classification.

Session II-3: Public law – the Three Rs in regulation addressing animal use

Session II-3: Oral presentations

II-3-104

Good regulatory practice: Directive 2010/63/EU, a missed opportunity?

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Consideration at WC6 of whether regulation drove, managed or monitored change concluded that regulatory frameworks for the use of animals for experimental and other scientific purposes should adopt a flexible approach to anticipate, promote and make provision for technical progress in science and animal welfare; and reflect the evolution of informed societal and political thinking. It was argued that regulation should focus on what must be achieved and why, rather than how it is to be achieved.

Directive 2010/63/EU, concluded in September 2010 and taking effect in EU Member States in January 2013, will shape the regulation of animal use for experimental and other scientific purposes, and public policy in Europe with respect to the 3Rs, for the foreseeable future. A superficial analysis of the structure and contents of the new Directive indicates a desire to adopt a flexible approach, and confirms that in a number of areas where it is foreseen there will be evidence to support improved science and animal welfare progress can be made by updating technical annexes. However, a more rigorous analysis identifies areas where the focus is on modest or ambiguous minimum provisions, frameworks and inputs rather than outputs and outcomes, and the need for a structured and well-resourced programme to properly maintain and update the technical annexes.

In the context of the 3Rs this presentation considers lessons learned from the interpretation and implementation of Directive 86/609/EEC; the drafting of Directive 2010/63/EU; its implementation by Member States; and the resources and systems required to ensure that the new EU regulatory system keeps abreast of technical progress, and informed societal and political thinking.

II-3-475

The Animal Welfare Act: a regulatory roadblock

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The Animal Welfare Act (AWA) became law after development of the 3Rs principles. However, these principles were not incorporated into the legislation. This is one of the fundamental reasons the AWA has become a barrier to the development and implementation of alternatives to animal testing in the United States. Though many people assume the AWA was adequately designed to protect animals used in testing, it has become abundantly clear that it was not. Attempts to amend and reform the AWA to require a more

serious and modern consideration of the welfare of animals have been unsuccessful, and worse, oftentimes counterproductive.

Further, the AWA does not incorporate a notion of ongoing scientific development in its regulatory regime. This is part of the reason legal analysis, regulatory science and risk management are mired in decades old notions. Concepts such as legal liability protection, intellectual property protection, private property and profit trump considerations such as animal welfare, shared scientific knowledge, scientific development and efficient and humane resource management.

This presentation will address some of the fundamental flaws in the AWA in order to learn from these mistakes as we look for regulatory changes that will incorporate the 3Rs principles and implement the National Research Council's vision and strategy for toxicity testing in the 21st Century. The future of chemical toxicity testing is being written now. Unless we change the dated legal and scientific formulas we have been using, the outcomes will not change.

II-3-522

How different countries control animal experiments outside recognised establishments

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The laws of different countries show considerable variety in controls and monitoring and enforcement approaches, but adherence to the 3Rs is a common theme. Scientific whaling provides a good example of how robust different systems are for ensuring application of the 3Rs in a difficult research environment outside of the normal controls of a research establishment. The activities may be in international waters and outside the scope of national legislation, inspection is difficult, and on site there is rarely staff with the animal's interest at heart. It shares features with research on wild animals and on farms. This paper will explore how different countries' laws on animal experimentation provide control on such work, and consider how good monitoring and effective enforcement could be obtained.

II-3-210

From mouse to machine: how have attitudes and individuals affected the progress of the Three Rs in shellfish toxin testing?

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European safety monitoring programmes to detect marine biotoxins in shellfish harvested for human consumption have relied heavily on the use of Mouse Bioassays (MBAs) because, until recently, these have been the only method acceptable under food hygiene legislation. However, these bioassays cause significant suffering to the animals used and are considered to have some serious scientific drawbacks. This combination of factors has driven a need for change in how such testing is performed.

Attitudes of those involved at all levels have been critical to successfully minimising animal use. Much of the drive for change has come from highly motivated individuals. At a local level, cooperation has been needed among animal technicians, the veterinary staff caring for the animals, scientists involved in both the monitoring programme and in developing alternative technologies, and laboratory managers (due to the staff and financial resource costs created by moving away from the *status quo*).

At a national regulatory level there have been issues with the conflicting requirements of legislation protecting animals used in scientific procedures and over-specification of methods in the Food Hygiene Regulations. This has necessitated negotiations between Government Regulators at science and policy levels to resolve conflicts. Lack of clarity in the process for validation of alternative methods has proved a significant obstacle. Decisions made by regulators have had the potential to impact on the shellfish industry, necessitating their involvement in the process of change.

Cross-disciplinary discussion and good communication has been essential at all levels in order to implement change. Trust has had to be developed and maintained. Individual personalities have impacted significantly on the speed of progress. Compromise has been necessary by all parties.

Session II-3: Poster presentations

II-3-188

New EU Directive 2010/63/EU on the protection of animals used for scientific purposes: Animal welfare aspects of the transposition into national law

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In the European Union, the new EU Directive 2010/63/EU on the protection of animals used for scientific purposes has been adopted in 2010 to replace Directive 86/609/EEC. EU Member States (MS) must transpose the provisions of the Directive into national law by November 2012.

From the animal welfare perspective the new Directive is disappointing in several respects: For instance, there will be no rigorous restrictions on the use of non-human primates and even experiments on great apes remain possible. Researchers may still use animals even where scientifically approved 3Rs methods exist. The authorisation procedure is far from being stringent and transparent. Generally, MS may not adopt stricter national rules.

However, the Directive provides standards that will improve animal welfare at least in some MS. All MS now must establish an authorisation system involving a cost/benefit analysis. Transparency and quality control will be improved by the requirement to publish non-technical project summaries and by retrospective assessment of selected projects. Some specific provisions of the Directive allow for stronger national rules, e.g. banning the use of great apes or long-lasting severe procedures. The Directive could also become a driving force for promoting the 3Rs as it demands specific respective activities on both the national and EU level.

European animal welfare organisations will continue to strive towards having the highest animal welfare standards taken up on national levels. We demand effective measures to implement and control a transparent, meaningful and accountable protection of laboratory animals.

II-3-191

Analysis of EU-legislation in terms of consistency and state-of-the-art regarding the implementation of the 3Rs in the data requirements to identify potential for further improvement

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Present and future EU legislation on the protection of animals used for scientific purposes (Directives 86/609/EEC and 2010/63/EU) requires that, wherever alternative methods recognised by EU legislation are available, they have to be used instead of animal tests. Unfortunately, this principle is not implemented to its full extent when it comes to risk assessment that chemicals and new products have to undergo prior to their authorisation and placement on the market. In a recent study, the Animal Welfare Academy screened data requirements of relevant EU laws and provisions regarding chemicals (REACH), biocides, pesticides and food safety and found that test methods as part of the risk assessment do not reflect the state-of-the-art of science and technology. Most of the data requirements we investigated still require testing on animals for many toxicological endpoints, even though 40 alternative testing methods accepted on EU or OECD level (ICCVAM, Mar 2011) are at hand. This unacceptable state of affairs is due to a multitude of reasons. These may range from shortage of manpower to implementing existing knowledge and expertise in the field of alternative methods to unclear and misleading statements on the applicability and state of validation of alternative methods. In conclusion, we strongly suggest a homogeneous EU-wide strategy for all areas involving risk assessment of substances with the aim to better implement the 3Rs and comply with the Directives 86/609/EEC and 2010/63/EU. As a positive side-effect, this would clearly simplify data requirements, save costs on various levels and improve product safety for consumers.

II-3-298

Regulatory changes and the resultant effect on alternatives consideration in the United States

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Guidelines and regulations regarding animal use in research, teaching, and testing emanate from several government agencies and humane organizations, the most influential and wide reaching being the USDA (United States Department of Agriculture), NIH (National Institutes of Health), and AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care). All three have made recent changes in their requirements and expectations, affecting both scientist and institution. This poster will outline the recent changes, and how these changes relate to animal care and the 3Rs.

Forefront among the changes are the updated USDA Animal Care Resource Guide, the proposed adoption and implementation by NIH of the Guide for the Care and Use of Laboratory Animals, 8th edition, and AAALAC's recent adoption of three resources to be used as standards for animal care program evaluation.

II-3-359

The Brazilian law that regulates animal use does not improve the reduction concept of 3Rs

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The Brazilian law number 11,794 that regulates animal use in scientific research and education states that reuse of the same animal is not allowed after reaching the main aim of the research project. Our group works with pyrogen, skin and eye irritation tests for the routine quality control of biologicals, medical devices, large volume parenterals, cosmetics, topical medicines and cleaning products, as well as for comparing *in vitro* and *in vivo* assays. Rabbits that did not receive a pyrogenic sample used to be reused in one of the irritation tests. Usually we use 1,200 rabbits per year for all of those activities. If the reuse of animals is banned, almost half of this number will be needed to perform irritation tests. It is understood that the rabbit pyrogen test is not a severe one, and can be classified as a mild assay, so it does not offer a high level of suffering to animals that prevents them from participating in another assay. Recently, the European Union reviewed the animal Directive and it is stated that "reuse should be balanced against any adverse effects on their welfare" and that "it should be considered on a case-by-case basis". This Directive also allows the reuse of animals if the previous procedure was "mild" or "moderate". So, it is strongly recommended that Brazilian animal law must be reviewed, in order to allow the reuse of animals in some circumstances where the good sense contributes to animal reduction.

II-3-362

Comparison of the pyrogen *in vivo* methods described in Brazilian and European pharmacopoeias: which one contributes to animal reduction?

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The Brazilian Pharmacopoeia follows the American methodology and differs from the European in the experimental design. This study compared how both methods can affect the final result and which one has the more stringent criteria. The agreement and/or disagreement between the results of pharmacopoeias were analyzed by: a) 44 samples of the sector with the result of repetition; b) hypothetical data using the threshold value of fever of 0.5°C; and c) 451 number combinations in a computer program simulating the first test (3 animals). If all animals presented temperature rise equal to 0.5°C, the Brazilian Pharmacopoeia would present result "pyrogen" after an 8 animals test, whereas the European Pharmacopoeia would present "non-pyrogen" after a 12 animals test. It demonstrates that European Pharmacopoeia does not consider the elevation of 0.5°C as an indicator of fever and may use more animals, besides being less stringent. Both the routine and the computer data showed that the Brazilian Pharmacopoeia was more rigid at low temperature rise (up to 1.15°C). Between 1.2°C and 2.6°C the pharmacopoeias had the same result forwarding the product to "go to next stage". The European Pharmacopoeia was stricter in high temperature rise (above 2.7°C). The results indicate that the methodological differences may generate uncertainty in the evaluations of the protocols according to the country where the product was manufactured and the interference in the interpretation of results may lead to a different conclusion about pyrogenicity of a sample. Besides, European Pharmacopoeia uses more animals, not contributing to Reduction.

II-3-369

The need for establishing a training and educational system for animal use and care personnel in Brazil

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In 2008, Brazil published Law 11,794 aiming to regulate the scientific use of animals. In Brazil, a significant number of the laboratory animal care personnel is autodidact, what many times may lead to biased procedures. The number of opportunities to study laboratory animal science have grown in the last decade nevertheless, the need for formal training and for continuing education systems are still a must. The Ethics Committee on Animal Use of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ) imposes that all proposals describe how the personnel involved in activities were trained, justifying the individual ability to perform the procedures described. When a proposal is approved by CEUA/FIOCRUZ, the involved personnel could be considered indirectly licensed to perform those activities. Taking FELASA procedures as an example, it is evident that people involved in working with animal breeding or experimentation should be trained and categorized. Effective ways of moving forward to implement this concept in Brazil are: i) harmonization of procedures related to animal science activities by ABNT, a Brazilian institution that publishes technical procedures; ii) inclusion of the training framework in the INMETRO (Brazilian accreditation institution) system; and iii) definition of the training program to be offered by accredited institutions throughout the Country. CEUA/FIOCRUZ members are convinced that once those actions are implemented the quality of animals used in science will be improved, issues of animal welfare will be taken care of and the approval process by the Brazilian CEUAs and its control by society and government will be improved.

II-3-374

Does Brazilian animal law really regulate animal use and improve the development of alternative methods?

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Brazil has recently published a new regulation (Law 11,794/08) that has thrown light on animal use in the country and opened the possibility for standardizing procedures and personnel training which, in turn, would improve the quality of animals used in science. If nothing else, this consequence of the law would widely justify the efforts to implement the new concepts and arrangements needed. Considering AAALAC International and other accreditation associations' quality standards, the Brazilian will develop partnerships with international corporations to develop and produce health products. The recent decision of the Brazilian Council for the Control of Animal Experimentation (CONCEA) to create an exception that favors industry autonomy concerning animal use, by excluding animals used in the production of biological products of the aim of the Law upsets the way forward to a controlled and good animal practice. This decision goes against the aim of the Law which is to regulate activities in teaching and scientific research, as stated, since the Law includes the production and quality control of biologicals as part of scientific research. Brazil, just 2.5 years after publishing the Law loses the opportunity to improve good animal use procedures and to raise the Brazilian animal user industry rating to internationally accredited and shows to the society that ends justify animal suffering and neglect. This exception will negatively impact on future and current international partnerships. If nothing changes there will be no need for the generation of alternative methods in the country since the industry won't need them.

II-3-441

Animal suffering in US laboratories: efforts to tackle this critical issue

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Animal pain and distress not only impact animal welfare, but can also confound experimental variables and lead to poor scientific results. Inadequate attention to and relief of distress and pain remain widespread in the U.S. The Humane Society of the United States (HSUS) analyzed violations of the Animal Welfare Act by animal research institutions in 2009 and found that the most common violations were related to the Institutional Animal Care and Use Committee, including requirements for justifying experiments involving pain and distress. The HSUS also published an analysis of noncompliance with federal regulations at federally funded research institutions over a three month period and found that the majority of the reported incidents resulted in animal pain and distress, and 75% in animal death. In order to increase attention to pain and distress overall, the HSUS is urging more than 500 U.S. colleges and universities to adopt internal policies to prevent animals in their laboratories from enduring severe pain and distress; more than 60 schools to date have done so. Current U.S. laws do not specifically prohibit procedures or conditions that cause severe distress and pain; however, the first-of-its kind legislation that would do so was introduced in the state of Maine in 2011. This presentation will describe the analyses of federal animal welfare violations to highlight the need to mitigate pain and distress, and will further describe HSUS's efforts to bring attention to the issues of pain and distress.

II-3-493

Exclusion of birds, rats, and mice from legal protection in the U.S.: a science policy case study

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The Animal Welfare Act (AWA) is the only U.S.-wide law that governs use of animals in research. With the 1970 amendments, coverage under the Act was extended to any "warm-blooded animal that the Secretary [of Agriculture] may determine is being used". However, in the process of writing the regulations to implement the law, the U.S. Department of Agriculture (USDA) chose to interpret that clause as having discretion to exclude the vast majority of warm-blooded animals used in research: mice and rats.

Animal protection groups objected and a federal judge called the exclusion "arbitrary and capricious". However, the USDA's determination remained in effect until a second judge's critical assessment prompted a lawsuit settlement in 2000 and USDA agreed to proceed with timely regulatory process.

The legislative and regulatory history of the AWA is generally one of expanding protections, but in 2002, leadership in the U.S. Senate allowed an amendment to the Act that explicitly and decisively reversed the USDA's agreement.

The case study provides a qualitative analysis of relevant policy considerations, drawing on court documents, legal articles and papers of the Alternatives Research & Development Foundation, which initiated the lawsuit against USDA. While Animal Law classes in the U.S. study this protracted debate and its legal outcome, key details, such as the effect on adoption of alternative methods, and opinion polls showing scientist support of regulation of these species, are often overlooked. The case study also makes recommendations for continued advancement of the AWA, including protection for birds, rats and mice not specifically excluded in the Act.

Session II-4: Implementing the Three Rs – alternatives to legislation

Session II-4: Oral presentations

II-4-582

Information retrieval on alternative methods to animal experiments – one of the factors that affect implementation of the Three Rs in research and testing

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High-quality research and the protection of animals used for scientific purposes both demand the application of best tools to ensure that the 3Rs are appropriately considered and realized. It is essential that each animal experiment is carefully evaluated regarding its indispensability. The decision of whether or not an animal experiment is indispensable is to be based on the state of the scientific art and a scientific examination whether the pursued purpose cannot be achieved by alternative methods. In this context searching high-value information on alternatives to animal testing is discussed as one of the key elements to prove the indispensability of animal experiments. *Indispensability searches* are composed of several steps *which are based on each other*. They should start by defining the scientific objectives of research projects, followed by choosing appropriate information resources, compiling relevant search terms, creating search queries and documenting the search process.

To improve information dissemination on alternative methods at national level the National German Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) pursues a three-fold strategy. ZEBET's strategy consists of (1) capture of and supply of information – via AnimAlt-ZEBET database, (2) education in systematic search procedures as part of courses accredited by the Federation of Laboratory Animal Science Associations (FELASA), and (3) research in retrieval technology. ZEBET's strategy aims at supporting well-informed scientists and well-informed competent authorities responsible for proving the indispensability of animal experiments.

II-4-064

Shaping Three Rs behavior through an accreditation program

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For 45 years AAALAC International has reviewed and accredited institutional animal care and use programs. Today, AAALAC International accredits more than 830 programs in 34 countries around the world. AAALAC is uniquely positioned to assess animal care and use programs using internationally accepted standards and the peer-reviewed literature. To be accredited, institutions must comply with applicable regulations and policies regarding the use of animals in research, testing and teaching, but must also meet high-order principles embodied by AAALAC International such as implementing the Three Rs, ensuring the input of a qualified veterinarian to the program, ensuring review of the proposed animal work by an internal or external oversight body, and protecting personnel through a well-designed occupational health and safety program. The AAALAC process addresses those overarching areas as well as methods of housing, including enrichment; methods of ensuring competency of personnel; micro- and macro-environmental factors that may impact animal welfare; and methods of assessing and mitigating pain and/or distress, to name just a few. In this manner, AAALAC fosters a spirit of teamwork among the institution's professionals involved in the creative development, the critical review and the final implementation of research animal proposals. Over its long history, AAALAC has promoted the incorporation of the principles of replacement, refinement and reduction into accredited programs. Specific examples of program deficiencies identified by AAALAC and corrective measures taken by institutions will be described as examples of the way in which an international accreditation program can enhance animal welfare and the quality of science.

II-4-436

Scientist's views on Three Rs: Comparison of Canadian and UK scientists

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We surveyed Canadian animal-based scientists to benchmark their current understanding of the Three Rs and to identify where our organization's Three Rs resources should be focussed. A similar survey had previously been carried in the United Kingdom (UK). Both surveys found that refinement is the least understood "R" (just 49% [n=304] of Canadian participants included the concept of "minimizing pain and distress" in their definitions of refinement). Neither country's scientists view replacement as achievable. Half of Canadian participants said they could not replace because animals are the subject of the research (e.g. field research) or similar to their UK counterparts they "need to look at whole animal systems" (the reason given by 77% [n=1,343] of UK participants). Many Canadian scientists feel they already reduce as much as possible and further reduction may compromise individual experimental protocols. However, applying reduction strategies to research programs may have support from scientists: 77% of UK survey participants (n=1,529) identified data sharing or collaboration between research groups as possibilities for reduction. Both surveys found that Three Rs training delivered by national animal use regulators is appropriate. In Canada 65% (n=414) of participants learned about the Three Rs through CCAC training modules and other resources. Similarly, 57% (n=1,529) of UK participants learned about Three Rs from Home Office training courses. Although different methodologies were used for these surveys, comparing results further develops our understanding of scientists' views on Three Rs and the resources needed to support implementation

Session II-4: Poster presentations

II-4-103

An advisory center for the 3Rs

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The need for a qualified point of contact to provide advice on the 3Rs has been voiced in Switzerland for the past few decades by authorities, industry and researchers alike. Swiss legislation is currently one of the most advanced national animal welfare legislations in the world, and clearly demands the incorporation of the 3Rs into research projects. Nevertheless, all parties concerned stress that a noticeable lack of implementation of the 3Rs exists; this appears to be not so much due to missing goodwill, but to a considerable extent to deficits in knowledge about existing and feasible methods. Therefore, in order to overcome these difficulties and beginning June 2011, a 3R point of contact is planned as a joint venture between CAAT-Europe, the Doerenkamp Zbinden Foundation (DZF) and, if possible, other organizations specializing on alternative methods. The new point of contact provides practical advice for researchers, industry, and national authorities, helps with conceptualization of projects with regard to the 3Rs, and offers "tailor-made" presentations for specific working groups. In all cases, the advisory center is obliged to strict confidentiality. In light of the new EU Directive 2010/63, comparable points of contact will be required in Europe, if the aspired strengthening of the concept of 3Rs is not to remain a mere well-meant intention.

II-4-143

Benefits of post-approval monitoring

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Institutions where research teams use animals must apply best practices of animal use, and, more recently, establish a post approval monitoring (PAM). At the CRCHUM¸ PAM visits for protocols began in May 2008. The result of those visits is presented in visits reports that are sent to the institutional animal care committee as well as to the research teams. These reports, whether consistent or not, allow to identify the strengths and weaknesses in practices of the use of animals and better concentrate on the elements to improve. During the visits, the PAM helped identify refinement points that could be put into place.

Advice and refinement techniques mentioned in the visits reports are prepared in collaboration with the veterinarian and animal health technicians from the animal facility. The person responsible for the post approval monitoring meets the visited teams in order to communicate the report results. She discusses refinement points and the best way to implement them. For example, thanks to the combined efforts of everybody, we have succeeded in eliminating the use of barbiturates in survival surgeries, in favour of safer anaesthetics. In short, the visits are a pledge of quality and uniformity of care and gradually, are becoming a required element in the process of optimizing the care and in the creation of personalized training.

II-4-183

Nanoparticles in cosmetics: Does EU legislation allow animal testing – or not?

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In 2003, as a result of enduring public pressure, stepwise bans on animal testing for cosmetic ingredients and products and their marketing if tested on animals were implemented in the EU Cosmetics Directive. Concurrently, nanomaterials are increasingly being used in cosmetic products. Cosmetics range amongst the most important application areas for nanotechnological consumer products. Consequently, the 2009 European Cosmetics Products Regulation introduces specific provisions for nanomaterials, requesting a high level of human health protection.

In 2007, the European Commission's Scientific Committee on Consumer Products published an Opinion on the Safety of Nanomaterials in Cosmetic Products. The Committee expresses concerns about insufficient hazard information on nanomaterials, recognizes large data gaps in risk assessment methodologies and emphasizes that only *in vitro* methods specifically validated for nanomaterials are permissible for safety assessment. Until today, scientific problems, e.g. lack of relevant reference data, stand in the way of meeting this goal. Furthermore, cosmetic substances, such as nano-form titanium dioxide used in sunscreen products, are also produced for other purposes, and therefore might have been tested according to the REACH chemicals legislation, which includes animal testing.

The presentation discusses the animal testing implications of the diverging provisions. Also taking into account ongoing efforts to develop non-animal (nanomaterial) safety testing strategies, it proposes solutions on how to meet the citizens' expectation to purchase "cruelty-free" cosmetics. Notwithstanding all hurdles, the animal welfare provisions of the European cosmetics legislation have the potential for a success story, preventing animal testing without diminishing consumer safety – also for nanomaterials in cosmetics.

II-4-224

The industrial applicability of *in vitro* methods: the role of the In Vitro Testing Industrial Platform (IVTIP)

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IVTIP is an association of 45 companies with an active interest in (i) supporting and applying the principles of the 3Rs (Replacement, Reduction and Refinement of animal testing) for compound discovery, product development and assessment, and regulatory testing, and (ii) promoting the adoption of the fourth R (societal Responsibility). Member companies are represented globally from the following sectors: consumer products, pharmaceuticals, chemicals, cosmetics, and independent contract research organizations. IVTIP has established close contact with the European Commission in relevant Framework Programmes, ECVAM and EPAA. Recently, it has initiated a close collaboration with ESTIV and CAAT-Europe in order to improve the flow of relevant knowledge between academia, industry and regulatory authorities, to stimulate the application of *in vitro* tests by industry and to facilitate their acceptance by regulatory authorities. IVTIP endorses the US NRC's "Toxicity Testing in the 21st Century" strategy as the ultimate replacement of animal experimentation for regulatory/safety testing and focuses on the implementation of innovative strategies. IVTIP provides international discussion forums to address selected topics (e.g., "Toxicity Testing in the 21st Century" (Antwerp, Belgium, 2009), "Integrated Testing Strategies" (Geneva, Switzerland, 2010), and "Limitations of 3D Tissue Models" (Monaco, 2011)), and to identify and discuss novel tools, approaches and technologies in terms of relevance and applicability. The outcome of these discussion forums are published as peer reviewed papers in relevant journals. IVTIP has become an important stakeholder in the ongoing discussions on new regulations involving *in vitro* testing, thereby ensuring effective dissemination through transfer of both technology and knowledge.

II-4-228

Promoting the use and development of alternative methods for regulatory purposes and in research – ECVAM's DataBase service on ALternative Methods (DB-ALM)

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Since 2006 the DB-ALM (http://ecvam-dbalm.jrc.ec.europa.eu), ECVAM's DataBase service for ALternative Methods, is publicly available, providing ready-to-use information as peer-reviewed data sheets. The database includes alternative methods at all stages of development and validation. For the time being its focus is on toxicity assessment methods but not restricted to it and can be widened to mode of action and other experimental approaches. Today, 152 method-summary descriptions and 130 INVITTOX protocols are included providing all information needed to use the tests. In addition DB-ALM provides 82 evaluations and details on formal validation studies, 9163 test results, 5321 bibliographic references and contacts on over 200 persons/institutions active in the field of alternatives. Today, the service has over 2200 registered users from 75 countries coming from academia, industry and regulatory authorities.

Making information on alternatives easily accessible is key during authorisation processes for animal experiments. ECVAM will therefore continue to enhance its DB-ALM, both with regard to content and user interface. In addition, ECVAM's Search Guide project will provide search procedures and user guidance to facilitate the location of information on any 3Rs alternative together with an inventory of relevant resources. Its publication as a handbook and on the Internet is expected for this year. In future all ECVAM information resources will be accessible through a central access point nicknamed for the moment "CALISTO: ECVAM's Center of ALternatives Information SysTems & Orientation". This will include a tracking tool for ECVAM validations to allow stakeholders to follow the process from initial submission of a test to ECVAM until the validity is finally confirmed – or not.

II-4-282

Bridging the gap between validation and implementation: replacing animal use in vaccine batch potency testing

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As technologically advanced, high-throughput techniques are developed that can replace, reduce or refine animal use, harmonization of validated protocols between international regulatory authorities is necessary to foster wide-reaching implementation. Because regulatory acceptance does not guarantee that an approved non-animal method will be adopted for use by manufacturers and regulators, PETA's Regulatory Testing Division (RTD) has developed a multi-component process that (1) confirms the acceptability of data from novel methods by regulatory authorities, (2) distributes information on available and accepted non-animal approaches via stakeholder alerts, (3) publicizes accepted non-animal techniques, and (4) confirms manufacturer implementation of these methods.

By engaging with regulators and manufacturers, RTD employs this process to promote best practices while effectively reducing the reliance on older animal-use-intensive methods. This poster outlines the application of this paradigm to the use of non-animal vaccine batch potency tests, including detailed case studies of RTD's approach to fostering regulatory and industrial integration of *in vitro* erysipelas and leptospirosis vaccine batch potency tests. Successes include (1) verification of acceptance by regulatory authorities, (2) verification of use by vaccine manufacturers, (3) deletion of *in vivo* guidance documents, and (4) elimination of barriers to obtaining waivers for Target Animal Batch Safety Testing.

II-4-299

Animal protection through participation in the Organisation for Economic Co-operation and Development: The ICAPO model

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The International Council on Animal Protection in OECD Programmes (ICAPO), comprising eleven organisations representing more than 30 million members and supporters in Asia, Europe, North America, was granted invited expert status on the Test Guidelines Programme at the OECD in 2002. Since that time, ICAPO has sought to ensure the widest possible integration of non-animal testing methods in the OECD, an influential international organisation that develops harmonised guidelines and programmes for the assessment of chemicals. Through the use of internal and external scientific and policy experts, ICAPO advocates the replacement, reduction, and refinement of animal use within existing and new test guidelines. ICAPO participates in relevant OECD meetings and working groups, comments on OECD draft test guidelines and other documents, and nominates outside experts to take part in OECD activities on ICAPO's behalf. Activity topic areas include endocrine disruptors, (Q)SARs, nanomaterials, environmental and human health test guidelines, and existing chemical assessment approaches. This presentation will discuss ICAPO activities within the OECD and resulting outcomes, as well as opportunities and challenges inherent in working within the structure of the OECD. It will also discuss the International Council on Animal Protection in Pharmaceutical Programmes (ICAPPP), ICAPO's sister coalition formed to promote animal protection in pharmaceutical guidelines developed through tripartite agreement among Japan, Europe and the United States under the International Conference on Harmonisation (ICH). Unlike ICAPO, ICAPPP does not yet have official status but nevertheless has successfully implemented 3Rs initiatives within pharmaceutical guidelines.

II-4-344

The use of nonhuman primates in research: The Association of Primate Veterinarians as an educational resource for enhancing primate welfare

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The Association of Primate Veterinarians (APV) was founded in 1973 and consists of over 400 veterinarians around the world engaged in nonhuman primate breeding, care and oversight in research, zoo, and sanctuary settings. The organization recognizes the continuing need for judicious use of nonhuman primate models in scientific research for the foreseeable future. Because of their advanced cognitive capacity, APV accepts that nonhuman primates are difficult to manage well in captivity, that research use should be highly scrutinized and limited to the most essential studies, and that accepted standards and conditions for management of these species in captivity must be constantly scrutinized and refined to enhance animal welfare. Unfortunately, significant differences exist internationally in acceptable standards for nonhuman primate care and use, which may result in deficiencies in animal well-being. To achieve international harmonization of high standards of care, APV has as its vision to promote excellence in nonhuman primate knowledge, care, and compassion for better health and science. A key role of the organization is to develop, educate, and disseminate best practices for refinements in nonhuman primate care and management. APV has developed and published a number of guidelines documents to assist veterinarians, researchers, and animal care committees to enhance animal well-being. The organization also promotes a number of other educational tools, symposia, fellowships, and international veterinary exchanges to further the knowledge of those working with these species. This poster will describe APV's role in educating veterinarians and the research community in refinement of nonhuman primate care and use.

II-4-373

Application of alternative toxicological methods in safety testing of perfumery and cosmetic products in Russia

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In 2010, the Single Customs Union between Russia, Belarus and Kazakhstan was set up. The countries' participants of the Customs Union accepted the basic document "Uniform sanitary-epidemiologic and hygienic requirements to goods subject to sanitary and epidemiological supervision (control)". This document confirms the use of alternative *in vitro* methods for testing medical articles and equipment, personal care products, baby goods, household chemicals, and perfumery and cosmetic products (PCP).

In the practice of PCP control in Russia alternative methods began to be applied widely under the active participation and collaboration of research centers of The Russian Academy of Medical Sciences, Preventive Toxicology Division from the Centre of Hygiene and Epidemiology in Moscow of Rospotrebnadzor, RNIITO Rosmedtekhnologij. The Russian association of manufacturers of PCP, COLIPA, and leading manufacturers of PCP, in particular Unilever & SEAC, supported Russian toxicologists in this direction.

The development of alternative methods for safety testing of PCP in Russia is conducted in a number of directions:

– Revealing correlation between *in vitro* toxicity indicators and different selective effects on the laboratory animals;
– The research of schemes for alternative testing;
– Research of toxicity of different kinds of products on several *in vitro* test objects simultaneously for revealing the most adequate models.

Human skin fibroblasts, cattle sperm cells, luminescent bacteria and the HET-CAM test are used as test models. Three alternative *in vitro* methods have been confirmed for safety testing of PCP in Russia. The method of ultrasound dopplerography on the HET-CAM vessels for irritation testing of PCP is currently at the "statement stage".

II-4-387

Implementation of *in vitro* replacement technologies in regulatory drug testing – an innovation systems perspective

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The replacement of *in vivo* methods by *in vitro* methods in regulatory drug testing is rare. The aim of this research is to identify barriers and drivers of the replacement of *in vivo* methods by *in vitro* methods in Europe.

We studied two cases. The first case is the Draize eye test. Since 2009, the *in vivo* test is partly replaced by *in vitro* methods. The second case concerns EPO potency testing. Since the eighties, financial and scientific efforts have been made to replace the *in vivo* EPO potency test with *in vitro* methods; however the efforts failed to deliver expected outcomes. The innovation systems approach is used to identify the drivers and barriers regarding replacement of *in vivo* methods by *in vitro* methods in regulatory drug testing in Europe, such as the presence or absence of legislative pressure, legitimacy, and funding. Combining and comparing the outcomes resulted in an overview of potential barriers and drivers, and an indication of which of these factors are critical for replacement of *in vivo* methods by *in vitro* methods in regulatory drug testing. Policy makers could use these results to formulate policies that enable the replacement of *in vivo* methods by *in vitro* methods in regulatory drug testing.

II-4-416

Animal protection laws and regulations in India

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India has one of the most comprehensive animal protection laws. India's Constitution, in Section 51A (g), prescribes that it is the fundamental duty of every Indian citizen to have compassion for all living creatures. The Prevention of Cruelty to Animals Act 1960, and the Breeding of and Experiments on Animal (Control and Supervision) Rules 1998, provide for avoidance of experiments on animals wherever possible, and where animal are used they shall not be subjected to any cruelty. These provisions are enforced by an independent Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), under the Ministry of Environment and Forests. The use of animals must be first reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

The other most significant legal provision to animals is embodied in the Indian Wildlife (Protection) Act, 1972. Bonnet monkeys, rhesus monkeys, sharks, freshwater frogs, etc., are given legal protection. In compliance with the directions of the Hon'ble High Court of Delhi dated 19 May 1997, the Central Board of Secondary Education has decided to make dissection of animals optional to the students of Senior Secondary Classes. The University Grants Commission (UGC) has set up an Expert Committee to look into the possibility of banning dissection of animals for studies in zoology in colleges and universities. But the problem lies in the practice and enforcement. If only the laws are properly implemented and practiced, the animals in India will be the happiest lot.

II-4-451

Enhancing implementation of the 3Rs in daily practice – which way to go?

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In the Netherlands, like in many other countries, researchers are obliged by law to apply 3R methods in research using animals. Finding and implementing the 3Rs, however, remains a difficult task according to the results of our local and national survey among researchers. In the national survey questionnaires were also sent to Animal Welfare Officers (AWO) and members of Animal Ethic Committees (AEC). These groups also indicated that they experience practical difficulties in their legal obligations to apply the 3Rs. Implementation of 3R methods seems mainly to depend on a positive 3R-attitude of the researcher, a motivating and cooperative working environment, and an efficient professional network.

During an intensive workshop, researchers, AWOs and AEC members elaborated on how 3R implementation in practice can be enhanced. It was the first time in the Netherlands that these different professions, affiliated with different organizations (including academia, industry and contract research organizations), came together to discuss this topic in depth. The workshop resulted in 6 consensus statements covering policy, education and daily practice.

During the 8th World Congress of Alternatives the recommendations, based on the consensus statements, will be presented including the state of affairs concerning the first spin-off activities. In part, these survey results led to a focus shift of our research group towards implementation of systematic reviews of animal studies, a transparent and thorough method for accumulating and analyzing all relevant animal studies. Other suggestions for improvement will be made throughout the whole research chain, from public to researcher, from editor to legislator.

II-4-469

Prioritising promising 3R research, a helpful classification scheme

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The Netherlands have known a strong research tradition in the development of 3R methods, with the ultimate objective to decrease animal use and animal suffering as effectively and efficiently as possible. Unfortunately, the (inter)national acceptance and implementation of many of these methods tend to lag behind and thus, 3R expectations may not be fully realized.

Improvement of the exchange of knowledge between fundamental and applied research would help promising, innovative 3R methods on their way from development to implementation and use. To improve knowledge exchange within such specific promising research areas demands an interactive, tailor-made and chain-based approach, including activities ranging from raising awareness among a new generation of researchers, to the implementation of 3R methods into (inter)national law and legislation. But how do we define the most promising research areas and developments for the 3Rs?

A classification scheme was drawn up by the NKCA to help governmental authorities, researchers, companies and NGOs to prioritise their ongoing and new research activities. The scheme includes a set of criteria to classify research areas/projects as promising or less relevant for the ultimate application of 3R-alternatives. The classification criteria were divided in three categories; the scale of the problem, chance of success, and impact on animal use. Based on ethical, scientific, political or practical factors, increased value can be assigned to some specific criteria within these categories.

The classification scheme will be presented in the poster. It will also be available on request, by contacting the authors.

II-4-476

Animal welfare and Three Rs education: Filling the gap in interdisciplinary studies

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Scientific developments and evolving humane standards are pointing more than ever in the direction of finding alternatives to using animals as test models. However, the educational systems responsible for introducing attorneys, veterinarians, life scientists, social scientists and others to the 3Rs concept and also to animal welfare laws and regulations is woefully inadequate. With limited exceptions, law schools offer minimal instruction in animal law, regulatory decision making or risk management. Veterinary schools fail to provide their students with sufficient training in recognizing and reducing laboratory animal pain, or with the scientific theoretical framework for doing so. Many other graduate science programs minimize the importance of understanding and embracing the 3Rs and other animal welfare principles, and very few address the legal and social implications of decisions related to animal welfare.

These educational failures are structural; they are not dependent upon a particular school or discipline. Therefore the changes which must be made are also structural. If changes do not occur within the educational training of the professional schools, their graduates will not be well prepared to address the legal, social, scientific, economic and international issues related to animal testing today.

This presentation explores some of the history of animal welfare education in law schools, veterinary schools and other graduate science programs; explains why the 3Rs have not become an essential part of American education; and provides suggestions for change.

II-4-482

Improving 3Rs information in research publications

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This presentation will argue the case for making researchers more 3R-aware by expecting clear objectives and information on experimental design, refinements and husbandry practices in research papers. Currently some papers even fail to describe adequately the purpose of the study (5% of the 271 papers scrutinised by Kilkenny et al., 2009) so neither referees nor readers can judge whether replacement alternatives could have been considered, or alternative, more efficient designs or refinements used. There is typically no discussion of why the particular animal, model, design or group size was chosen, nor of what was done to minimise severity. It is usually not clear how the animals were selected for the experimental manipulations, how they were caged, or what if any environmental enrichment there was. Additional information available online could easily indicate why an option was chosen as well as giving details of what was done, and some of this might be expressed succinctly enough to be in the main text. Provision of such information would stimulate researchers to think of alternatives, and disseminate ideas for improved design and refinements applicable to a particular area of study more widely than local ethical review or networking.

Reference

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II-4-519

The American Veterinary Medical Association's Animal Welfare Committee: Educating veterinarians and the public regarding best practices for animal welfare

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The American Veterinary Medical Association (AVMA) represents a diverse group of over 81,500 veterinarians. A strategic priority for the AVMA is to be a leading advocate and authoritative resource for animal welfare, both for the veterinary community and the public at large. Veterinarians should be willing and capable to speak authoritatively on animal welfare and the principles of the 3Rs in all environs where animals are used. The AVMA's strategically diverse Animal Welfare Committee (AWC) and Animal Welfare Division (AWD) are charged with proactively identifying animal welfare concerns and opportunities, critically evaluating information including stakeholder concerns, and determining what actions might be most appropriate and effective to address concerns. In cases where no or insufficient information exists, the AWC recommends plans to address the knowledge gap in animal welfare. To educate veterinarians about refinements in veterinary medical care, the AWC has developed a set of policies that addresses many aspects of animal use, as sporting animals, food sources, research subjects, and companion animals. These policies are regularly reviewed and updated by the AWC to incorporate up-to-date evidence for enhancing animal welfare. The AVMA also sponsors national and international educational sessions and symposia related to animal welfare, as well as publications and projects that significantly impact animal care and use globally, including the AVMA's Guidelines on Euthanasia, Model Dog Care Act, and a planned Model Animal Welfare Veterinary Curriculum. This poster will address the role of the AVMA AWC and AWD in educating veterinarians and the public about animal welfare refinements.

II-4-524

AltTox.org: Communication platform for 21st Century Toxicology

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"21st Century Toxicology" deserves 21st century communication tools. AltTox.org is an interactive, online resource for professionals interested in advancing toxicology to better protecting human health or to reduce reliance on animal use. AltTox is intended to supplement more conventional means of sharing information, such as books, journals, and static websites. Its scope goes beyond that of websites that cover the activities of individual institutions or organizations. At the same time, it maintains a sharp focus on *in vitro* and *in silico* methods and relevant integrated testing strategies, and does not dilute its coverage by addressing other toxicological methods, fields of biomedical science, or areas of alternative methods. AltTox users include scientists, regulators, advocates, politicians, and others in industry, government, academia, and non-governmental organizations worldwide. The site provides relevant, concise, and up-to-date content, written in accessible language, as well as an interactive community platform (AltTox Forum), an extensive set of essays on "The Way Forward," a calendar of upcoming meetings, and listings of a variety of helpful resources. The website averaged over 8,000 visitors per month over the past year (an increase of 25% from the previous year) and nearly 17,000 page views per month. The website has a global reach, with 40% of visits from North America, 32% from Europe, and 22% from Asia. AltTox currently has approximately 60 "Way Forward" essays, over 1,200 subscribers to our monthly newsletter (AltTox Digest), and over 300 registered members of the AltTox Forum. Interested parties are encouraged to visit and contribute to AltTox regularly.

II-4-549

Programming study on 3R alternatives; how to focus 3R efforts in the Netherlands

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In 2010-2011, the Netherlands Knowledge Centre on Alternatives to animal use (NKCA) published the Programming Study on 3R alternatives, which was aimed at finding out how and in which research fields in The Netherlands, the knowledge exchange between fundamental and applied research needs improvement in order to get promising, innovative 3R-alternatives from development to implementation and application. The following aspects of stimulating 3R-alternatives are included: 1) priorities in ongoing and future activities (research, development and implementation); 2) recommendations for a more integrated approach of 3R-alternatives development; 3) the international context; 4) creating a favorable 3R research climate. Also included are the chains and legal frameworks of different application domains, to identify areas where (inter)national harmonization is desirable/essential for the implementation of 3R methods. A classification scheme (presented in a different poster) was drawn up to classify research areas/projects as promising or less relevant for the ultimate application of 3R-alternatives. Within fundamental research, the most promising research fields are: 1) development of human medicines; and 2) research towards cancer and other human diseases; and in applied research: 1) quality control of human medicine and biologicals (including sera and vaccines); and 2) toxicological risk assessment. Improved knowledge exchange within these areas demands an interactive, chain-based approach, including activities ranging from raising awareness among researchers to the implementation of 3R methods into (inter)national law and legislation. A strong dialogue on this subject between policymakers, research, corporate businesses and society will be needed. Our report is available on request by contacting the authors.

II-4-593

A new class of biomimetic, *in silico* models designed for increasing research efficiency while reducing animal use

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The new class of biomimetic, *in silico* models is fundamentally different from models used by the European Commission's BioSim Network (http://biosim-network.eu/). BioSim's plan, using established methods (simulation models of cellular and pharmacological processes constructed using continuous mathematics), was to obtain a deeper understanding of pathological and pharmacological processes, with the ultimate goal of achieving more rational drug development coupled with reductions in the need for animal experiments. We explain why the approach was stymied. Our objective has been the same, but our approach and methods have been designed specifically for achieving a reduction in the need for animal experiments. We build analogues of biological wet-lab counterparts: concretized, explanatory hypotheses about the mechanistic consequences of xenobiotic interventions built using object and agent oriented software components (Hunt et al., 2008, 2009; Hunt and Ropella, 2011). Interchangeable components link coarse-grained systemic phenomena to fine-grained molecular details. We draw on two examples (*in silico* livers and epithelial cells) to explain how the biological wet-lab side of the R&D process might function when these models and methods are fully implemented. Accumulated mechanistic knowledge is easily measured and visualized in action. Components within analogues validated for many compounds can use programmed "intelligence" to automatically parameterize for, and respond to, a new, not previously seen compound based on its physicochemical properties. Encouraging exploration of this new model class (by the R&D community) will help make clear how its scientific use will lower costs and expedite achieving R&D objectives. Animal use will be reduced because *in silico* experimentation will focus the scientific question being asked, and that will incrementally reduce the need for exploratory animal experiments.

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II-4-596

Effecting change in animal welfare at a national level – The role of the Canadian Veterinary Medical Association

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Refining animal welfare practices is a key priority for the Canadian Veterinary Medical Association. The organization is a national science and ethics based resource for veterinarians, veterinary students, provincial and federal governments, and the public regarding animal welfare standards for all species. Through the actions of its Animal Welfare Committee (AWC), the CVMA seeks to educate members and others regarding acceptable practices for animal care and use, leading to enhancement of animal well-being. The CVMA's AWC achieves their goals in a number of ways. Leadership and active advocacy for enhancing animal welfare is demonstrated through development of position statements that consider current knowledge and scientific evidence to refine treatment and care of animals. These position statements are developed with member consultation and advice from pertinent stakeholder groups. In addition, the CVMA's AWC develops and disseminates educational tools, such as posters on various topics, for veterinarians, students, and the public, which are strategically geared to create shifts in thinking about currently accepted practices; maintains a roster of spokespersons to address national animal welfare issues, publishes peer-reviewed papers on current animal welfare topics; and develops codes of management and husbandry practice for species not covered by national producer organizations. The CVMA takes an active advocacy and lobbying role at a national level with a number of nongovernmental and governmental organizations and committees to effect policy changes in accepted best practices for animal welfare. This poster will explore the CVMA's role in effecting enhancements for animal welfare in Canada.

II-4-605

Canadian Association for Laboratory Animal Medicine: Promoting research animal welfare coast-to-coast

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The Canadian Association for Laboratory Animal Medicine/ L'Association Canadienne de la Médicine des animaux de laboratoire (CALAM/ACMAL) was founded in 1982 and is the national organization that represents the interests of Canadian laboratory animal veterinarians working to support the humane care and use of animals used in research, teaching, and testing. The vision of the organization is to be recognized and respected as leaders in laboratory animal welfare. The central document to the CALAM/ACMAL vision is the Standards of Veterinary Care, which was last updated in 2007. The document emphasizes that CALAM/ACMAL and its individual members have a responsibility to provide leadership in developing best practices for the humane care and use of animals in research, teaching, testing and production, with due consideration of the 3Rs: replacement of animals used, when possible; reduction of numbers of animals used; and refinement of techniques and procedures employed. CALAM/ACMAL recognizes that the well-being and welfare of animals used in research, teaching, and testing are the main focus for all laboratory animal veterinary roles and responsibilities. For laboratory animal veterinarians, animal welfare includes physical and behavioral aspects of an animal's condition, evaluated in terms of environmental comfort, freedom from pain and distress, and provision of appropriate social interactions. The organization promotes a number of educational tools, symposia, and fellowships to veterinary students and veterinarians to facilitate the exchange of knowledge and harmonization of standards of veterinary care for Canadian laboratory animal veterinarians. This poster will describe CALAM/ACMAL's role in promoting research animal welfare.

II-4-713

REACH regulation: Ensuring safety of industrial enzymes – is animal testing necessary?

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Enzymes for technical applications have to be registered under the EU chemicals regulation, REACH. The enzyme industry has to ensure that production, handling and use of the enzymes is safe throughout the supply chain for consumers, workers and the environment. Enzymes are produced by fermentation and categorized as UVCB's (Unknown/Variable composition, Complex reaction products or Biological materials). Identity and sameness is based on enzyme identification according to the specific catalytic activity of the enzyme.

The safety documentation of an enzyme product consists of two elements, safety of the enzyme protein and safety of the non-enzymatic constituents derived from the fermentation process. Apart from being potential respiratory sensitizers and the minor risk of skin/eye irritation for some proteases, enzymes in general have a low risk profile. Safety of the "other constituents" is therefore in focus and is closely linked to the safety of the production strain used for the fermentation. However, when an enzyme of known catalytic identity has been produced by a well-characterized, non-pathogenic production strain following good manufacturing practices then this enzyme should be regarded as safe for use. According to our experience, future toxicological testing of enzymes can be avoided by applying read-across and data waiving without extensive *in vivo* toxicology programs. Risk assessment includes exposure assessment related to the few possible hazards mentioned above. If required, relevant *in vitro* alternatives will be applied for specific issues.

In conclusion, hazard characterization of biological substances like enzymes requires an alternative approach compared to the hazard characterization of "classical" chemicals.

II-4-715

A partnership between the Australian National University and the MAWA Trust leads to the establishment of the Australian Centre for Alternatives to Animal Research

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As a result of the implementation of successive editions of the *Australian Code of Practice for Care and Use of Animals for Scientific Purposes*, and representative participation in the *World Congresses on Alternatives and Animal Use in the Life Sciences*, the Australian National University (ANU) has made significant progress with implementation of the 3Rs. This is true of refinement methodologies, the replacement of animals in teaching, and research animal reduction through the Ethics Committees' recognition of statistical validity requirements for experimental animal numbers. Progress, however, towards the aim of animal replacement in fundamental biomedical research, which is widespread at the ANU, is slow.

The Medical Advances Without Animals Trust (MAWA) operates an independent medical research trust fund and is committed to advancing methodologies that replace the use of animals and animal products in biomedical research. To stimulate greater interest and activity in this regard, MAWA awarded funds to the ANU for a Fellowship and the appointment of an Associate Professor in Alternatives to provide scientific leadership in replacement research. A key objective of the ANU and MAWA partnership is to establish *The Australian Centre for Alternatives to Animal Research* (ACAAR) to support the development of non-animal research alternatives across Australia. The ongoing research focus associated with the Centre will be directly on human biology and thus has the additional advantage of encouraging the translation of fundamental medical advances to the clinic. The ANU based research programme will begin by developing alternative methodologies using international expertise in computational biology and bioinformatics.

Session II-5: Validation of Three Rs alternative methods

Session II-5: Oral presentations

II-5-561

Validation of the 21st Century Toxicology Toolbox: challenges, opportunities, and the way forward

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Increasing efforts are being directed at finding improved innovative methods for assessing whether chemicals may cause adverse health effects. Collectively referred to as the 21st Century Toxicology Toolbox, these methods includes a wide range of tools that increasingly incorporate understanding and detection of the molecular, genetic, structural, and cellular perturbations of pathways and mechanisms that may lead to adverse health outcomes. Applications include toxicogenomics, metabolomics, proteomics, cell based assays, biochemical activity profiles, and computational models. These tools are used to create complex biological activity profiles, with an expectation that these will eventually predict toxicity and safety without the use of animals. Much of this profile data will initially be used for prioritizing chemicals for further testing in validated test methods, decisions on product development, as mechanistic data to inform weight of evidence decisions on chemical safety, hazard, and risks, and to reduce uncertainties in risk assessment. Using such data to make regulatory risk assessment decisions will require validation to demonstrate that the proposed decision strategies can provide equivalent or improved protection of consumers and workers compared to existing test methods. Flexibility in the validation of these new tools and strategies is essential, and will vary depending on the intended purpose, applicability domain, and existing data for the proposed tools. Consideration and use of appropriate validation strategies early in the test method development process is expected to expedite acceptance of new tools and approaches that will provide improved predictions of safety and hazard and reduce and replace animal use.

II-5-688

Post-approval validation issues: Experience with the 3T3 NRU *in vitro* phototoxicity assay

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Validation of alternative toxicological tests is historically done by comparing results generated by the alternative test against results generated by the accepted "gold standard". However, due to many limitations the number of compounds that can be used for validation remains rather small in comparison to the entire chemical universe. It is therefore logical to assume that the practical application of a validated test will generate information that can and should be used to review the test performance and to improve the original method, if needed.

Such a "post-validation" review was initiated in 2009 by EFPIA, when it informed ECVAM that it would have data from industry that indicate that the 3T3 NRU phototoxicity test, when applied to oral intake, generates an inacceptable high number of false positives, hence triggering a large number of confirmatory animal tests. ECVAM then proposed to organise a workshop with EFPIA where industry could present its data and phototoxicity experts could discuss how to approach the issue.

This workshop took place in October 2010 and the workshop report will most likely be published before the WC8. However, this paper will not address the specific finding of the workshop but discuss the overall usefulness of such reviews and the conditions to make these a success.

As a second example some cell transformation assays will be discussed for which ECVAM proposes that standardised protocols, developed in the course of a prevalidation study concluded in 2010, should be used for future application of that type of test. ECVAM also invites test users to provide ECVAM with the test results in order to allow a future review of the methods.

II-5-289

Reduction of animal use through validation of a chemical method of detection for paralytic shellfish toxins

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Shellfish toxins are produced by algae and accumulate in filter-feeding shellfish. Paralytic shellfish toxins (PSTs) are particularly serious since toxin levels can rise quickly and cause death. The Canadian Food Inspection Agency (CFIA) analyses more than 11,000 samples of Canadian shellfish for PST levels each year. Until recently the reference method for PSTs was a mouse bioassay (MBA) which used three mice/sample for a total of approximately 40,000 mice/year. In 2005 a collaborative, multi-year project was initiated with the National Research Council Canada to develop and validate a chemical PST method to replace the MBA.

Available methods were evaluated. The best alternative was optimized for a high-throughput regulatory laboratory and used in parallel with MBA testing for one summer as a pilot project to overcome challenges with high sample load. A single lab validation was then completed at the CFIA Dartmouth Laboratory, followed by approval from Canadian and US officials to use this method for screening during a transition period. During this transition period a small percentage of samples received by the laboratory (10%) and all results requiring regulatory action were confirmed with MBA. Animal use was decreased by 75%. To gain international acceptance of the new chemistry-based procedure a collaborative study was carried out among labs from ten countries. This study confirmed the method could be implemented successfully in different laboratories and has led to the method being granted official method status by AOAC International. Animal testing for PSTs in all CFIA labs has now been reduced or eliminated.

II-5-203

The limited value of acute toxicity tests in safety assessment

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A collaboration, led by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and AstraZeneca, analysed data from 70 compounds across therapeutic areas and demonstrated that acute toxicity studies had no value in assessing risk before the first clinical trials in humans. Additionally, consensus between clinicians, toxicologists, regulators and directors of Poison Centres has been reached that acute toxicity studies are not used for managing overdose of pharmaceuticals and are of little value in treating human poisoning from chemicals. Therefore, the last remaining driver for acute toxicity studies for pharmaceuticals has been removed.

The impact of the pharmaceutical sector initiative has stimulated efforts to explore the value of acute toxicity testing for other sectors. An expert working group, led by the NC3Rs, has highlighted circumstances where acute toxicity testing of non-pharmaceutical chemicals is redundant and may be avoided.

In addition, the European Partnership for Alternative Approaches to Animal Testing (EPAA), has established a multi-stakeholder team (including AstraZeneca, NC3Rs, ECVAM, the Humane Society and representatives of industry sectors) which has demonstrated that the primary regulatory driver for conducting acute toxicity studies across non-pharmaceutical sectors is for classification and labelling. Further work into the value of acute toxicity studies for classification purposes is ongoing.

These collaborations demonstrate the opportunities provided by creating a forum for a wide range of stakeholders to review whether animal toxicity studies are providing the information needed to make assessments of risk to human safety. The results will enable consensus to be reached on how to reduce the number of animals used and make the drug and chemical development process more efficient.

Session II-5: Poster presentations

II-5-192

Developing regulatory acceptable *in vitro* alternatives to established *in vivo* assays

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In the last 15 years there has been a significant change in the safety assessment paradigm that has seen the introduction and regulatory acceptance of an increasing number of alternative *in vitro* methods as replacement for, or supplement to, existing *in vivo* test strategies. This has been driven by both the search for scientific advancement and ethical considerations. Within the EU, in particular, this has also been driven by changes in the regulatory legislation relating to chemical (REACH) and consumer products (7th Amendment to the Cosmetics Directive). A major challenge in developing alternative methods is the need to validate against a known human endpoint and not *in vivo* animal data. In this presentation we will discuss some of the assays (e.g. dermal absorption, skin and eye irritation, phototoxicity, drug transporter and hepatic metabolism) that have been developed over the last 15 years. We will highlight how these have assisted in achieving the goals of the 3Rs and discuss some of the issues facing the validation of new assays for more complex toxicological endpoints.

II-5-321

InVitroJobs – communication network and job platform presents "Working group – a Portrait"

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Against the backdrop of European legislation (REACH, Cosmetics Directive, new EU Directive on animal experiments), the development of replacement methods gains particular significance. The Federal Association People for Animal Rights has creating the internet platform InVitroJobs, providing support via the publication of job offers, thesis assignments and internships for budding scientists and students interested in working with animal-free methods. InVitroJobs also offers a forum to many international and renowned working groups with promising approaches to replacing animal experiments in research, development or service.

Since its online launch in 2009, InVitroJobs has grown continuously, with job offers, working groups and online visitors on the rise (over 90,000 visitors so far, more than 400 per week). Approximately 50% come from Germany, followed by visitors from the United States. 15 percent of all clicks were job offer-related, followed by searches for in-depth information on *in vitro* and *in silico* working groups.

As the results of the working groups' research is often known only to professionals, and above all because there is a need for an overview of contents and methods in the area of replacement methods, InVitroJobs has recently started a regular description of scientists and their innovative research, "Working Group – a Portrait". The focus is on newly-developed methods, their evaluation and a forecast as to which animal experiments they can replace. The presentation of the first working group immediately generated considerable interest in participation. This indicates the need for public education and presentation.

II-5-371

An evaluation of the Reconstructed human Epidermis (RhE) method for predicting skin corrosivity of chemical products with extreme acid pH

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The purpose of this analysis was to evaluate the Reconstructed human Epidermis (RhE) model as an *in vitro* method to predict skin corrosivity (OECD 431) for acid products with extreme pH (< 2) when compared with *in vivo* data and the AISE Method (The Worst Case Table) of classification. Extreme pH can be a useful predictor of irritation but may lead to over classification in weakly buffered systems. Our objective was to determine whether the RhE model could accurately identify corrosive and non-corrosive acid products. When compared with the *in vivo* data, 2/7 products tested using the RhE method predicted the same skin classification. The skin classification of the remaining five formulas was over-predicted when compared with the *in vivo* data. There were no products for which the RhE under-predicted the skin classification when compared to the *in vivo* results. When compared with the AISE Method (which considers the results of the EU conventional method calculation and pH/acid reserve), 8/23 products tested using a RhE method predicted the same skin classification. The skin classification of the remaining fifteen formulas was over-predicted when compared with the AISE Method. There were no products in which the RhE under-predicted the skin classification when compared to the AISE method. Overall, the RhE did not reliably identify non-corrosive formulations when compared to either the *in vivo* data or the AISE method. This presents significant challenges under hazard classification guidelines such as the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) which recommends testing with a validated *in vitro* method to confirm a non-corrosive classification for an extreme pH product.

II-5-534

Update on validation status and industry utilization of normal human 3D (NHu-3D) tissue models in toxicology

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Progress in *in vitro* tissue engineering over the last several decades has contributed to a significant decrease in animal use for industrial risk and safety assessment of products and ingredients. MatTek Corporation has a long-standing involvement in development, production and validation of animal alternative *in vitro* models produced from normal human cells (NHu-3D models). The currently available suite of NHu-3D models includes: Skin (EpiDerm, MelanoDerm, EpiDerm-FT), Ocular (EpiOcular, EpiOcular-FT), Airway (EpiAirway, EpiAirway-FT), Vaginal (EpiVaginal, EpiVaginal-FT), Oral (EpiOral, EpiGingival, EpiGingival-FT) and Dendritic Cells (DC-100). EpiDerm NHu-3D skin models are widely utilized in preclinical toxicology and efficacy applications during industrial product development (e.g. skin penetration, skin lightening, irritation, corrosion, ingredient efficacy and wound healing). In addition, the EpiDerm model is formally validated by ECVAM and accepted by OECD for regulatory use in skin corrosion (OECD TG 431, 2004), and irritation (OECD TG 439, 2009) and is pre-validated for phototoxicity testing. The EpiOcular model is widely utilized for ocular irritation studies during industrial product development. The EpiOcular EF50 assay is currently an EPA accepted method for testing of antimicrobial cleaning products. In addition, an EpiOcular eye irritation test (EIT) is currently undergoing formal COLIPA-ECVAM validation for regulatory use. Additional NHu-3D models and assays for toxicity, allergenicity, genotoxicity, and other animal alternative applications are under development. Thus, NHu-3D models have made significant contributions to reduction of animal use in industrial product development and regulatory testing. MatTek will continue to devote focused efforts toward further elimination of animal use in biological science and regulatory testing.

II-5-645

Multi-study validation trial for cytochrome P450 induction providing a reliable human-metabolic competent standard model or method using the human cryoHepaRG® cell line and cryopreserved human hepatocytes

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The Institute IHCP-JRC of the European Commission organised under the auspices of ECVAM a stakeholder meeting in the field of Toxicokinetics and Metabolism in which the basis for the current multi-study validation trial for cytochrome P-450 (CYP) induction was established. The main objective of this trial is the provision of a reliable human-metabolic competent standard model or method for use in integrated testing strategies. It was agreed to initially assess the potential for CYP induction at clinically relevant doses in selected *in vitro* test systems since CYP induction is a sensitive indicator of *de novo* protein synthesis. In this way human clinically obtained data on CYP induction could be used as the reference data. Based on the new legislation for cosmetics (Directive 2003/15/EC) and chemicals (REACH Regulation 1907/2006/EC) emphasising the need for alternative methods and integrated non-animal test strategies concerns have been raised over the need for regulatory purposes of a reliable and relevant human metabolic competent source modelling the process of xenobiotic biotransformation.

The CYP induction validation trial will assess the reliability (reproducibility within- and between-laboratories) and relevance (ability to assess *in vivo* human CYP induction) of two test systems (cells in culture) with a challenging set of test items (chemicals) for which high quality *in vivo* data are available. In this validation study the test systems cryopreserved HepaRG® and cryopreserved human hepatocytes will be used. Five test facilities are involved including, the CRO's Pharmacelsus and Kaly-Cell as the lead laboratories, the pharmaceutical companies Astra Zeneca and Janssens Pharmaceutica, a division of Johnson and Johnson and the European Commission JRC ECVAM *In vitro*-Methods Unit and Systems Toxicology laboratories. The result of this validation trial will be the starting point for a novel *in vitro* platform for assessing biotransformation and toxicity.

Session II-6: Setting limits and resolving conflicts between the Rs

Session II-6: Oral presentations

II-6-533

The 3Rs principle – mind the ethical gap!

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Over its 50 years of existence, the 3Rs principle has become a tremendous success. As an idea it is widely known and generally well accepted, it has made its way into many legislative texts and guidelines and it is often referred to in communication with the public and with potential critics of animal experimentation. The principle seems to unify the concern for better science with the concern for higher ethical standards. And the principle seems so clear and comprehensive that it is tempting to believe firstly that its full implementation is merely a practical and technical matter and secondly that once it is implemented it only takes good communication skills to achieve a wide public consensus about laboratory animal use. However, we think that both these beliefs are deceptive.

Firstly, we argue that underneath the seemingly clear surface of the 3Rs principle are both ambiguities and dilemmas which have clear ethical significance. Thus it is unclear what counts as reduction – is it in absolute or in relative numbers? Also the choice of species gives rise to questions in relation to refinement – is it always a refinement to move from a "higher" to a "lower" species? Between the 3Rs there are some obvious dilemmas. In fact, those working with the development and implementation of the 3Rs have more or less divided into two tribes – one focusing on replacement of live animal experiment and the other on reduction and refinement of actual animal experimentation – with rather limited contact between the two. Another obvious dilemma is between reduction and refinement where often researchers must choose between trying to cut down on the number of animals used or to limit the amount of discomfort or suffering imposed on the affected animals.

Secondly, we argue that there is a need for a more explicit ethical discussion concerning how to deal with the aforementioned dilemmas. This discussion will both link up with a wider discussion regarding our right to make use of animals to further human goals, and it will relate to specific principles regarding animal use. For example there may be a tension between a principle aiming to minimize the number of animals harmed and a principle of fairness focusing on improving the lot of the animals most badly affected.

Our main conclusion will certainly not be to question the usefulness of the 3Rs principle. Rather it will be a call for a greater awareness of the underlying ethical issues and the need for an explicit discussion of these issues.

II-6-140

A new approach to replacing primates in biomedical science: accessing the views of scientists

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The use of primates in biomedical science has always been a contentious and vehemently debated issue. Yet the arguments on both sides have largely remained unchanged and there has been little progress towards replacing primate models. Presented here is an ongoing PhD project, using a novel multidisciplinary approach, which includes interviewing relevant experts to try to unravel and understand the multifaceted factors involved in the debate. The overall aim is to assess the feasibility of phasing out primate models by investigating two fields of research in particular, schistosomiasis and Parkinson's disease.

The unique insight provided by this approach will be used to discuss how scientists justify their selection of experimental model and choice of research area. Some of the preliminary findings from the interview data will be presented. These will illustrate how factors such as personal ethics, regulation, scientific relevance, and potential health benefits influence how those conducting the studies view the feasibility of replacing primate models in these two fields of research.

II-6-108

Beastly bias and species choice

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Concerns about the welfare of animals are dependent on the assumption that they are sentient, that is, that they have feelings, and those feelings matter to them. Species choice in research is usually determined by scientific reasons, or availability. However, where species choice is possible, European Directive 6106/1/10 requires that animals with the least capacity to experience pain, suffering, distress or lasting harm be used. Within the UK, particular justification is currently required for the use of non-human primates, equids, dogs and cats, and the use of New-World Monkeys over Old-World Monkeys is favoured.

However, species choice on welfare grounds is not a simple matter. Should a fish always be used in preference to a mouse? A mouse to a primate? A bird to a rat? The reality is that it is not easy to determine a species' capacity or relative capacity to suffer. Criteria used to argue for sentience such as encephalisation, complexity and behaviour are not reliable indicators and there is no evidence that pain perception varies. Some argue that primates should be given special status because of their cognitive abilities, but complex behaviours possessed by primates are also found in fish.

Legal controls rightly take account of public views, but these may not always be correct in animal welfare terms. Those making species-choice decisions need to be aware of legislation, but also should make as explicit as possible reasons for considering that one species would suffer more than another in a particular study.

Session II-6: Poster presentations

II-6-497

Endpoints for humane sacrifice in non-clinical safety studies

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The drug development process requires regulatory toxicity studies in animals to assess non-clinical safety aspects. Such studies monitor the potential adverse effects of new drug compounds. It is the ethical responsibility of the scientific community to define the upper acceptable level of adverse effects, while striking a balance between scientific and ethical demands. We established internal guiding principles that describe endpoints for humane sacrifice and assist decision-making when the upper acceptable level of adverse findings is reached. Our guiding principles are based on national and international guidelines and help in the assessment of signs leading to humane sacrifice in experimental animals. The attainment of common guidelines regarding the recognition of animal suffering and appropriate response increases the validity of data from animal studies.

II-6-558

What can regulatory toxicology and other scientific disciplines learn from Three Rs approaches used in the shellfish toxin testing arena?

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European safety monitoring programmes to detect marine biotoxins in shellfish harvested for human consumption have historically relied heavily on the use of Mouse Bioassays (MBAs). MBAs have a number of potentially serious limitations including variability and false negative and positive results. Importantly, in common with many other toxicological assays, shellfish toxin MBAs cause significant animal suffering.

Over the last 15 years, UK laboratories have used a number of strategies for refinement, reduction and finally replacement of these MBAs. Tactics to reduce animal suffering and numbers have included reduction of duration of tests and the number of animals used for each sample, the use of anaesthesia, the use of defined clinical endpoints (rather than death) and alternative pre-screening methods. Using a combination of these approaches, a steady reduction in the use of mice has been achieved, thus significantly reducing animal suffering.

However, such changes have been accompanied by the threat of infraction proceedings for technical non-compliance with European food hygiene legislation. Over-specification in these regulations did not allow for implementation of the Three Rs even where equivalent, or greater, public health protection is afforded by the alternative. A more flexible European Food Safety Regulation was finally delivered in 2010. This required funding (for alternatives research) and prioritisation (for the scientific validation) combined with pressure from enlightened regulators as well as from animal welfare interests.

Can these lessons be usefully applied in other regulatory areas? This presentation will explore the possibilities and consider examples.

II-6-581

The replacement of animals in shellfish biotoxin testing: a global perspective

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Earlier this year, European Union member countries voted to phase out the use of the Mouse Bioassay (MBA) for testing marine biotoxins and replace it, for the majority of biotoxins, with the non-animal LC/MS (Liquid Chromatography-Mass Spectrometry) method. The decision for replacement came after years of pressure from various stakeholders, highlighting the scientific limitations of the lethal test, which causes "substantial" suffering to animals used and displays direct nonconformity with the tenets of EU Directive 86/609 for the protection of animals used for scientific purposes[1]. Nonetheless, there remains much disparity among EU member states in their eagerness to replace the MBA, which currently consumes 600,000 mice[2] in Europe every year, and this becomes even more complex when the situation is observed globally. In a North American context, the use of the MBA is still permitted for testing certain marine biotoxins despite readily available non-animal, scientifically robust methods. The lack of global harmonisation on this issue is problematic when the MBA has been described by the European Food Safety Authority (EFSA) as "inappropriate with inherent uncertainty, variability and poor specificity"[3] for the testing of most biotoxins, and thus cannot be relied upon to ensure safety to consumers. This presentation will articulate a strategy for a harmonised international shellfish- monitoring programme.

1 Article 7: Member states "[....] *shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal*".
2 Intergroup on the Welfare and Conservation of Animals. Report of the 264th Session. Biotoxins and shellfish safety. 8 July 2010.
3 EFSA CONTAM Panel Scientific Opinions, available at: http://www.efsa.europa.eu/en/scdocs.htm

Theme III
Incorporation of the Three Rs in Education and Training

Session III-1: Innovative teaching in the life sciences

Session III-1: Oral presentations

III-1-101

Is animal free teaching in the life sciences better teaching?

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Traditionally life science disciplines such as physiology and pharmacology employ, as part of a mix of teaching methods, practical classes based on experiments on whole animals or isolated organ or tissue systems. Such classes are designed to meet specific learning objectives: re-enforcement of (existing) student knowledge; and teaching a variety of generic and preparation-specific laboratory skills, and experimental design skills. They also provide vehicles for teaching data handling, scientific communication, and team working skills and of course they promote staff-student interaction. Over the last 20 years, at least in the UK, the number of life science students entering universities has increased significantly and to cope with such numbers courses have reduced the number and diversity of laboratory classes, sometimes replacing them with more innovative, often technology-based approaches.

This presentation will introduce Session III-1: "Innovative teaching in the life sciences" and will cover the various types of technology-based alternatives currently available to teachers and how they meet learning objectives. Methods of successful integration of resources into mainstream teaching will be described together with data drawn from a number of studies to compare the educational effectiveness of computer-based learning with more traditional methods.

It will provide an overview of how technology has changed the way life sciences are taught, how innovations have been implemented and explore whether today's courses meet learning requirements.

III-1-062

Challenges of using alternatives to animals in laboratory classes in physiology: the Spanish experience

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Physiology is taught in all the life sciences, including medicine, pharmacy, biology, veterinary medicine, biochemistry and others. Teaching physiology entails both theoretical and practical classes, and the latter have traditionally involved the use of animals. Traditional practices include dissection, the muscle-nerve preparation of frogs or rats, the study of the effect of hormones on sex glands in rats, study of the heart in frogs, study of intestinal absorption in rats, and many others.

In recent years many efforts have been made to develop methods that do not use animals, such as models, mannequins, computer programs, and others. Similar efforts have been made to encourage members of the educational community to adopt such methods. Nevertheless, efforts to replace traditional practices using animals by these alternative methods have encountered many difficulties, especially the reluctance of teachers to change.

Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes clearly states: "The use of animals for scientific or educational purposes should therefore only be considered where a non-animal alternative is unavailable". Based on this, there is no justification to use animals, since there are many developed and marketed alternatives available.

In this talk we will examine the reality of the use of animals for educational purposes in Spain, the efforts to change the minds of teachers, and the opinion of students on this matter.

III-1-205

Alternatives to animals in teaching: Experience in an Indian medical school

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In the 1990s, the practical classes in pharmacology for the undergraduate medical course in JIPMER, Pondicherry, India included 15 animal experiments. It was mandatory for students to carry out animal experiments to pass the final examination. With the advent of the new curriculum prescribed by the Medical Council of India (MCI) in 1997, the number of animal experiments was reduced to seven. CAL software on the effect of drugs on dog blood pressure was developed in-house and introduced into the course, and it replaced the use of dogs completely. A set of clinical pharmacology exercises was also introduced as alternatives. As the feedback from students was encouraging, a few more computer simulated experiments developed in-house were introduced to replace some more animal experiments. At present, five live animal experiments and two computer simulated animal experiments are conducted. The MCI in 2010 clarified that animal experiments can be replaced with suitable alternatives. This paved the way for complete replacement of animal experiments in teaching but due to difficulties in implementing the alternatives, the pace of replacement is slow. Our department has developed, distributed and used CAL packages from the late 1980s and set up a CAL laboratory in the last decade. It is expected that with the impending revision of the medical curriculum by the MCI, all animal experiments will be replaced soon. The results of a survey conducted among the teachers on the usefulness, acceptance and barriers with respect to the use of alternatives to live animal experiments will be presented.

III-1-413

Animal use in pharmaceutical drug discovery and development – current status and future directions

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The pharmaceutical industry researches and develops new, safe and efficacious medicines for patients. The process that leads to the development of a new medicine is long and complex and involves the use of animals at multiple points of the drug discovery and development process. While *in vitro* assays play a significant role in high throughput screening of potentially active molecules during the early stages of the discovery process, studies with animal models are essential to elucidate disease mechanisms and demonstrate efficacy. Furthermore, regulatory agencies require evaluation of the safety of potential medicines in animals before entry into humans and then later in the drug development process. Therefore, to successfully bring forth new medicines to patients, the pharmaceutical industry requires staff with a wide range of skills in the conduct of *in vivo* studies with both rodent and non-rodent species.

However, many pharmaceutical companies are committed to finding and implementing approaches that aim to Refine, Reduce and Replace (3Rs) the use of animals in discovery and development programs for new therapeutics. Through recent advances in scientific knowledge and technologies, alternatives to testing on animals, such as the use of physiologically based pharmacokinetics modeling, is increasingly being adopted as part of the drug discovery and development strategies.

While these methods enable reduction in the number of animal experiments and in the number of animals used in each experiment, they do not completely eliminate animal use in pharmaceutical development. Several examples will be provided to demonstrate how these approaches have been used in conjunction with animal studies during the drug discovery and development process.

III-1-448

Educating the next generation of *in-vivo* scientists: Meeting the needs of industry & academia

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The 2007 Association of British Pharmaceutical Industry report "*In-vivo* Sciences in the UK; Sustaining the supply of skills in the 21st Century" highlighted the need to continue to provide an education and training in *in-vivo* sciences in order to ensure that the United Kingdom has sufficient *in-vivo* scientists with the necessary skills to maintain its position as a world leader in biomedical research. The report also concluded that it is essential that this training is provided at the earliest possible opportunity.

This presentation will argue for the need to provide a select cohort of undergraduate students who intend to follow a career in biomedical research, either in industry or academia, with an education and practical experience in integrative studies. It will also argue that all students on undergraduate courses in the biomedical sciences should be provided with some exposure to isolated tissue experimental preparations in the course of their studies. However, any such training must be accompanied by a substantial training in the ethics of the use of animals or animal tissues in research, complimentary experimental techniques and the principles of the 3Rs.

The presentation will also include the perspective of an undergraduate pharmacology student who has completed an *in-vivo* Industrial Placement year, a final year *in-vivo* techniques module and utilised an *ex-vivo* preparation in her Final Year project.

Session III-1: Poster presentations

III-1-250

Live zoology and digital technologies as effective alternatives for animal use in zoology curriculum: A success story from MDS University Ajmer, India

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A large number of animals are killed every year for laboratory exercises all over the world. This has caused great biodiversity loss and some animal species have even become endangered due to such practices. While analyzing the curriculum of zoology of most universities of the world, it has been observed that animal dissection has been overemphasized in schools, colleges and universities in the name of understanding external and internal organization of the body. Very little emphasis has been put on studying animals in nature. With the advancement of digital technologies and excellent documentaries on animal life, we took on a mission to replace dead zoology with live zoology at undergraduate as well as post-graduate levels at MDS University, Ajmer (Rajasthan), India and framed a new curriculum having exercises such as studying species in nature, their current status, threats, behavioral ecology, identification and monitoring based on sound spectrum software (sonotaxonomy), understanding internal organization, molecular biology and physiology using software. The feedback response from students and teachers is very good. The highest regulatory bodies in school education (CBSE/NCERT) and higher education (UGC) have appreciated the idea and soon the recommendations are to be implemented in the remaining educational institutions in India. The result of implementation of this progressive curriculum based on live zoology is such that some new records, species and behavioral patterns have been identified by the young students. The change can potentially motivate a large number of students to opt for zoology programs. We are endeavoring to orient teachers and resource persons in the country and outside the country with the modern curriculum and related techniques.

III-1-307

In vitro toxicology training programs at Mahatma Gandhi-Doerenkamp Center (MGDC), India: a status report and a review

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Mahatma Gandhi-Doerenkamp Center (MGDC) has been established in India by DZF, Switzerland, to motivate the teachers and students to take to "alternatives" in place of animals in life science education along the lines of the 3Rs principle. Additionally, it is also a mandate of the Center to inculcate non-animal methods in toxicity testing and research. The latter is aimed to be achieved by providing intensive training to the stakeholders in *in vitro* toxicology. A beginning has been made at MGDC in this direction. Three 10 day workshops on "Methods in Animal Cell Culture Techniques & *In vitro* Toxicology" have been conducted. The participants include scientists, technicians, university and college teachers, research scholars and graduate students. Enrollment to each workshop is limited to 16 to ensure hands-on to each participant individually. There being no prototype course structure, the MGDC has designed one and covers exercises in primary and established cell line culture,

enumeration of cells, cell viability assays, genotoxicity testing, apoptosis/necrosis assays, determination of ROS, and molecular end points. This is a pilot attempt to motivate the stakeholders to change to humane science and sophisticated approaches to toxicology. More than the appreciation of the trainees, this series of workshops has sparked tremendous enthusiasm to change to a more rationalized science in terms of *in vitro* toxicology. This presentation analyzes the feedback and reviews the opinions. It is also the aim of this presentation to obtain the suggestions of the WC8 participants for improvement.

III-1-385

First ethics ranking of universities in Germany

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SATIS (Latin: enough!) – the project for humane education of People for Animal Rights Germany (PARG) performed the first ethics ranking to analyze the use of animals and needs of alternatives in education and to create a guideline for high-school graduates and students, accessible online at www.satis-tierrechte.de.

We used a questionnaire-based telephone survey and called the responsible teachers of all German institutes of biology (70), medicine (35) and veterinary medicine (5) to ask which animals or alternatives are used in practical classes and if students are able to conscientiously object. The university ranking was then established by means of specific ethical criteria.

73% of the teachers were prepared to answer the telephone interview. Five percent of all teachers showed a positive interest in the project and asked for advice and information. These will be initial points for our future activities in implementing alternatives in education. Compared to earlier surveys of SATIS, several institutes for human medicine cancelled the use of animals. No degree without animal use exists for veterinary medicine or bachelor of science biology. Apart from possibilities like passive working in groups students do not have the chance to complete their study if they conscientiously object. We are however working at a political level to introduce conscientious objection into German law. The new EU Directive (2010/63/EU) and the German animal welfare law require that existing alternatives have to be used and we are advising academia and administration with regard to the implementation of alternative methods.

III-1-468

The 4th R

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In India under the guidance of the CPCSEA we impart the Three Rs as part of the teaching and training of medical students. Recent trend is to include the 4th R – rehabilitation. Presently the funding agencies in the public sector insist on allocating funds for the rehabilitation of the animals intended to be used in experimentation. The teaching at our institute seeks to highlight the 4th R. The results of encouraging the 4th R will be presented and discussed in the light of socio-religious tenets of Asian Indians of this country.

III-1-507

Master students' feedback on 3Rs education approach

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Although the implementation of the 3Rs in scientific research and education is fundamental nowadays, few are actively involved in teaching alternatives to animal use in Italy and consequently students taking life science courses are generally "under-exposed" to the 3Rs and related topics. We report here the critical evaluation provided by students after attending the 2010-2011 course "Alternative Methods to Animal Use in Toxicology" within the framework of the Master's Program in Veterinary Biotechnology Sciences at the Faculty of Veterinary Medicine in Milan. The students found the course to be helpful in terms of gaining a better understanding of the 3Rs and in improving the quality of science through teaching that was inspiring and interesting. The broader overview provided by the course of the current alternative approaches was believed to be of crucial importance to the *curricula*. The students deemed that rapid development and implementation of alternative approaches in research and testing needed to be supported by familiarity with the techniques, and that practical classes could provide opportunities for the acquisition of skills applicable to a future career characterized by a modern and advanced approach. The course also offered opportunities to interact with research institutes and associations which are directly involved with the 3Rs. Students reported the need to further enrich the didactic material available such as videos, computer programs and specific textbooks. Finally, considering the interdisciplinary nature of the topic and the lack of established standardization, collaboration between teachers of disciplines involving the 3Rs through exchange of content and experiences was considered to be fundamental.

III-1-509

Investing in humane education: Provision of alternatives across India

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InterNICHE has worked with Indian teachers, students, campaigners and others since 2002, during which time a range of national and local projects have been initiated and supported. These have included outreach visits and seminars, demonstrations and training in alternatives, replacement-based grant projects, the establishment of a national alternatives library, and work at the level of government and academic councils. Within these, distribution of alternatives has played an important role, both to familiarise end users and other stakeholders with alternatives and to achieve replacement in specific practical classes. By funding the development of new software and negotiating with producers, InterNICHE has been able to offer many alternatives for free or at cost. Recent distribution includes at the 2009 national zoology alternatives seminar; the 2010 national conference of the CPCSEA, the government agency for animal experimentation; the Dissection Committee of the UGC, who help define the curriculum for university zoology departments; and the 2010 national animal protection network conference. Often an alternatives pack, featuring a collation of software from across the disciplines, has been distributed. Software specific to one discipline has also been sent directly to teachers; the Indian Journal of Pharmacology disseminated 4000 copies of pharmacology freeware. By mid-2011, with support from the Marchig Animal Welfare Trust, InterNICHE had distributed products whose normal purchase value exceeds US$ 500,000. With the UGC abandoning the requirement for dissection in zoology studies from 2011 onwards, a major new project is helping to provide alternatives to the estimated 17 million animals used annually in this field.

Session III-2: Innovative training in human and veterinary medicine

Session III-2: Oral presentations

III-2-395

The potential of humane teaching methods within veterinary and other biomedical education

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Both historically and in many regions today, animal use resulting in harm or death has remained prominent within veterinary and other biomedical education, in disciplines such as surgery, physiology, biochemistry, anatomy, pharmacology, and parasitology. Less recognized are the harms that may also be experienced by students and staff who participate in such animal use. These range from hazardous exposures to toxic chemical preservatives, to psychological and cognitive phenomena which may adversely affect learning and attitudes towards animal welfare. However, in recent years many non-harmful alternatives have been introduced within courses internationally. These include modernized computer simulations, high quality videos, "ethically-sourced cadavers" such as from animals euthanized for medical reasons, permanently preserved specimens, models, mannequins, advanced surgical and clinical skills simulators, non-invasive self-experimentation, and supervised clinical experiences. Published educational evaluations have demonstrated that humane alternatives achieve superior or equivalent learning outcomes such as the acquisition of clinical or surgical skills or theoretical knowledge, around 90% of the time. However, many educators remain unaware of the potential offered by humane teaching methods, or of the evidence relating to their educational efficacy. Accordingly, this presentation reviews the development of humane teaching methods and the published literature examining their educational efficacy. The contemporary and future potential of alternative teaching methods is also illustrated using selected examples.

III-2-398

A veterinary student's perspective concerning educational animal use and the potential for humane alternatives

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Veterinary students internationally, as well as students from other life and health science disciplines, are provided with a range of learning tools. In particular, the use of animals offers a unique privilege, allowing students to practice technical or clinical skills and learn through physical exploration and investigation of living organisms. Yet, the animals obtained may not always be ethically-sourced, and the purposes for which they are supplied may not always be humane, or educationally necessary. Significant animal welfare benefits accrue when institutions or courses cease the killing of animals for teaching purposes. However, the sourcing of living animals and cadavers may continue to pose ethical and welfare problems. The necessity and effectiveness of these modes of teaching therefore require further examination, especially given the availability of alternative teaching tools and methodologies that safeguard the welfare of animals. Tools such as video demonstrations or computer simulations may potentially be available yet disregarded in lieu of traditional teaching methods. Student attitudes may also be conditioned in favour of harmful animal use. Often, alternative technologies are perceived as supplementary to the use of animals, rather than accepted as viable replacements to advance student learning and contribute to reduction of animal use. Within Australia, the University of Sydney has been at the forefront of efforts to introduce humane veterinary curricula. This presentation provides the perspective of a University of Sydney veterinary student on the potential application to veterinary education of current and developing humane alternatives, and the advantages and drawbacks these may present.

III-2-071

Expected frequency of use and proficiency of core surgical skills in entry-level veterinary practice: 2009 AVMA General Practitioner and ACVS Diplomate Core Surgical Skills survey results

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Recent studies rank general surgical skills as the most important skill, procedure, or area of knowledge that new graduates need to know by the time of graduation. However, veterinary teaching hospitals can no longer provide consistent caseloads for students to obtain crucial hands-on surgical experience. Additionally, for ethical and financial reasons, non-survival procedural laboratories are being phased out, so overall student surgical experience is even more limited. Consequently, surgical curriculums are transitioning from a procedures-based strategy to a skills-based approach for teaching surgery. The primary goal of the skills-based approach is teaching skills fundamental to performing any surgery, rather than teaching specific procedures expected of entry-level veterinarians. Unfortunately, no definition has been provided to date regarding what constitutes these fundamental surgery skills. To help identify and define these core surgical skills, and to determine expected entry-level frequency and proficiency for these skills, the authors considered it critical to solicit broad input from the veterinary profession to assist in formulating these definitions and parameters. Pursuant to establishing this type of profession-wide consensus, and to validate and initiate production of surgical training modules aligned with current professional input and opinion, the authors conducted two national surveys, the results of which now form the structure and sequence for producing skills-oriented e-learning instructional courses that we anticipate could be adapted into current surgical training curricula within veterinary institutions. This session will provide a review of the literature, survey methodology and present the results of the ACVS specialist survey, and the AVMA general practitioner survey.

III-2-508

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

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A new student movement for alternatives in veterinary education and for humane veterinary practice has been founded in Egypt. Cairo University Vets for Alternatives (CUVA) was established in 2010 through student self-organisation, with a vision to enhance education and training with humane alternatives. This followed the 1st North Africa and Middle East Seminar on Alternatives in Education and Training and subsequent InterNICHE outreach to faculty and students. Membership of CUVA includes over 400 students and junior teachers, facilitated by the social networking site Facebook. A workgroup on clinical rotations has developed collaborative projects with shelters and veterinary outreach organisations to help animal patients in cities and in villages. This can increase their practical experience and develop an animal welfare awareness that is often lacking in graduates. The Egyptian revolution of February 2011 gave rise to many welfare challenges for animals, particularly horses and camels, due to the disruption to the tourist trade. A month-long outreach project with CUVA involvement provided targeted animal care. A work group on body donation programs plans to provide cadavers that are ethically sourced according to the InterNICHE policy. Both clinical work and body donation programs can potentially replace the killing and animal experiments within anatomy, pathology, clinical skills and surgery training. The empowerment achieved through CUVA's establishment and activity has now led to a focus that reaches beyond alternatives, with a new awareness of the role of the veterinarian in a wide range of animal care issues. CUVA aims to help initiate campaigns at other faculties in Egypt and across the region.

III-2-516

Elimination of live terminal surgeries in Canadian veterinary practice: The case of the Veterinary Skills Training and Enhancement Program (VSTEP) curriculum change at the Ontario Veterinary College (OVC)

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The Veterinary Skills Training and Enhancement Program (VSTEP) is a program at the Ontario Veterinary College (OVC) designed to upgrade the skills of foreign-trained veterinarians living in Canada. While the OVC has offered for years the optional use of non-harmful alternatives to the terminal surgeries performed on live animals as part of the traditional veterinary medical degree, the VSTEP included mandatory terminal surgeries on live animals. As a result of a campaign, these surgeries were eliminated in September 2010. This presentation will explore the ethical issues surrounding the surgeries in the VSTEP and the difficulties encountered by students asking for alternatives, through the experience of a recent VSTEP student and InterNICHE member, Dr. Anya Yushchenk. The presentation will also examine how an alternative program can be successfully implemented, and other positive change brought to a veterinary curriculum, by way of the combined effort of activists and professionals. Finally, practicalities regarding the successful elimination of terminal surgeries from the curriculum will be discussed using as an example the rescue from slaughter by Animal Alliance of Canada of purpose-bred sheep.

Session III-2: Poster presentations

III-2-113

Course on alternative methods to animal use in toxicology in the veterinary faculty of Milan

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In Italy the Three Rs, even if experiencing growing demand and starting to interest students, is a topic included only occasionally in the education of life science undergraduates. If existing, it is restricted to *ad hoc* lessons and rarely included in institutionalized post-graduate or continuing education courses. Even rarer are Three Rs focused courses at the university level in professional degree programs. In the first semester of the 2010-2011 academic year, a mandatory course entitled Alternative Methods for Animal Use in Toxicology was established as part of the curriculum of the two-year Masters Program in Veterinary Biotechnology Sciences, organized by the Faculty of Veterinary Medicine in Milan. It is a 6-credit course totalling 48 hours, 24 theoretical and 24 practical. The aim of the course is to teach the Three Rs by giving a critical methodological view of the use of alternative methods in toxicology, helping students to gain a better understanding of the Three Rs concept, thus educating and training people able to work with the Three Rs in toxicology. This pilot course covers different aspects of the Three Rs, ranging from a general introduction to the illustration of new and innovative techniques that support the Three Rs in toxicological research. It includes hands-on training and an informational website exclusively dedicated to this topic. Very positive feedback has been received from the students about this initiative.

III-2-128

Ophthalmic artery super-selective catheterization of the pig as a training model with possible implications in retinoblastoma treatment

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Purpose: To develop a technique for local drug administration in a porcine model with potential translation to retinoblastoma chemotherapy treatment.

Methods: The ophthalmic artery catheterization was carried out in an anesthetized animal under anticoagulation. A 5-French arterial sheath was placed in the femoral artery and a 5-F catheter was guided into the common carotid artery to the maxillary artery. The ophthalmic artery was super-selectively catheterized (OAI) using a microcatheter. Serial angiograms were performed. Chemotherapy (topotecan) was delivered in a pulsatile fashion. The microcatheter was removed and systematic procurement of vitreous and plasma samples started immediately. Two animals were systemically administered (IA) with the same dose of chemotherapy through the external carotid and plasma and vitreous samples were obtained.

Results: The ophthalmic artery of the 4 animals was successfully catheterized by means of the super-selective ophthalmic artery technique. Maximum total topotecan concentration in the vitreous (median, range) after OAI and IA was 131.8 ng/ml (112.9-138.7) and 5.4 ng/ml (4.7-6.1), respectively. Systemic

exposure for topotecan was low for both modalities of administration with a median (range) value of 10.6 ng*h/ml (6.8-13.4).

Conclusion: We were able to develop the super-selective ophthalmic artery catheterization in a porcine model. Topotecan was infused using this technique and vitreous drug levels were 24 times higher than those attained after IA infusion of the same dose of chemotherapy. Topotecan systemic exposure was low and comparable between drug administration techniques. These results show the selectivity of the infusion to attain the ocular structures with potential implications in retinoblastoma treatment.

III-2-434

New replacement alternatives used for training students in veterinary medicine in the Netherlands

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Over the last 12 months, the Faculty of Veterinary Medicine, Utrecht University, The Netherlands and the Dutch Society for Replacement of Animal Testing (ds RAT, Proefdiervrij) have discussed possibilities to further reduce the use of laboratory animals for teaching and training veterinary students. The deliberations have led to the signing of two formal agreements between both parties.

Until recently animals, mainly dogs and cats, that were frozen or formaldehyde fixed after euthanasia upon arrival were used for teaching knowledge of anatomy in practical classes at the Faculty of Veterinary Medicine in Utrecht. A similar approach was used for practical classes in which students were trained in surgical skills. The Utrecht University and ds RAT agreed to join forces to introduce a body donation program aiming at a full replacement of laboratory animals by pets euthanized for health related causes. After roughly half a year the initiative already can be called a success.

Ds RAT has also agreed to substantially support the Utrecht University to further develop plastinated models of animals to replace the need for carcasses. This assistance allows for the production of a number of educational boxes containing a variety of detailed body parts of different animal species for teaching and training veterinary anatomy. Both projects are a typical demonstration that parties that may have conflicting points of view can nevertheless find ways to join efforts so as to reach goals of mutual interest.

III-2-488

Implementation of a body donation program and use of software: Replacement in veterinary anatomy in Peru

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In 2009 the Laboratory of Animal Anatomy and Wild Fauna (LAAFS) of the Faculty of Veterinary Medicine, National Major University of San Marcos (FMV-UNMSM), Peru implemented the use of ethically sourced animal cadavers in a pilot body donation program. The Department of Animal Anatomy has 55 students per year who use cadavers of different animal species, including dogs, for dissection and acquisition of manual skills with the use of surgical equipment. InterNICHE donated to the LAAFS the Virtual Canine Anatomy software, enabling the partial replacement of the use of animals for dissection from 60 animals to 12 dogs per year. In a survey, 91.1% (41/45) of students requested to use the software more frequently, and 84.4% (38/45) were able to identify anatomical structures more easily. The 12 bodies of dogs were acquired ethically as defined by the InterNICHE policy, from veterinary clinics and in the same clinic FMV-UNMSM, in which they had died or were euthanized for terminal diseases, cancer, poisoning and car accidents. The establishment of the body donation program required awareness from veterinarians working in the clinics to explain to the companion animal guardians the need for a culture of donation of cadavers. Authorization for donation is provided by the guardian or witness. The FMV-UNMSM supports administratively the LAAFS with the transportation expenses to collect the cadavers from the clinics and for embalming, and continues to change its educational approach from a conventional to a more humanitarian and effective one using alternative tools and approaches.

III-2-492

Curricular transformation at St Petersburg State Veterinary Academy

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Collaboration between InterNICHE and the Department of Pharmacology at St Petersburg State Veterinary Academy in Russia began in 2005 following alternatives promotion by InterNICHE at veterinary congresses and across Russia. Demonstration and loan of alternatives, along with presentations and meetings with Academy officials, teachers and students, led to great interest in humane teaching approaches. Economic considerations and recognition of the importance of computer literacy also played a role in developing more openness to new and modern teaching methods. With support from the International Association Against Painful Experiments on Animals (IAAPEA) a multimedia laboratory was established and InterNICHE provided computer software and a training mannequin. Further material to support successful implementation of the learning tools was produced by InterNICHE and the department, including a translated version of pharmacology software and a manual on its use. The annual use of over 1000 animals in the department was ended and a formal agreement was signed to confirm the transformation. Widespread media coverage across former Soviet countries demonstrated that change and innovation had brought major benefits to the educational process. Visits and donations to other departments, and recognition of the benefits of humane education have now led to virtually the whole Academy abandoning animal experiments. A conflict between progress at the Academy and outdated demands for animal use from the Russian government's Academic Methodology Unit is being addressed.

III-2-510

The use of formal agreements to achieve replacement in education: The experience of Russia and Ukraine

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By 2011, nearly 40 formal agreements had been signed between humane education campaigners and universities across Russia, Belarus and Ukraine. Since 2005, when InterNICHE signed the first contracts with St Petersburg State Veterinary Academy and Velikie Luki State Agricultural Academy, adopting a strategy of formalising the collaboration with universities to achieve specific replacement-focused objectives has proved effective. InterNICHE National Contacts perform the majority of outreach, alliance-building and follow-up with universities, and liaise internationally to combine the strengths and resources of the localised and the global in the process. InterNICHE pioneered the use of agreements in Russia, and InterNICHE and Doctors Against Animal Experiments (DAAE) (Germany) collaborate for those in the Ukraine. Typically the Agreements are signed by the Dean or Rector of the university along with the InterNICHE Co-ordinator and DAAE Project Manager. They usually detail the animal use to be replaced, and refer to curricular change at the level of department, faculty or whole university. Resources such as computer hardware, models, mannequins and software alternatives are usually provided by InterNICHE and DAAE. Media interest in the curricular transformation has been high. In many cases the agreements bring to an end the animal experiments and dissections and secure the implementation of alternatives; in others the project confirms and consolidates an existing change and secures implementation. By 2011, together they are saving an estimated 40,000 animals (vertebrates and invertebrates) from being killed annually. As one tool in the broader campaign for humane education, the successful use of agreements reflects a growing acceptance of replacement of animal experiments in education in former Soviet countries.

III-2-517

International harmonization of education and training standards for laboratory animal veterinarians

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Animal-based research and education is increasingly an international enterprise that draws significant public attention. The welfare of research animals, quality of scientific data, and institutional reputation significantly depend on assurance that veterinarians managing and overseeing research animal care are adequately trained and qualified. Yet, the knowledge and experience of veterinarians serving in this role can vary widely and globally, education and training available to veterinarians in laboratory animal medicine ranges from specialty board certification to on-the-job exposure. The International Association of Colleges of Laboratory Animal Medicine (IACLAM) has determined that even within its member Colleges there is considerable variability in training programs, credential review, recertification procedures, and examination composition. New graduates from veterinary colleges often have not received adequate education and training in research animal care, medicine, or management. Inadequate training can adversely jeopardize animal health and welfare, as well as personnel and facility safety, and the entire institutional research enterprise. The World Organisation for Animal Health (OIE), in collaboration with IACLAM and the National Academies' Institute for Laboratory Animal Research, recently assessed the laboratory animal veterinary community's perspective on harmonizing global veterinary training and education in laboratory animal medicine. This was based on discussion groups convened during several major laboratory animal science meetings in Europe, North America and Asia in 2010. A total of 106 individuals representing 27 countries participated. Topics addressed included roles of laboratory animal veterinarians; core knowledge and practical work-related skills required for proficiency; acceptable approaches for imparting core knowledge; types of experiences suitable for instilling work-related skills; and type and amount of training necessary to attain proficiency. The concept themes from the discussion groups will be addressed.

III-2-521

How formal training influences researchers' awareness and attitudes to animal use in biomedical research

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Even with existing regulation and supervision of animal use, the individual researcher's responsibility is still decisive in implementing the 3Rs. Training in laboratory animal science aims to raise researchers' awareness and increase their knowledge, but its effect on scientists' attitudes has not so far been systematically assessed. Participants in six FELASA Cat-C courses (N=150), held between 2008 and 2010 in Portugal, were surveyed in a self-administered questionnaire. Questions related to the 3Rs and their application, attitudes to animal use and ethical review of animal experiments. One year later, respondents were asked to answer a similar questionnaire (53% response rate) with added self-evaluation questions on the impact of training. Prior to training, most researchers (62%) were completely unaware of the 3Rs of animal research (23% claimed to know but failed to name them; 15% correctly named the 3Rs), a problem the courses effectively overturn, with 98% of respondents being able to name the 3Rs one year after. Moreover, the actual implementation of the 3Rs in their research rose considerably (from 30% to 60%). There is, however, a degree of reluctance to acknowledge Replacement, since participation in the course did not change perceptions of the current and future need for animal use in research. Based on this and other data from the surveys, our presentation will focus on the importance of formal training not only as a means to increase knowledge and develop technical skills, but also to raise awareness to ethical aspects of the use of animal models of research.

III-2-700

International Harmonization of Education and Training Standards for Laboratory Animal Veterinarians

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Animal-based research and education is increasingly an international enterprise that draws significant public attention. The welfare of research animals, quality of scientific data, and institutional reputation significantly depend on assurance that veterinarians managing and overseeing research animal care are adequately trained and qualified. Yet, the knowledge and experience of veterinarians serving in this role can vary widely and globally, education and training available to veterinarians in laboratory animal medicine ranges from specialty board certification to on-the-job exposure. The International Association of Colleges of Laboratory Animal Medicine (IACLAM) has determined that even within its member Colleges there is considerable variability in training programs, credential review, recertification procedures, and examination composition. New graduates from veterinary colleges often have not received adequate education and training in research animal care, medicine, or management. Inadequate training can adversely jeopardize animal health and welfare, as well as personnel and facility safety, and the entire institutional research enterprise. The World Organisation for Animal Health (OIE), in collaboration with IACLAM and the National Academies' Institute for Laboratory Animal Research, recently assessed the laboratory animal veterinary community's perspective on harmonizing global veterinary training and education in laboratory animal medicine. This was based on discussion groups convened during several major laboratory animal science meetings in Europe, North America and Asia in 2010. A total of 106 individuals representing 27 countries participated. Topics addressed included roles of laboratory animal veterinarians; core knowledge and practical work-related skills required for proficiency; acceptable approaches for imparting core knowledge; types of experiences suitable for instilling work-related skills; and type and amount of training necessary to attain proficiency. The concept themes from the discussion groups will be addressed.

III-2-701

Surgical training applied to experimentation: a course without the use of live animals

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The main objectives of surgical training are to learn how to consider the patient as a whole and to acquire perfect technical skills. Often neglected, the latter allows a decrease of the impact of surgical trauma, decreased morbidity and even mortality. Insisting on the importance of a thorough evaluation of the patient, from the physiological point of view, as well as in terms of discomfort and pain, this set of courses aims to increase the technical competence of novice or confirmed trainees.

The program focuses on:

– Mastering the surgical context from the preparation of the equipment and tools to aseptic techniques.
– Numerous practical training with alternative supports
– Interactive discussion and analysis of selected surgical procedures and procedures proposed by trainees.

The twenty-two hours of training include 90% practical and tutored courses with a maximum of 20 attendees* per session. Teaching is ensured by veterinarian surgeons (DVM), lecturers or research scientists (Ecole de Chirurgie de Lyon). Teaching material, documents and state of the art alternative supports (patent pending) are specially developed. This formation has been approved by the Swiss "Association des Vétérinaires Cantonaux" and has been submitted to the French "Commission Nationale de l'Expérimentation Animale". This poster was awarded the first prize by the "Association Française des Sciences et Techniques de l'Animal de Laboratoire" (AFSTAL) during the 2011 meeting in Marne La Vallée – France.

*In France an initial "Niveau 1" or "Niveau 2" training is required.

Session III-3: Development of non-animal teaching/training models

Session III-3: Oral presentations

III-3-712

An ethical scoring system for the production and assessment of alternatives in education and training

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Assessment of non-animal alternative tools in education and training is a process that must address a range of pedagogical, ethical, environmental and economic issues. Within the ethical field, criteria include whether and how animals were used in the production process, broader ethical issues presented by content and design, and potential for replacement of harmful animal use. To encourage humane production of alternatives and to facilitate assessment and implementation, InterNICHE has developed an ethical scoring system that forms part of a comprehensive review and assessment process. The potential for international implementation and associated widespread replacement is scored through judging an alternative's ability to meet and exceed the teaching objectives of a conventional practical class, and its accessibility, opportunities for translation and other criteria. The pedagogical and training aims of an alternative, with both explicit lessons and implicit messages, also play a role in the scoring, with alternatives developed for acquisition of knowledge and skills for the purpose of animal care scoring higher than those for the purpose of animal experimentation. When an alternative reflects progressive teaching approaches and technological innovation, and when holistic representations rather than instrumental use of animals are made, it also scores higher. In the production of video and software alternatives for anatomy practical classes, the use of animal cadavers that are ethically sourced according to the InterNICHE Policy on Alternatives and Animal Use in Education and Training would contribute to a higher score than a product that used killed animals. The use within physiology and pharmacology software of existing data or mathematical algorithms rather than data derived from new animal experiments would also contribute to a higher score. Examples of the process of ethical scoring for a number of existing alternatives will be presented, with an exploration of the criteria and their weighting. The process is being applied to alternatives detailed in the InterNICHE book and database "From Guinea Pig to Computer Mouse" (2nd ed.), available and updated on-line at www.interniche.org, and is being discussed with producers to improve the nature and quality of new products.

III-3-115

Ex vivo pulsed heart model for cardiac surgical and interventional product development and training

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An *ex vivo* pulsed heart model was developed to simulate a beating heart in an operating room environment. The heart and aorta of the pig was obtained from a local abattoir. The use of discarded organs increases the overall use of the animal and reduces the need for dedicated research animals.

The objective was to develop a clinically relevant beating heart model with direct visualization of the intra-cardiac structures for development of surgical and interventional products as well as education and training. The heart was perfused with saline that circulated along the natural blood flow pathway with inflow through the left atrium and outflow through the aortic valve. The pump function of the heart was achieved through a changing left ventricular volume that developed a left ventricular pressure pulse. The preload and afterload were created from separate hydrostatic columns and reservoirs that also provided system compliance and pulse damping of pump induced artifacts. Solid-state micro-pressure transducers were located in the left atrium, left ventricle, and aortic root to provide real time pressure monitoring and phase relationships. Left ventricle volume and pressure modulation were controlled with an external piston pump through an apical balloon cannula.

The model simulated an operating room environment with anatomical orientation of the heart and aorta on the surgical table, cardiac surgical instruments, ultrasound imaging equipment, and cardiopulmonary bypass extra-corporal pumps. Laparoscopic cameras positioned in the left atrium, left ventricle, and aortic root provided direct visualization of intra-cardiac structures such as the mitral and aortic valves, atrial appendage, intraventricular septum, chordae tendinea, papillary muscles, etc. High-resolution cameras provided imaging of native valve function, surgical techniques, prosthetic devices, and the coronary ostium for catheterization.

The *ex vivo* pulsed heart model simulated a beating heart in an operating room environment for product development and training. The use of harvested porcine hearts may be reanimated to approximate *in vivo* conditions for evaluation of device performance while reducing the use of live animals for research.

III-3-146

Transparent laparoscopic simulator with adjustable physiological conditions for product development and surgical training

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Laparoscopy dates back to around 1901, when it was reportedly first used in a gynecologic procedure performed in Russia. The popularity of the technique as a diagnostic and treatment tool has increased dramatically since the early 1980's to today where it is a widespread procedure for various surgeries. A novel bench top model is presented that simulates the anatomical and physiological conditions present during laparoscopy.

The objective was to create a bench top laparoscopic simulator with adjustable physiological conditions to aid in the design and development of laparoscopic devices and to be used as a surgical trainer.

The laparoscopic chamber approximates the volume and aspect ratio of the human adult abdominal cavity during distention, providing the physical constraints of conventional laparoscopic surgery. Environmental conditions of insufflation pressure, temperature, and humidity are also included, eliminating the need for dedicated research animals. Multiple access ports accommodate both 5 and 10-12 mm trocars in the anterior and lateral planes allowing placement of cameras and instruments.

The Transparent Laparoscopic Simulator enables rapid prototype evaluations and training of medical professions without the use of dedicated research animals. Environmental conditions in the chamber may be created to simulate a broad range of patient conditions by controlling insufflation pressure, temperature, and humidity. The transparent walls of the chamber provide direct visualization of the device being used or the test being

performed, instead of indirect imaging with a camera and monitor as in conventional laparoscopic surgery. Alternatively, the chamber may be covered to hide the interior and allow for training with the use of a conventional monitor. The full field of view provides the investigator an unobstructed three-dimensional assessment. The laparoscopic chamber has been used for new product development, surgical training and design validation.

III-3-291

STARR Trainer, an alternative to live animal usage, for product development and surgeon training

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Trans-Anal Rectal Resection (STARR) is a surgical treatment for obstructed defecation syndrome (ODS), a condition of chronic constipation associated with anatomical anomalies of the pelvic floor and rectum. Ethicon Endo-Surgery developed two staplers to enable surgeons to perform this STARR surgery. These staplers allow the surgeon to resect full thickness rectal wall via a minimally invasive trans-anal approach. The goal of this study was to develop a STARR trainer as an alternative to live animal usage for both product development and for use during surgeon training.

Ex vivo methodology was explored as a means to study staple line integrity and staple line burst pressure during product development. A stand was developed as a tool to suspend harvested porcine tissue, distal colon and rectum, in the correct orientation and tension to perform suture placement, device orientation and tissue resection.

The stand, STARR Trainer, allowed easy and consistent placement of the staple lines for staple line testing. It was easy to view traction suture placement, in folding of the tissue into the staple anvil and the final staple line. These features also made the stand a useful tool in training surgeons. Animal usage for training was reduced by 67% after implementing the STARR Trainer.

The STARR Trainer is an effective alternative to animal use for product development and surgeon training.

III-3-442

The use of rat skull model for teaching learning methods for bleeding

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It is necessary to collect blood from small rodents for different experimental or diagnostic procedures, and this requires training and the use of anesthetized animals for students to learn the various techniques of bleeding. Plastination (P) is the most important technique for preservation of biological specimens.

Our objective was to propose the use of the skull and plastinated heads for the training of students, without sacrificing animals.

The P methodology consists of slowly replacing tissue fluids and a portion of the tissue lipids with a polymer under vacuum. The results are clean, dry, odorless and durable real biological specimens. It keeps the dissected specimens from deteriorating. Waste adult rats were obtained and heads and skulls were prepared by thermal maceration and mechanical removal of soft tissues. Each skull was bleached with hydrogen peroxide. Other heads had the skin left intact and other heads were retired. They were fixed in 10% buffered formalin. After dehydration with acetone, silicone infiltration was performed.

Rat skulls were obtained with and without soft tissues to demonstrate the anatomical relationship and provide a training model for the collection of blood from the retro-orbital sinus, as well as the facial vein.

The advantages of this model is the elimination of the use of live animals, reduction of trauma induced in animals during learning, reduction in the anxiety caused to students when handling live animals. The rat head is an ideal model for education, skills development and refinement of the bleeding technique.

III-3-459

Simulation of animal experiments using mannequins, chemical sensors and computer software

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The computer assisted learning (CAL) of animal based experiments has substantially contributed to replacement of animals in education and training. However, CAL does not enable the user to develop skills like surgical techniques, dose administration techniques, preparation of drug solutions and recording of biological responses, which are acquired by performing a wet lab experiment. These skills play a crucial role in actual research activities. Hence, to reinforce the experimental details, there is a need to enrich CAL techniques by combining them with mannequins and chemical sensors. Such combination can lead to better virtualization of the animal experiment and help students develop maximum experimental skills.

The teaching aid developed by us completely simulates the invasive blood pressure recording procedure in rats and dogs. This module can be used to routinely teach/learn/demonstrate the experiment of invasive blood pressure recording in rat/dog in the same way as it is performed in wet lab experiments. Using this artificial rat/dog along with dummy drug solutions and related software, effects of different drugs on the blood pressure can be demonstrated. "Exam mode" incorporated in the relevant software can be used for conducting exams on these experiments.

The combined use of mannequins, sensors and CAL can simulate the animal based experiments in totality and can prove to be a complete replacement for a few experiments on animals in education and training.

Session III-3: Poster presentations

III-3-047

Focus on animal welfare, the role of the 3Rs in the wellbeing of animals on the African continent

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Since the 1970s, the concept of the 3Rs (Replacement, Reduction and Refinement) has had a major influence on the field of laboratory animal science. Refinement refers to improvements to housing and care and procedures, which minimize actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable. Reduction refers to the lifetime experience of the animal. There is evidence that refinement not only benefits animals, but also improves the quality of research findings. Reduction refers to methods which minimize animal use and enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals, thereby reducing future use of animals. The main aim of this paper is to highlight the plight of animals on the African continent; it focuses on the abuse of animal rights by those who innocently violate these rights and those who don't believe that animals too have rights.

III-3-215

Outcomes of efforts of Mahatma Gandhi – Doerenkamp Center (MGDC), India, to replace animal dissections in life science and biomedical science education

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The Mahatma Gandhi Doerenkamp Center for Alternatives to Use of Animals in Life Science Education was established in July 2009 by the Doerenkamp-Zbinden Foundation, Switzerland, with sensitization and motivation of stake-holders to replace use of animals in education as the principal mission goal. This Center works to achieve excellence in life science education by introducing value-based learning systems. Realizing the removal of countless animals from the wild and subjecting them to the gruesome dissection for purpose of understanding animal anatomy and evolution, the Center has fixed university and college teachers as the principal target group to reach, since they are the ones who decide on the curriculum. The Center conducts localized workshops/seminars for these teachers at different places across the country. Within a very short span of time the Center has organized about 20 such programs. These are one full day programs starting with addresses by a team of academics who are emotionally attached to the concept of alternatives. The participants are told how archaic dissection is as a learning tool, how the large scale removal of animals from their natural habitats can potentially disturb the ecosystem and hamper the biodiversity, how this practice defies the animal protection laws, and how students are turned to become violent towards the animals, etc. The participants are motivated to turn to humane and value-added science education by adopting several alternatives. A momentum and an expectation are built when the participants are given an on-screen demonstration of the various digital alternatives. In the afternoon the participants handle the computer mouse to learn animal anatomy, physiology, etc. The programs culminate in a discussion session where doubts and apprehensions are discussed and students are asked to record their feedback. In light of the enormous success, several universities have already revised their zoology/life science curriculum and the digital alternatives have started finding place in it. This presentation will review the feedback of the participants and the initiative from the regulatory authorities of higher education in India to support the efforts of MGDC in this endeavor.

III-3-465

Replacement of animal use in medical physiology

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The replacement of animal use in medical education with non-animal methods and techniques often yields both ethical and technical advantages. Particular emphasis is related to ensure the efficiency and validity of computer simulations regarding animal use replacement. The aim of the study was to clarify the medical student attitude toward computer simulation during the laboratory practice in medical physiology.

89 medical students (31 male and 58 female; 20 ±2 years old) at Skopje's Faculty were given questionnaires regarding the computer program: Renal function in humans by Sheffield BioScience Programs, UK. The students usually used PCs as follows: regularly 57%; fairly often 17.9%; sometimes 17.9%; and 1.1% rarely with learning purpose of 51%. At the end of the laboratory classes the students had to answer with: strongly disagree (SD); disagree (D); neutral (N); agree (A); and strongly agree (SA).

To study physiology using animal experiments the students answered with: A 40.4%; SA 20.2%; D 8.9%; and SD 14.6%. The majority of the students preferred working with computer simulations in small groups: 47.1% A and 38.2% SA, and with its good data presentation 70.7% A and 10.1% SA. For replacement in animal use 16.8% A and 20.2% SA, but D 24.7% and SD 19.1%.

From the obtained results we may conclude that the medical students accept the computer simulation in medical physiology due to good data presentation, studying together in small groups but still don't completely accept to leave the conventional experiments within animal use.

III-3-487

Strategies for replacement in the medical physiology laboratory in Centro Universitario de la Costa (Puerto, Vallarta) of Universidad de Guadalajara

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The approach in the formation of human resources in health sciences at the Centro Universitario de la Costa, has led us to try to give to the medical and nursing students an integral formation that includes human development as an assignment, free to be taken by any of these students. This has led us to search for alternatives to the use of animals for the teaching of medical physiology.

Since 2008, we acquired the Biopac Student Lab, which has allowed us the opportunity for self-experimentation, using the students as volunteers, to observe the most common physiological variables, and strengthen the learning in the laboratory. Therefore, we have developed a group of outstanding students, which have created a physiology manual with detailed protocols to implement different practical activities in the physiology laboratory.

In 2011, we received a new Biopac Student Lab Advanced and an upgrade of the current Biopac Student Lab thanks to the support of Interniche. With all this, we can reinforce the strategy to replace the use of animals in our lab and start using it in other departments of the Centro Universitario and other campus on the university net. The combination of a program of human development and the challenge of using alternatives in medical education as extracurricular activities will give us a plus in the formation of human resources in health sciences.

III-3-496

International practical training on *in vitro* methods

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With the recent advancements in the adoption of a considerable number of alternative methods to animal testing by the OECD, and the global harmonization of the world commerce, the importance of providing education on the adopted alternative methods throughout the world is becoming increasingly necessary.

In particular, the specifics of the *in vitro* test method protocols, the importance of ensuring good laboratory practices, proficiency, reliability and relevance of the method for regulatory purposes, calls for an education preferentially based on practical demonstrations and/or hands-on-training.

Such training is essential to scientists performing *in vitro* tests, and is also key for regulators to gain the necessary understanding and make critical assessments of the *in vitro* data. Furthermore, such training could favor standardization of regulatory assessment and decisions on hazard properties of chemicals across the world.

As an example, a practical workshop is currently being organized in Brazil by IIVS, SeCAM, University of Goiás and a local organizing committee comprised of representatives from government, regulatory agencies, academia, industry and scientific associations. Such an inclusive composition allows for transparent and open discussions on the aims and goals of the workshop. The practical training will be provided by IIVS on assays that are currently accepted at the OECD level, and will focus on the technical steps of the adopted *in vitro* protocols (highlighting the critical steps for data assessment), good laboratory practices, and finally the interpretation of results for regulatory purposes. Details on the concepts and organization of the practical trainings will be provided.

Session III-4: Replacement alternatives and teaching objectives – determining if and when student learning objectives require the use of animals

Session III-4: Oral presentations

III-4-158

New innovative elements in the FELASA Category C course for researchers: towards a more effective literature search and systematic reviews of animal studies

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Thorough analysis of already existing literature and data is a prerequisite for executing well designed animal experiments. In this way, the maximum amount of information for animals (and humans) will be derived from previously performed studies, and unnecessary duplication is prevented. Systematic reviews are the most suitable way to carry out such a thorough analysis, since all relevant studies are identified, appraised, selected and data extracted to generate new data. Within clinical research, systematic reviews are common practice (evidence-based medicine). This is not yet the case within animal research. Because systematic reviews contribute to (1) better quality science, (2) implementation of the 3Rs and (3) better patient safety it is important to apply them. To introduce this evidence-based approach into animal research, we have implemented education on the basic principles of systematic reviews of animal studies into our FELASA category C courses over the last 2 years. Special attention is given to the development of comprehensive literature search strategies in a hands-on practical. We have also expanded the education on systematic reviews to a special dedicated 1 EC course for Master Biomedical Science students. The education is considered – both by teachers and students – to have an added value and to be a necessary part of education of future researchers. The content of the education, number of students and the evaluation reports will be discussed in the presentation. We suggest that these topics be included in all FELASA category C courses.

III-4-479

Replacement and *in vivo* learning objectives in European competence training

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The FELASA Accreditation Board reviews courses and curricula which follow FELASA guidelines for the four European competence categories (A-D). Laboratory animal specialists (D) will have gained both theoretical and practical competences in laboratory animal science, which may or may not involve the use of animals. Caretakers (A) must be well-acquainted with the behaviour of animals, observing signs of illness or poor welfare, and correct procedures for handling and restraint; training is inevitably practically-based, preferably under guidance of skilled practitioners. Category B persons, who conduct experiments, must work sensitively and with minimal impact on animal well-being. Although the use of animals can be minimised, at some point the technical expertise associated with working with sentient animals needs to be acquired under close supervision. Category C persons, who design and oversee experiments, do not always need to acquire practical expertise in their work, but they must clearly understand its impact on the biology and ethology of the animals being used. Although this can be taught using a basic theoretical approach, often with considerable success, it may be necessary to present these attitudes within a context in which animals are actually used. Survey findings (Howard, 2000; Carlson et al., 2001) suggest that students proposing to enter careers involving whole-animal research gain a great deal from supervised work with animals whilst attending FELASA-type courses; for those not entering such employment in the near future, the case for attending *in vivo* practical classes is less apparent.

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III-4-209

Reducing the number of animals used in teaching and training of graduate students and scientists – possibilities and limitations

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When educating graduate students and scientists that are supposed to independently design, conduct and perform animal experiments (FELASA category C persons), it is essential for the students to practice various procedures on animals. Previous investigations have shown that animal handling and procedures are among the topics that students of this category appreciate the most and also request more practice in. This is, however, in conflict with the general striving of reducing animals in education. Thus, it is highly important to identify in which cases the use of live animals is necessary, and in which cases it is not. At our department, we offer FELASA accredited category C courses that contain practical hands-on exercises in handling, injections, blood sampling and surgery, where live animals are used. Practical exercises in anesthesia and behavior, however, have been replaced with video-based exercises, where animals have been filmed. This has turned out to be very successful, and this presentation will describe the educational advantages of this strategy, how it has been perceived by the students, and to what extent it has reduced the number of animals used. The presentation will also bring to discussion the possibilities and limitations of replacing live animals in general, when teaching FELASA category C persons.

III-4-583

A survey of animal use and alternatives in higher education in Europe

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A study was conducted in 2010 to determine the extent to which animals were still being used or had been replaced by computer-based alternatives across a selection of universities from 10 European countries: UK, France, Germany, Spain, Italy, Poland, Holland, Slovenia, Czech Republic and Macedonia. Response rates varied between 73.1% (UK) and 26.3% (Romania) thus making valid comparisons difficult.

A questionnaire, designed to collect information on the use of innovative technologies in teaching physiology and pharmacology and containing questions relating to animal was delivered online using the UK Bristol Online Survey service.

Universities in the UK, Spain, and France had the highest average levels of animal usage with the highest total use in the UK. Spanish universities used the highest number of mammals and those in the UK the most amphibians and guinea pigs for teaching. Of the four eastern European countries surveyed, Romania had the highest use of animals in teaching.

Computer-based alternatives (both commercially available and freeware) were used to some extent by all countries. Romania, Spain, and Poland had the highest reported use with Macedonia, Italy and France the lowest. Major barriers to the introduction of alternatives were "resources not available in local languages"; "difficulty finding resources"; "lack of money to purchase resources" and "available resources don't meet learning objectives".

Major factors which would persuade academic staff to introduce alternatives were: "published evidence of effectiveness" and "recommendation from a colleague". In western European institutions students' objecting to the use of animals in teaching was an important driver.

III-4-409

Alternatives to animal testing in the faculty of veterinary medicine of the National Autonomous University of Mexico

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The use of non-human animals is the most accepted way to obtain knowledge and psychomotor skills needed for future professionals in the academic system. The UNAM is not the exception and the Faculty of Veterinary Medicine mistreats non-human animals in different ways causing pain, stress and injury. In recent years, there have been doubts in the minds of many students and teachers about the unethical way the medicine principles are being taught and they are forced to commit these acts of abuse against animals, feeling their sensitivity and moral or ethical principles violated, and therefore their integrity. Dissections and/or vivisection send the wrong message to students. Instead of learning the anthropocentric and specist values, which claim that non-human animals are the subject of moral consideration, they learn that life is disposable and that nonhuman animals can be used at will. Consciously or unconsciously these acts remain qualities such as sensitivity and compassion to the future professionals. The alternatives are ethical educational media and should completely replace the harmful use of non-human animals and be used in combination to achieve the educational objectives. The use of alternative methods allows the acquisition of the desired knowledge plus ethics and respect for life should be the trend to continue throughout the world.

This work considers the ethical conflicts which students enter as they are forced to harm animals and their right to conscientious objection, and provides proposals of the existing methods to replace the use of animals in education.

Session III-4: Poster presentations

III-4-117

Perception of animals used in education and research in Brazil by students and professors

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Animal use in education and research has raised ethical questions in society. The objective of this work was to study the way students and professors see animal use. Thus, a study was performed on the perception of animal use by 70 students and 17 professors of five areas – biology, engineering and management, humanities (philosophy, physical education, languages and pedagogy), social sciences (accounting sciences) and law – of 17 university programs, through an open and objective questionnaire. Regarding animal use in teaching, the interviewees agree (63%) with the use of living animals, mainly the mouse, in veterinary medicine and biology, even though they report not being aware of their purpose. In an open question about the use of animals for teaching purposes, part of the interviewees (48.5%) would not use them, but only 18.5% cited the use of alternatives, whereas 41.5% had no restrictions. Of the latter, 20% alleged the need to use animals. In research, the use of white mice was considered correct and fundamental for drug production (27%), especially by the interviewees of the biological areas (85%). The results reflect the traditional vision that the benefits of using animals surpass the costs for their welfare. However, it is necessary to continue the present study and increase the sample size.

III-4-136

The opinion on the use of animals in higher education in Brazil: comparison across programs and between first and last year students

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The use of animals in education and research is an ethical and polemic issue. Currently, the Brazilian legislation is in transition with the recently approved Law no. 11,794 (2008). In this study we evaluated the ethical matters in the use of animals at the Federal University of Paraná through a qualitative analysis by using questionnaires and interviews. The objective was to compare the opinion of interviewees from different programs and freshmen versus veterans. The analyzed group was composed of 101 students and 20 professors of biology, pharmacy, medicine and veterinary medicine programs. Approximately half of the students (44.6%) do not know the legislation that regulates the use of animals in education. Regarding the use of alternative methods by the professors, most of them believe that it cannot perfectly approach the learning goals. Professors who use animals for teaching represent 35.0% of the interviewees and for research 55.0%, of which 13.3% practice vivisection. Some alternative methods are used by 70.0%, but only 38.9% of the professors and 29.7% of the students trust them. The students in biology (90.9%) and veterinary medicine (73.3%) are more likely to be concerned with animal welfare in education and research than those in other programs. Students nearly graduated present similar knowledge of legislation as freshmen, and the majority of the interviewees (68.9%) do not believe that alternative methods present high quality. Results suggest that it is necessary to extend the discussion on alternatives to animal use in the academic environment.

III-4-383

Teaching in pharmacology and 3Rs: problems and ways forward

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The advance in the discovery of drugs has increased the quantity of scientific available information to be evaluated, creating new challenges in the selection of the knowledge to being given in the university classroom. Drug-discovery research requires accurate quantification of the affinity, selectivity and biological effects of new compounds. From *in vitro* assay, it is possible to get information indicating its potential efficacy and safety but *in vivo* studies are essential for determining whether the *in vitro* activity does in fact, translate to the *in vivo* situation. Integrative pharmacologists, who understand the potentials and the risks inherent to a pharmacological mechanism with the ability to build intellectual and technical bridges between molecular, cellular and intact organisms, are needed to evaluate new drugs. However, there is currently a severe shortage of pharmacologists with the skills needed to carry out *in vivo* studies in medical research. Economical and ethical factors have led to a decline in the teaching of *in vivo* pharmacology but this knowledge and appreciation of integrated responses must be given proper emphasis in any pharmacology courses for undergraduate and postgraduate students. Videos, experimental design, statistical analysis, and data handling exercises related to animal experimentation used in the educational process, could improve animal welfare and the quality of biomedical research and testing in our country. Although economical obstacles must be overcome, this will provide a mechanism by which most biomedical science students can become aware of issues related to the use of animals in research.

III-4-407

Education and animal experimentation, ethics in higher education

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We aim to promote respect for animals in the existing national laws and regulations on their handling and use and to promote quality and humane education.

Animals are used for education that puts them in a situation that enables us to observe and obtain information but this situation does not consider their welfare. They are giving us an invaluable service, but against their will. They are beings capable of suffering and some scientists have difficulty recognizing their suffering. Therefore, we believe that education provided in the Faculty of Zaragoza, UNAM, must not forget the humanitarian part in education. We must not confuse our students, we ask them to follow the laws and rules that exist but on the other hand many teachers ignore them to take the shortest route when we use animals in class, regardless the emotional characteristics of students, teachers or animals themselves. Ethics does not excuse us for doing so. Teachers need to build the transcendental value system that forms the basis of the academic vocation, individually and as a group. In many cases, they are resistant to chance. They believe the best is the use of animals and software reduces the quality of teaching. However, there are several studies comparing the effectiveness of alternative methods to traditional methods. Therefore, we need to make teachers aware of the principles of the Three Rs – reduction, refinement and replacement; this last one the goal of our university.

III-4-603

Training for Reduction

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A recent survey has shown significant deficits in experimental design and reporting in studies published in quality biomedical journals across a range of disciplines (Kilkenny et al., 2009). The faults are so widespread that they would be expected to pervade those elements of regulatory work where guidelines do not stipulate a design. The FRAME Reduction Steering Committee is attempting to correct the inconsistencies. At a FRAME/LASA conference bringing together researchers and statisticians it was clear that a major problem was that even when statisticians were consulted their lack of appreciation of the biomedical questions and constraints made it difficult for them to advise on the best design. FRAME has brought together a group of teachers with both biomedical and statistical understanding to offer training for younger researchers that enables them to avoid the common errors, to appreciate how to select efficient designs, and to pose biomedical questions in a way that helps statisticians provide best advice. Several courses have now been run, which have attracted participants from across Europe and received excellent ratings. Pre- and post-tests confirm the enhancement in understanding achieved in only a few days. This understanding should have an impact in reducing animal numbers and waste of people's time and resources in the studies in which the attendees are involved and follow-up has indicated that this is the case.

Reference

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Session III-5: Introducing multi-media to the curriculum

Session III-5: Oral presentations

III-5-320

The usage of alternatives at the Norwegian School of Veterinary Science

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In March 2009 the Norwegian Reference Centre for Laboratory Animal Science and Alternatives at the Norwegian School of Veterinary Science opened a multimedia room / training clinic. It contains a large number of alternatives that can be used in the education of the veterinary students and the veterinary nurse students. The room has 4 laptops that are equipped with a number of programs made for teaching anatomy, pathology and physiology. There are also many CDs and DVDs with teaching material in laboratory animal science, ethics, anatomy, handling of laboratory animals, anesthesia, analgesia, surgery, necropsy and more. The room also has a number of models used for training practical clinical techniques. Today the room is being used frequently by the students in both mandatory education and also in their spare time. Some of the alternatives can completely replace animals being used in teaching. In addition, usage of the alternatives gives the student skills and confidence, so that when the student does the procedure on a live animal for the first time they do it faster and better, which leads to better animal welfare.

III-5-659

Teaching surgical techniques in the twenty-first century

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The use of microsurgical techniques is increasing all over the world, necessitating the training of more and more people in microsurgical skills. Traditionally, live animals have been used for this purpose, and in rather large numbers, as it often takes seventy or more attempts for a person to learn the necessary skills. Students are faced with tough problems at the same time, to master the hand-eye co-ordination techniques whilst simultaneously assuring the animal's welfare. On top of that the knowledge on the animals' anatomy is most often disappointing. The result is too often the untimely death of the animal.

The Microsurgical Developments Foundation has a clear policy of reducing, refining or replacing animal use wherever possible. We wanted to apply this admirable policy to the problem described above. Our basic innovative idea was that in the same way that physical models have been used throughout the ages to teach anatomy; it should be possible to build a suitable life-like model for the teaching of anatomy (MD 3-D Anatomical Rat Model), whilst the surgical procedures could be trained using the MD PVC-Rat. Students could separately learn all anatomical structures involved and the different skills needed before moving on to live animal experiments. Several aspects of both models will be discussed, together with the results that our students obtain.

III-5-440

Personalized resources on human and animal biology: Lessons from U.S. veterinary medical education

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The vigorous transition to alternatives occurring within United States veterinary medical education, especially with multimedia approaches, has been spearheaded by the creative leadership of educators developing new resources and teaching methods, a widespread movement with contributions from all veterinary schools. Many previous uses of animals in veterinary teaching have been replaced with alternatives. The explosive growth in subject matter that must be learned by veterinary students, combined with economic pressures, has stimulated these developments. This presentation provides background on the power of web-based instruction for students' personalized curricular access, with delivery of the specific information whenever needed, even in bite-sized pieces, much as students expect to acquire their own personal music collections.

As one example from the University of California, Davis, oral descriptors annotate individual histology slides, providing simple, personalized instruction. The teacher's familiar voice guides the student in looking at complex visual material, with a pointing arrow and zooming into areas of interest, leading the viewer to understand what is seen. Superb resources such as these are available to students whenever needed. Rather than students sitting as passive receptacles in a large classroom lecture, they are given a personal tour at a time of their choosing, with the option of returning for a refresher whenever useful.

Such a concept is available for translation to other learning settings. Building personalized listings of efficacious resources appropriate for the situation, and providing them online, places learners at the forefront in choosing what to learn.

III-5-402

Development of the 3Rs platform website in Korea for exchanging knowledge and sharing examples of best practice to replace laboratory animal use in education

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This year will mark the 250th world anniversary of veterinary education. Veterinary science was introduced into the Korean curriculum in 1906. There are ten veterinary medical schools in Korea. Two new Korean laws legislating animal welfare and the humane use of animals in science came into effect in 2008 and 2009. Both laws, the Animal Protection Law and the Laboratory Animal Law impose the Three Rs principles of replacement, reduction and refinement on procedures using animals and require ethical committee review prior to conducting animal experiments in research, testing and education. The joint project of the Royal Society for the Prevention of Cruelty to Animals, UK and the College of Veterinary Medicine, Konkuk University, Korea has set up systematic procedures to promote awareness of moral and ethical issues based on sound science from 2008 to 2010. Our research discovered that the need for development and implementation of well-proven alternatives to animals in education is clearly recognized by a majority of the veterinary professors and students. At this early stage, alternatives to the use of laboratory animals are often viewed as supplementary educational teaching aids, rather than replacements for animals. For a teacher with a busy teaching schedule and a traditional curriculum already in place, the prospect of adopting new and unfamiliar materials with a language barrier could be daunting. We are undertaking the development of the 3Rs platform website in Korea to provide user friendly alternative teaching resources collaborating with global experts and the Alternatives Research & Development Foundation.

III-5-511

A collaborative multi-language website and database for alternatives in education and training

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The new InterNICHE website under development at www.interniche.org is a content-rich resource designed to facilitate the implementation of humane education and alternatives. Using the open-source Drupal software, the Content Management System (CSM) and Framework (CMF) allows for a development process that is module-based and customisable so as to meet the needs of both developers and end-users. Information and other resources from InterNICHE and from teachers, students, campaigners and others from across the world can be effectively shared through such a collaborative international project and user-friendly system. The site is extensible, so further functionality to support such sharing, and to meet needs as they arise, can be added module by module. The translation functionality facilitates the localisation of information and news resources, and encourages participation and sharing from the diverse community that is involved in curricular transformation. Introductory information is available in over 100 languages, and translations of existing and new text can be uploaded with ease. The role-based access defines which users can view, edit and publish data, thereby better serving all users, including InterNICHE as a network and organisation. The many roles available encourage participation according to chosen degree of input. Various searchable databases such as the Alternatives File from the book "from Guinea Pig to Computer Mouse" (InterNICHE, 2003) provide rich seams of collated information, with database updating and translation opportunities provided through registered access. Management of other InterNICHE resources such as the network of Alternative Loan Systems is also supported. Version control supports management of the evolving information resource base. The site itself is hosted by an ethical communications collective.

III-5-500

Modular delivery of core surgical skills instruction in veterinary medicine

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Based on the results of two recent national surveys conducted by the authors about core surgical skills and proficiencies expected of entry-level veterinarians, an initiative has begun, the goal of which is to create a complete set of interactive core skills trainers in a digital multi-media modular format to be delivered through a centralized e-learning platform. Advantages of these modules include: guided/self-paced skills acquisition in a low stakes on-line format; potential reduction of required live animal experiences since students will have the necessary learning resources to acquire the core skills essential for multi-species surgical practice; inherent flexibility and re-usability of the e-learning format will allow for integration of modules into a variety of curricular plans. Developing teaching formats that maximally leverage faculty teaching resources, while allowing earlier opportunities for students to practice critical skill sets, such as those required to become competent entry-level surgeons, is vital to advancing ethical surgical teaching programs and graduating high quality veterinarians. These trainers will help ensure that learners are able to maximize the increasingly limited number of cadaveric and live-animal hands-on training experiences to their fullest potential. This session will highlight our first skills trainer, "Surgical Instrument Handing and Atraumatic Use." The developmental stages of this trainer and the finished module will be presented as a proof of concept. The course will contain a variety of interactive multimedia materials produced specifically for the course including: mini-lectures with 3D illustrations and demonstrations, interactive activities and assessments, course notes, hands-on laboratory exercises, and a course evaluation.

Session III-5: Poster presentations

III-5-086

The new alternative laboratory for training and teaching

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The new alternative lab (AltLab) was opened in the summer of 2009 thanks to donations from the Norwegian Animal Protection Alliance and the University of Bergen. This has given an opportunity to teach and train students, researchers and animal technicians. The AltLab contain several animal models, multimedia programs and surgical items. In the AltLab, users can learn intubation, blood sampling and different surgery techniques like transplantation of veins and organs, and injections. The AltLab also contains a library for selected books, DVDs and multimedia programs.

III-5-435

Teaching human biology and health in pre-college

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Continuing a historic tradition and following national and state standards for teaching human and animal biology and body systems, elementary and high school science teachers employ anatomical specimens and models in laboratory activities. Students explore the anatomical models or specimens and gain a perspective on their own bodies that can include a growing understanding of health maintenance. These high school classes offer most students their last formal opportunity to consolidate knowledge about their own bodies and health care. High motivation accompanies these activities that include or simulate dissection and even physiological processes. Students gain an opportunity to integrate knowledge concerning the basic biology of the human body and how that relates to specific experiences of family members with various medical conditions.

We provide a convenient guide to free teaching resources on human and animal anatomy that have been peer-reviewed by science teachers for use in intermediate and secondary schools. Point-and-click access leads to free, refereed web-based resources for teaching about human body systems. Sites were selected from focus group reviews by teachers. A related webquest presents activities and teacher guides that make use of the websites, all available at: http://www.vetmed.ucdavis.edu/Animal_Alternatives/goanatomy.html

Complementary to these web resources are models of human and body systems and organs available free from The Science Bank at Animalearn through an easy-to-use loan program. The models and manikins, plus additional CD-ROMS and software, are rated for educational level and are loaned at no cost by Animalearn.

III-5-505

The impact of decentralised alternatives libraries on campaigning for replacement

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Libraries of alternatives offer hands-on experience of non-animal teaching and training tools. They enable borrowers to assess products and become familiarised with the range of available alternatives. InterNICHE established the first international alternatives library, known as the Alternatives Loan System, in 2002. It comprises a wide range of software, models, manikins and training devices chosen for their pedagogical value and potential for replacement. The alternatives cover all disciplines within medicine, veterinary medicine and biology. Borrowers range from teachers through ethics committees to campaigners. The library makes alternatives more accessible and provides a resource for conferences, exhibitions, outreach tours and training. The loans have facilitated implementation, as demonstrated by subsequent purchase and use of alternatives, and replacement of dissections and animal experiments. The positive impact of the resource and the growth and capacity of the InterNICHE network led to the establishment of further libraries in Russia, Ukraine, India, Mexico, Peru, Kenya and South Africa. Each resource has brought the benefits of the international library to the country, with further advantages. Being localised, it is more practical and economic, facilitating significantly increased access. Being managed by the InterNICHE National Contact or Partner, it empowers through new responsibilities; and with an important resource to offer, it strengthens their position nationally. Reflecting further decentralisation and provision of localised resources, each InterNICHE National Contact and Partner across the world also now has a set of 30 software alternatives. This equips even more campaigners with small but valuable software libraries that complement the Alternative Loan Systems.

III-5-506

Alternatives seminars and multimedia exhibitions: Global outreach and support for humane education initiatives

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An increasing number of seminars on humane education and alternatives to dissection and animal experiments have been organised by InterNICHE and its partners across the world. In combination with oral presentations and workshops, multimedia exhibitions with demonstrations of alternatives from across the disciplines comprise an essential part of the seminars. The multimedia exhibitions have also played a contributory role at other events. This paper details major events by region or country (Latin America, Europe, Africa, Middle East, India, China); by nature (type of outreach, degree of training); and by focus (faculty and discipline, participants). It situates each event within the context of local, national and international humane education initiatives and the growing movement for curricular change.

Session III-6: Training animal-based scientists

Session III-6: Poster presentations

III-6-046

Refinement in practical works in FELASA accredited course at ENVT

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The FELASA training course 011/05, organized at ENVT for numerous years, was accredited on 14 February 2006 as C level. This course entitled "Use and care of laboratory animals", i.e. "Utilisation et Protection des Animaux de Laboratoire" (UPAL) is accredited as level I in the French regulation. Based on the FELASA annual reports, the main features characterising this course will be summarized and analyzed

UPAL organises two annual sessions, in March and September. From 2006 to 2009, a total of 273 students attended the courses, with an equal repartition between the two sessions (135 and 138 students in March and September, respectively). Continuing education candidates represent the major part of students, comprising researchers from public and from private institutes. The staff included 17 teachers, half coming from private or public institutes (Sanofi-Aventis, Galderma, Pierre Fabre, Charles River, Janvier, Safe, INSERM, INRA); the other half from Veterinary Schools (Toulouse and Alfort). Electronic evaluation forms, asking general questions but also rating each presentation on the pertinence of the subject and on the quality of the presentation allowed fine analysis of the feedback of each course.

For wet-lab practice, students were divided into two sub-groups (composed of 15 to 20 students each). Over the years 2006-2009, we have improved the practical works in order to

– Reduce the number of animals;
– Refine their use, by switching from injectable to inhalation anesthesia and morphine analgesia;
– Re-use of animals for other pedagogic or scientific purposes instead of using another set of animals.

III-6-421

las-online.eu – a trilingual approach to refinement through laboratory animal science (LAS) education

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The welfare of animals used for research purposes depends on the professional competence of all personnel involved. High quality education in LAS lays the foundation that this competence is built upon as such courses that not only teach good scientific practice but help create awareness for the needs and the welfare of laboratory animals. Teaching in itself can be regarded as refinement as it ensures that the principles of the 3Rs are acted upon. However, no definite regulations regarding training programs are in place. Harmonizing LAS education would contribute to the welfare of animals by relating a common standardized set of minimum requirements, such as stated in the FELASA Guidelines, Annex V of the EU Directive 2010/63/EU or "The Guide". Language barriers might hamper the creation of such programs. A multilingual basic curriculum could help to further the harmonization process. Here we report on a trilingual online platform (EN/FR/DE) for teaching laboratory animal science (las-online.eu). Topics are based on the FELASA category B guidelines. Multimedia content, such as videos, animations and pictures help to prepare and review practical training, such as the zoom in section about rodent anatomy. Teaching content from a variety of sources can be easily imported and made available to the LAS community. The platforms' availability in different languages and the integration of other countries' legal requirements pertaining to animal research make it accessible to a broader user group. Training as a means of refinement may not reduce the number of animals but directly impacts the animals' wellbeing.

Theme IV
Animal Welfare for Refinement and High Quality Science

Session IV-1: Indicators of animal welfare to implement refinement

Session IV-1: Oral presentations

IV-1-072

Affective states and the assessment of laboratory-induced animal welfare impacts

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Animal welfare is increasingly understood in terms of a wide range of affective states or feelings animals may experience. Some of these can be assessed and others, as yet, cannot. Reference is made to a comprehensive system for ranking the impacts of scientific procedures on the affective states or feelings that can be assessed. To date, the predominant focus has been on pain and distress. It is argued that, in addition to these, other negative subjective and emotional experiences including the following should be considered: thirst, hunger, nausea, breathlessness, dizziness, debility, weakness, sickness, anxiety, boredom, fear, frustration, helplessness and loneliness. Moreover, negative impacts on positive emotional states or experiences such as satiety, vitality, reward, contentment, curiosity and playfulness should also be evaluated. The purpose here is to reinforce the principle that because animals may have bad or good experiences at our hands, we have an obligation to treat them considerately (at the very least); this translates into minimising the harm we do to them and maximising the good. More effective harm minimisation should result when the Three Rs are applied to mitigating this wider range of negative emotional experiences, and when other measures are adopted that promote specific positive emotional states or the general wellbeing of animals. It is anticipated that this broader perspective will enhance caring and empathetic attitudes towards animals among investigators and members of Animal Ethics Committees or Animal Care and Use Committees.

IV-1-610

The sensitivity of animals and application of the Three Rs

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It may be impossible to fully understand the point of view of a non-human animal, yet we are duty bound to come as close as possible. To this end, we try to determine the right level of anthropomorphism, closely study their natural history, and mentally add or subtract sensory or psychological "modules" from the equivalent human experience. In practice, however, humans typically care for animals as if they sense only what we sense, and understand their existence in our terms, if at all. Even when a

species is known to respond to particular light levels and cycles, to human presence and expectations, or to stimuli outside our perceptions, this may not result in husbandry changes. Indeed, we might try to eliminate these factors, as if experiences outside our reach deserve only to be eradicated. Established abilities, such as how a pigeon returns home or a dog senses cancerous cells, were neglected for decades because we were ignorant of the mechanism and were probably unwilling to tackle such a void in our understanding. Our protocols might change considerably if we countenance the possibility that animals know their own fates, see into our minds, and have access to everything that happens around them; or if we tried seeing animals as participants in, rather than subjects of, their fate. Would we be more careful, more frugal, and more likely to seek alternatives if one of the groups that we had to sincerely explain the research to was the animal itself?

IV-1-609

Reliance on behavior as a metric of animal welfare

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A first-tier method of assessing an animal's welfare is often a judgment based on observable behavior. Several factors may confound the observation and interpretation of the behaviors observed, which could result in missed opportunities to implement the Three Rs, especially refinements. Alternatively, the refinements initiated to address a perceived problem may not correlate with the issue because of misinterpretation of the observed behaviors. Typically, research animals are observed during normal working hours while routine activities are ongoing in the animal room. Yet, the benefits and limitations to assessing animal welfare at this time have not been analyzed across species. Also, factors such as the skill level of the individual making the observations must be considered in a system of reliance on behavior to detect welfare issues. Perhaps most importantly, responses of animals to experimental procedures, their environment, etc. vary among individuals and according to species, age/maturity, gender, physiological and pathological state, environment, phase of response to a stimulus, and other factors. Thus, any tendency to extrapolate assessment criteria among individual animals, strains and species is fallible. Yet, appropriately gathered and interpreted, behavior observations can be an important and practical tool to assess welfare and validate that refinements implemented address not only extant behavioral concerns but the underlying welfare issue. A framework for implementing a pragmatic approach to using behavior as a welfare indicator will be discussed.

IV-1-069

Complementary roles for systematic analytical evaluation and qualitative whole animal profiling in welfare assessment for Three Rs applications

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Application of the Three Rs involves prospective evaluation of potential negative welfare impacts of scientific procedures, while assessment of the success of Three Rs applications requires retrospective evaluation of the actual impacts. Two complementary approaches to animal welfare assessment are available to assist with this: Systematic Analytical Evaluation (SAE) and Whole Animal Profiling (WAP). SAE aims to comprehensively anticipate functional disruptions, rank scientific procedures according to their actual negative impacts, and guide the development and application of methods to mitigate such impacts. A key focus of SAE is to assess the likely impacts on subjective mental states, adduced from objective behavioural, physiological and pathophysiological knowledge. In contrast, WAP involves observers scoring subjective impressions of appearance, demeanour and behaviour in terms of overall welfare status at the time of the evaluation. Conclusions based on qualitative WAP have been validated using key quantitative behavioural and physiological indices of welfare status. These two approaches are complementary. For example, WAP can be used to verify welfare impacts anticipated using SAE, whereas SAE may be used to elucidate the factors that contribute to a particular welfare state identified using WAP. It is suggested that combining both approaches in assessments of laboratory animal welfare will facilitate more thorough evaluation of welfare status, enable immediate or future mitigation strategies to be identified, and thereby enhance application of Three Rs measures.

IV-1-520

Behaviour changes during rat euthanasia may be a poor indicator of aversion

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Laboratory rodents are commonly euthanized with carbon dioxide (CO_2), but approach-avoidance, avoidance-avoidance, and total dwelling time studies have shown that rats find exposure to the inhalant anaesthetic isoflurane less aversive than exposure to CO_2. The aim of this study was to describe gross behavioural changes during euthanasia with CO_2 and isoflurane to determine how well these behavioural changes relate to the aversion experiments. Thirteen male Sprague-Dawley rats weighing (mean ±SD) 466 ±69 g were euthanized with CO_2 delivered at a flow rate of 23% cage volume per minute, and 13 male Sprague-Dawley rats weighing 452 ±65 g were euthanized with 4% isoflurane delivered in oxygen at 23% of the test cage volume per minute. Trials were video recorded and rat behaviour was scored for activity (number of transitions between quadrants) and rearing (two front paws off the ground) from 90 s before gas delivery began until rats ceased all purposeful movement. The frequency of each behaviour was recorded in 10 s intervals. Activity increased in both treatments after gas delivery began, with no difference in peak activity between treatments (mean ±SE; CO_2: 2.5 ±0.3 vs. isoflurane 2.7 ±0.6; $t = 0.24$; $P = 0.81$). Rats showed a higher peak frequency of rearing when exposed to isoflurane (1.2 ±0.2) than when exposed to CO_2 (0.8 ±0.3; $t = 2.98$; $P = 0.007$). Given that multiple experiments have shown that isoflurane is less aversive that CO_2, we conclude that the behavioural differences are due to an excitatory phase during induction with isoflurane. These results illustrate that observations of gross behaviour during euthanasia may be a poor indicator of aversion.

IV-1-490

Impact of simple environmental improvements on affective behavior, physiology and immune system reactivity of C57BL/6 and BALB/c mice

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The objectives of this study were to evaluate the long term effects of enrichment and housing on BALB/c and C57BL/6 mice, with particular consideration of the effects on behaviour, body weight, fecal corticoid levels, and immune system function and responsiveness (changes in complete blood cell count and response to lipopolysaccharide challenge). The study was 20 weeks in duration and involved 160 mice; 20/sex/strain/housing paradigm. Mice (5/cage) were randomized into one of two housing paradigms in solid bottom caging: contact hardwood chip bedding, or contact hardwood chip bedding + cotton nesting material + clear amber tube + 10g wood wool + 1 Cheerio 3x/week. At 20 weeks, C57BL/6 mice were heavier than BALB/c mice and enriched mice had mild but consistently greater body weights than unenriched mice. Significantly less barbering was noted in enriched female C57BL/6 mice than unenriched B6 females. Mice were videotaped monthly and behaviours were scan scored. Dominance behavior was significantly more frequent in unenriched than enriched cages. Abnormal and aggressive sexual behavior was observed in male BALB/c mice and increased over time, with significantly increased intensity in mice in unenriched cages. There were no significant housing differences for stereotypic behavior, eating, locomotion, or positive social behaviours. Feces were collected monthly and fecal corticoid metabolites were extracted and evaluated. BALB/c mice had significantly higher levels of fecal corticoids than C57BL/6 mice for both sexes. Unenriched BALB/c males had significantly lower fecal corticoid levels during the dark phase than their enriched counterparts, suggesting a blunting in Circadian corticosterone release. Within each strain and sex, there were no differences in hematologic parameters for mice housed in either caging paradigm at month 1 or 5. Further, there was no consistent effect of housing paradigm on WBC and lymphocyte subset

relative ratios in response to LPS injection, although strain- and sex-specific differences were noted. In conclusion, consistent provision of simple environmental improvements to B6 and BALB/c mice led to production of larger mice with consistent decreases in cage aggression, barbering, and abnormal sexual behaviour. Inexpensive cage improvements do not significantly alter many physiologic parameters within a particular sex or strain of mouse but may improve overall animal well-being, in particular, through a reduction in cage aggression.

Session IV-1: Poster presentations

IV-1-061

Assessment of post-surgical pain in mice using species-typical burrowing behavior

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Detection of persistent pain of a mild-to-moderate degree in laboratory mice is difficult because mice do not show unambiguous symptoms of pain or suffering using standard methods of short-term observational or clinical monitoring. This study investigated the potential use of burrowing performance – a spontaneous and highly motivated behavior – as a measure of post-operative pain in laboratory mice. The influence of minor surgery on burrowing was investigated in adult C57BL/6J mice of both genders in a modified rodent burrowing test (displacement of food pellets from a pellet-filled tube) within the animal's home cage. Almost all (98%) healthy mice burrowed (mean latency 1.3 h, SEM 0.5 h). After surgery without pain treatment, latency of burrowing was significantly prolonged (mean Δ latency 10 h). Analgesic treatment using the anti-inflammatory drug carprofen (5 mg/kg bodyweight) decreased latency of burrowing after surgery (mean Δ latency 5.5 h) to the level found in mice that had been anesthetized (mean Δ latency 5.4 h) or had received anesthesia and analgesia (mean Δ latency 4.6 h). Analgesia during surgery was associated with a significantly earlier onset of burrowing compared to surgery without pain treatment. A distinct gradation in burrowing performance was found ranging from the undisturbed pre-operative status to the intermediate level following anesthesia/analgesia and surgery with analgesia, to the pronounced prolongation of latency to burrow after surgery without pain relief. In conclusion, post-surgical impairment of general condition, probably mainly attributable to pain, can be conveniently assessed in laboratory mice on the basis of the burrowing test.

IV-1-120

Defining metrics to measure and communicate progress of 3Rs investments and activities – European Federation of Pharmaceutical Industries and Associations (EFPIA)

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In order to make a self-assessment of how its members have implemented the 3Rs, EFPIA conducted a survey with input from 18 companies, representing a range of therapeutic areas and geographical locations. One conclusion from this survey was that there were no commonly agreed key performance indicators (KPIs). Therefore, measuring and communicating the impact of 3Rs activities is very difficult. The current situation does not reflect all the 3Rs initiatives within industry. It is important to stress that the implementation of the 3Rs is a continuous effort, integrated in science as it advances.

The questions "how much do you spend?", "how many animals did you save?", and "how does it improve science?" are the most frequently asked in public and internal company debates on animal use. Establishing KPIs in relation to the implementation of the 3Rs is a challenge, as direct links to projects and the business are not visible. The total numbers of animals used in R&D-projects is not a valid KPI. This number is influenced by multiple variables such as discontinuation or launch of projects, opening or closing research labs, or changing regulatory requirements. The impact of 3Rs developments can be viewed from different perspectives (e.g. ethical, scientific, resource). Thus a single KPI covering all perspectives might be unrealistic. The EFPIA group on Research and Animal Welfare collated examples of potential KPIs to try and define a common set of indicators which could be used by its members in order to provide evidence of the benefits of 3Rs implementation.

IV-1-144

Assessment of intraplantar FCA-induced mechanical hypersensitivity using dynamic weight bearing

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Chronic pain affects one in three people over the course of their lifetime and is very poorly treated. Animal models are an integral part of pain research; however, current models tend to rely on evoked responses. There is belief that non-evoked responses may be a more relevant behavioural readout, as the animal responds in a more natural manner.

The standard incapacitance model involves placing a mouse, under light restraint, in a small (4x4 cm) Perspex chamber with each hind paw on a single pressure transducer. However, this places the mouse in an unnatural situation which may produce additional stresses in the animal. Dynamic weight bearing (DWB) is much less invasive, as the animal is placed in an enclosed area (11x11 cm) on top of a sensor containing 1936 pressure transducers and is allowed free movement. This enables assessment of the animal's natural stance during testing, which may be more clinically relevant.

Here, using the DWB test, it has been shown that intraplantar injection of Freund's Complete Adjuvant (FCA) (30 µl, 1 mg/ml) produces a significant ($P < 0.001$) reduction in weight placed through the injured hind paw for 4 days post FCA injection compared to vehicle (mineral oil, 30 µl) treated mice, which is indicative of inflammatory mechanical hypersensitivity. At 48 h post FCA injection, celecoxib (30 mg/kg p.o.) significantly ($P < 0.05$) reduced this FCA induced mechanical hypersensitivity compared to vehicle (1% methylcellulose 10 ml/kg p.o.) treated mice. This finding is in keeping with data previously acquired using the standard incapacitance model, but here only 6 mice were used per group rather than the normal 12. The DWB test is a refinement of the standard model and has the potential to reduce animal usage by half with no loss of data integrity.

IV-1-160

CO_2 and inhalent anaesthetics for the induction of euthanasia in mice: a comparative study

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The use of carbon dioxide (CO_2) for euthanasia in mice has been strongly criticised concerning animal welfare. Alternatives have not been sufficiently tested. Here, we investigate distress induced by exposure to CO_2, isoflurane and sevoflurane. NMRI mice were exposed to 100% CO_2 with different filling rates – 20% ($CO_2$220), 60% ($CO_2$60), and 100% ($CO_2$100) of chamber volume/min – or isoflurane and sevoflurane in different concentrations (Iso2%, Iso5%, Sevo4.8%, Sevo8%). We recorded behaviour and vocalisations during induction until surgical tolerance (ST) or during 5 min of air exposure (control). Then, mice were decapitated and glucose, adrenaline and noradrenaline were measured.

ST was reached fastest after exposure to $CO_2$100, followed by $CO_2$60 < Iso5% < Sevo8%. 37.5% of the mice did not reach ST within 5 min while exposed to Iso2% and Sevo4.8%. With $CO_2$20, 75% of the mice did not reach ST. Compared to control, changes in behaviour were apparent regarding grooming, arousal, escape behaviour and excitatory phenomena. No audible or ultrasound vocalisations were detected. Glucose concentrations had risen in Iso2%, Iso5%, and Sevo4.8% groups compared to control. Adrenaline and noradrenaline concentrations were increased in $CO_2$60 and $CO_2$100 treated mice compared to all groups.

Even though $CO_2$60 and $CO_2$100 induce narcosis faster than isoflurane and sevoflurane, the increases of adrenaline and noradrenaline point towards a higher perception of distress. Further investigations (histopathology of respiratory tract) are in progress to conclusively determine if isoflurane and sevoflurane in higher concentrations can be recommended for the induction of euthanasia in mice.

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IV-1-236

The effect of transportation on the physiology and behaviour of rats

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Transportation of laboratory rodents unavoidably causes stress. Nevertheless, very little is known about the effects of transportation and how long it takes for the animal to recuperate. To obtain reliable scientific results from experiments using animals, their physiological status needs to be normalized / stabilized to a condition which can be defined as baseline. Using stressed animals is likely to result in considerable and unintended effects on research results. We investigated physiological and behavioral parameters before and after transportation, as well as in transported and non-transported animals. Blood samples were taken for analysis on plasma corticosterone, glucose and creatine kinase. Physiological measurements were performed by means of telemetry, measuring heart rate, blood pressure and activity. Behavior was measured by means of home cage observations. Besides measuring these parameters, a study was dedicated to the effect of temperature fluctuations during transportation on the core body temperature of rats.

Temperature inside transportation boxes strongly correlated with body temperature. Significantly decreased body weight, and water and food intake were observed on the day of transportation in transported animals. Plasma corticosterone levels were increased up to at least 16 days after transportation. Female control rats showed decreased glucose levels compared to transported females on the day of transportation. Blood pressure and heart rate showed a lasting decrease after transportation. Grooming increased, while social interactions and locomotor activity decreased after transportation. With these studies, we have demonstrated that there is a long lasting effect of transportation on physiological and behavioural parameters.

IV-1-270

An interactive tool used to improve early recognition of health problems in mice

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Early recognition of a health problem allows prompt reporting, fast intervention and appropriate management of an animal in pain or in distress. To facilitate early detection of health problems, we built an extensive collection of photographs and videos illustrating physical and behavioral clinical signs as well as abnormal phenotypes observed in our experimental mice and mouse colonies. We used a screenshot software to highlight and annotate the photographs which allows quick recognition of the condition. In addition, several photographs of the same condition show the progression of clinical signs. This extensive collection (over 250 photographs and videos) is available through our internal network for consultation by the veterinary staff, animal care technicians and husbandry personnel. Specific pictures are posted on animal room doors to alert the animal care personnel of a possible health or welfare problem concerning a specific strain of mice. The collection is used as a teaching tool for our staff, investigators, interns, students and members from external institutions. We are developing an interactive webpage on our internal network, where it will be possible to search for pictures, appropriate terminology, explanations and references, using a medical term or an animal model. We observed that trained individuals increased the number of cases reported and improved their signalling of health problems which allowed early evaluation of the animals by the veterinary staff. This interactive tool has become a key element in improving the animal welfare for the mice housed in our institution.

IV-1-292

Eliminating pain and distress in ocular safety testing: use of topical anesthetics, systemic analgesics, and humane endpoints

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Current EPA and OECD test guidelines for the rabbit eye test only allow the use of topical anesthetics when the user demonstrates that such pretreatments do not interfere with the test results. This requirement often results in topical anesthetics and other pain relieving medications not being used. ICCVAM subsequently evaluated the potential impact of using topical anesthetics, systemic analgesics, and humane endpoints to avoid or minimize pain and distress when the rabbit eye test is required for ocular safety testing. ICCVAM concluded that balanced preemptive pain management should always be provided when the rabbit eye test is conducted for regulatory safety testing. This should include pre-treatment with a topical anesthetic and a systemic analgesic, followed by post-treatment with systemic analgesia until lesions resolve or the study is terminated. ICCVAM recommended regular monitoring and recording of all clinical signs that may be indicative of pain and/or distress, as well as recording of the nature, severity, and progression of all eye injuries. ICCVAM also recommended several additional types of ocular damage that should be used as humane endpoints to end studies earlier. US agencies have endorsed these ICCVAM recommendations, which will effectively eliminate pain and distress in most *in vivo* ocular safety testing situations, thereby significantly refining animal use. These refinements should be routinely used whenever the rabbit eye test is still required. A proposal to revise OECD TG 405 with these modifications is currently under consideration.

IV-1-296

Assessment of the effects of meloxicam on polyclonal antibody production and related adjuvant-induced inflammation in New Zealand White rabbits

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Complete and Incomplete Freund's Adjuvant (CFA/IFA) are often used in the production of antibodies to specific molecules and can cause local inflammation and granulomatous reactions which can be painful if abscess formation or skin ulceration occurs. Treatment of significant skin lesions may include the use of analgesics. We investigated whether the non-steroidal anti-inflammatory drug (NSAID) meloxicam, dosed for analgesia, would impact antibody production. We hypothesized that meloxicam treatment would not impact on antibody response. Thirty New Zealand White (NZW) rabbits received immunization with adjuvant over 7 months (1 CFA inoculation/3 IFA boosts). Half of the rabbits were randomly selected to receive meloxicam (0.75 mg/kg BID for 3 days, followed by 0.3 mg/kg BID for 7 days) in piña-colada flavored tablets, while the remaining received placebo tablets. This dose of meloxicam was well-tolerated with no clinical indications of gastrointestinal ulceration or nephrotoxicity. There was no significant difference between groups in food consumption, body weight, clinical pathology parameters, and number of nodules or ulcerated lesions. There was also no significant effect of treatment (n = 15/treatment group) on antibody titer levels determined by linear regression with an optical density target of 0.5 [$t(28) = 0.1579$, $p = 0.8757$], optical density target of 0.1 [$t(28) = 0.5031$, $p = 0.6189$], or evaluation by 3 times the background reading [$t(28) = 0.3560$, $p = 0.7245$]. Plasma IFN-gamma and IL-12, two cytokines associated with Th1- and Th-2-type immune responses, were evaluated by rabbit-specific ELISA. In summary, analgesic doses of the anti-inflammatory drug meloxicam do not alter antibody production in a traditional immunization protocol for polyclonal antibody production.

IV-1-309

Refinements in dog housing and husbandry, and the link with quality of science

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Dogs have special protection under the UK legislation on the protection of animals used in scientific research. It is critical that their welfare is maximized, and that the most reliable and valid scientific results are achieved from their use. Whilst the link between good welfare and good science is often made, housing and husbandry practices are often advocated without a sound scientific understanding of their welfare implications. In collaboration with academia and industry, we are developing a project to examine the link between Refinements in dog rearing, housing and husbandry and quality of scientific output, measured in terms of repeatability of data, and between-dog variability. AstraZeneca has recently built new facilities and has incorporated many design features aimed to improve dog welfare. These include pens with raised platforms, glass panels to improve visibility and reduce noise from barking and access to both indoor and outdoor runs with a variety of structural enrichments. In this presentation, we describe Refinements in housing and husbandry in the new facility and present preliminary data on physical, behavioural and cardiovascular measures on telemetered dogs. We describe our plans to incorporate further Refinements throughout the life of the dogs, especially in enhanced socialisation with humans, habituation to procedures and positive reinforcement training, and how these impact on quality of scientific data output.

IV-1-360

The effect of behavioural state and cage environment on responses to euthanasia with isoflurane or carbon dioxide in BALB/c mice

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Two common euthanasia agents, isoflurane and carbon dioxide (CO_2) cause aversion and stress. In addition, "procedural" stressors (handling, placement in an unfamiliar environment, etc.) may add to the overall stressfulness of euthanasia. To investigate this we carried out euthanasia in BALB/c mice (24 males and 24 females, 8 mice/group) with isoflurane (5% isoflurane in 20% cage volume/min oxygen) or CO_2 (20% cage volume/min) using three protocols:

A. euthanasia in empty cages into which mice were placed immediately prior to euthanasia, analogous to normal procedure;
B. home-cage euthanasia whilst initially sleeping; mice were acclimatised to the cage for ≥24hr and provided with bedding and nesting material;
C. home-cage euthanasia as above, but carried out during wakefulness.

Gas flow initiation caused sleeping mice to awaken in 13.5 ±1.3 s for Isoflurane and 10.1 ±1.2 s for CO_2 (difference not significant, $P = 0.077$). Time exposed to a euthanasia agent whilst awake was significantly shorter for mice exposed to Isoflurane (56.3 ±2.8 s) during sleep than for CO_2 (86 ±4.2 s) or animals which were awake when exposed to Isoflurane (77.4 ±3.6 s) ($P < 0.001$). Mice exposed to isoflurane showed significant increases in behaviours potentially associated with excitation and stress such as running and ataxia. Running occurred only during isoflurane exposure and more frequently amongst un-acclimatised mice than mice exposed in home cages ($P = 0.01$).

In conclusion, isoflurane appears to be less alerting than CO_2, taking longer to cause awakening. Initiation of euthanasia during sleep can significantly reduce the amount of time to which animals are exposed to euthanasia agents during wakefulness, and hence potentially reduce the time animals are stressed. Home-cage euthanasia reduces some signs of agitation or excitation.

IV-1-393

Environmental enrichment influences the results in behavioral tests

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An enriched environment is suggested to increase the welfare of captive animals. We tested whether such an environment can influence the performance of laboratory rats in tests commonly used in behavioral neuroscience. Eleven adult rats were individually housed in standard shoeboxes with litter and food/water ad lib; 11 other rats were housed in shoeboxes each containing hardwood blocks, Kraft paper towels and a non-toxic PVC tunnel. Rats were tested after 12 weeks. Compared to the non-enriched condition, the enriched environment showed no effects on the Sucrose Preference Test, Emergence Test, Open Field and Elevated Plus Maze. However, the enriched environment was associated with decreased swimming time in the Forced Swimming Test and decreased exploration time in the Novel Object Test. These data indicate that environmental enrichment can influence the baseline of laboratory rats on behavioral tests aimed at cognitive performance.

IV-1-461

Welfare assessment in swine in biomedical research – suggestion for a welfare assessment standard for research facilities

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The swine (*Sus scrofa*) is becoming an increasingly attractive experimental animal species as an alternative to rodents and non-human primates. Despite the fact that a considerable amount of biomedical research has been done on swine, hardly any studies include systematic welfare assessment of laboratory swine. In order to quantify and control laboratory swine welfare, a practical tool is needed. The purpose of this presentation is to suggest a welfare assessment standard for research facilities, primarily based on an exposition of ethological considerations relevant for the welfare of swine in biomedical research. The tools for porcine welfare assessment presented suggest a method for monitoring the welfare status of individual laboratory swine, which is intended to improve practical scoring of the welfare of individual swine, the interpretation of the findings, as well as communication between researcher and animal caretakers.

IV-1-466

The TIN score: assessment tool for distress in laboratory mice

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The ability to assess well-being in laboratory mice is crucial to maintaining quality research. If mice are distressed or in pain, protocols can be adjusted and humane endpoints can be determined more accurately. Lack of well-being might be assessed by observing decreases in natural behaviors. We previously observed that a new piece of nesting material, dropped into a cage of healthy mice, will be incorporated into the nest within 10 minutes – a normal response we called a TIN score (= Time to Incorporate Nest material). If mice completed this task within 10 minutes it was considered a positive TIN score. We observed mice before and after undergoing procedures ranging from mild to severe and determined their TIN score. We found that mice undergoing mild procedures such as osmotic pump placement and ovariectomy showed no change in their TIN score one day after the procedure. However, mice took much longer to regain their baseline TIN score if they underwent more severe procedures such as carotid injury surgery. Differences in TIN score also identified a difference in recovery rates of single versus group housing conditions in the carotid injury group. Additionally, we found we were able to discern differences in diabetic mice that were sick prior to surgical procedures. In summary, our preliminary evidence suggests that the TIN score is a potentially useful tool for assessing well-being in laboratory mice and further testing should be done on this method in order to expand the clinical significance of its application.

IV-1-467

Environmental enrichment for NTP studies

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Environmental enrichment has been described as any measure that promotes expression of species-specific natural behavior and inhibits abnormal behaviors. Enrichment is beneficial for the psychological and physical well-being of animals. Recent modifications to the National Toxicology Program (NTP) animal care and use program represent an important effort to include environmental enrichment in NTP rodent studies. These modifications fulfill the Guide for the Care and Use of Laboratory Animals (Guide) and AAALAC International requirements, enhance animal well-being by providing sensory and

motor stimulation, improve quality of experimental data, and allow animals to have choices and control over their environment. Within the framework of NTP study requirements, social and physical enrichment were considered appropriate enrichment options. Within this context, social enrichment of group housing allows animals to perform social behaviors such as grooming, vocalization, and play. The social nature of rodents readily allows for successful group housing of male and female rats and female mice. However, to offset behavioral issues in male mice, group housing requires introduction of mice in stable environments at weaning. Physical enrichment devices allow animals to control the stressors in their environment by enabling species appropriate behavior, e.g., nesting for mice, gnawing or burrowing and perching for rats. As the NTP moves toward instituting an enrichment program for future studies, several factors are being considered that will address the need to provide enrichment for animals on study without compromising the scientific question(s) under study.

IV-1-494

Rat aversion to isoflurane and carbon dioxide

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Laboratory rats are commonly euthanized with carbon dioxide (CO_2). The inhalant anaesthetic isoflurane appears to be less aversive than CO_2, but little research has directly compared rat aversion to the two gases. We used an aversion/avoidance experiment to compare aversion to isoflurane and CO_2. Albino Sprague-Dawley rats were given the choice between staying in a dark compartment filling with CO_2 (n = 8) or isoflurane (n = 8), or escaping to a compartment with a light intensity that rats find slightly or highly aversive (300 or 1600 lux). The flow rates of each gas, respectively, were the ones recommended for euthanasia to mimic real euthanasia. When tested at the high light level, none of the 8 rats left the dark compartment filling with isoflurane, but 6 of 8 rats left the dark compartment filling with CO_2 (Fisher-Exact test, P=0.004), indicating that aversion to CO_2 is higher than aversion to isoflurane. At the lower light level, 6 of 8 rats left the dark compartment when exposed to the isoflurane, and 8 of 8 left when exposed to CO_2 (N.S.), suggesting that isoflurane is moderately aversive. When rats were re-exposed to the two gases, all left the dark compartment, regardless of light level. However, rats remained longer when exposed to isoflurane (23 s ±3 s) versus CO_2 (15 s ±3 s; $F_{1,14} = 5.46$, $P < 0.035$). Together these results indicate that isoflurane is less aversive than carbon dioxide to laboratory rats, and thus preferable for use in euthanasia.

IV-1-564

Use of thermography as refinement indicator of animal welfare

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Thermography is the technique of measuring natural thermal radiation from body tissues of clinical interest. It is safe, requires no control of environmental conditions, and is non-invasive. The technique has been used for different applications, among others to study inflammatory processes in joints, and skin temperature in connection to pain or pruritus. This paper describes different application areas of thermography in laboratory animal protocols, and focuses on the use of digital infrared thermal imaging in a rat collagen induced arthritis model as a method for evaluating disease progression and pain. The presentation gives other examples of the use of thermography in laboratory animal science contributing to welfare evaluation of the animals.

Session IV-2: Farm animal research and the Three Rs

Session IV-2: Oral presentations

IV-2-652

Beyond harm reduction: good lives for farm animals

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During the European enlightenment, intellectuals attempted to use logic and empirical investigation to replace traditional beliefs. The Enlightenment arguably gave rise to modern science and the Industrial Revolution. It also provided the idea of balancing harms and benefits as a basis of ethical decision-making, and ultimately underlay the harm-reduction approach that is widely applied in the ethics of animal research.

Reacting against the new emphasis on science and industry was the Romantic Movement whose adherents valued emotion ahead of reason, and valued a simple, natural life ahead of technology. Although the rationalist/empiricist tradition still thrives in the world of science, Romantic values are prominent in Western societies today, perhaps revived during decades when pollution, climate change, genetic engineering and other developments have led to renewed skepticism regarding science and industry.

Seen through the eyes of the Romantic Movement, the keeping of animals would require more than harm-reduction and cost-benefit analysis. Rather, it would require that animals kept for human purposes have "good lives" in the sense of being healthy, being able to enjoy life, and living under circumstances that match their nature and adaptations. Given that Romantic values are deeply rooted in modern Western thought, public acceptance of animal research, and of livestock production practices, requires that these values be taken into account to at least some degree.

IV-2-611

Species-specific approaches are needed for effective implementation of the Three Rs in farm animal research

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Comprehensive, high quality standard operating procedures (SOPs) developed for individual species can provide guidance for novice researchers, inexperienced designated veterinarians and lay members of animal ethics committees. Those with regulatory responsibilities for experimental animal facilities sometimes have greater experience with laboratory and companion animals than with farm animals, and this can result in attempts to apply unnecessary or inappropriate conditions to the management of farm animals before and during laboratory or field studies. Biologically a rat is not a dog is not a pig is not a sheep. The purpose of this paper is to briefly outline, as an example, the development of a *Policy on the Care and Use of Sheep for Scientific Purposes Based on Good Practice* by Australian and New Zealand experts in sheep biology, behaviour, experimentation, husbandry and welfare, which led to the formulation of institutional SOPs for sheep. The areas covered include: teamwork and training; applying the code of practice; appropriateness of animals for the purpose, including their selection, acclimatisation and training; minimising stress; pain relief; facilities; confinement; movement of animals; and monitoring. On the basis of this experience guidance will be provided on the development of such SOPs.

IV-2-075

Positive reinforcement training in large experimental animals

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The use of large animals such as mini pigs, pigs and ruminants in animal experimentation often includes the need for some kind of force or restraint to perform a variety of procedures, e.g. moving the animal, different dosing regimens and clinical examinations. In animals of a certain size, these procedures are not only highly aversive to the animals but they also often force animal caretakers and technicians to work in ergonomically undesirable ways. To avoid or at least minimize the negative impact of such procedures on animals and technical staff, implementation of positive reinforcement training (PRT, aka clicker-training) is a promising tool, combining cognitive enrichment of the animals, improved animal-human relations and less straining working procedures for the staff. The use of PRT may in some cases even be cost-effective. PRT may present the possibility to do research that would otherwise not have been possible. Implementation of PRT has been carried out with success in both the pharmaceutical industry, in non-clinical safety testing and at the University of Copenhagen. Several procedures have been trained successfully and resulted in a more smooth and gentle working procedure during for example intra nasal dosing, subcutaneous dosing of high volumes, rectal probe measures and flushing of catheters.

This presentation will explain the theories behind PRT, namely classical and operant conditioning, and highlight the usefulness of the method using video examples demonstrating how clicker training has been used to train the animals to actively cooperate with the technicians during the above mentioned procedures.

Session IV-2: Poster presentations

IV-2-306

Farm animal research in Canada's private feedlot industry

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Feedlot Health Management Services Ltd (FHMS) is a private industry company providing animal health management, nutrition, and production consulting services, as well as individual animal data collection and management tools, to commercial feedlots in Canada and the United States. FHMS has a professional consulting team consisting of veterinarians and animal scientists with expertise in animal health and welfare, epidemiology, nutrition, production management, pathology, meat science, applied research, and economic modeling. At present, FHMS provides professional services for beef feedlots with an annual throughput of between one and two million animals. Research is a key pillar supporting our business model, providing value to clients through generation of data for high-level evidence-based decision making. At present, FHMS conducts over 50 studies annually, including: disease investigations; new technology assessments; product safety, efficacy and licensing studies, pathogen and antimicrobial resistance surveillance; individual animal and small pen research pilot studies; and large pen commercial field trials. Working with an emphasis on the Three Rs, FHMS conducts cattle research with the overall goal of enhancing animal health and welfare while providing results that are relevant and applicable to commercial feedlot producers. Based on over 25 years of feedlot research experience, large-scale field trials are often required to accurately reflect the disease dynamics and animal behaviour that occur in commercial production settings. With research results that are immediately applicable to Canadian and global beef industries, FHMS continually strives to enhance animal health and welfare and to be a proactive leader in these areas.

IV-2-319

Rumen Simulation Technique (RUSITEC) – an *in vitro* alternative for fermentation studies in cattle

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RUSITEC, a laboratory device for simulating the rumen (cattle stomach) environment was utilized to assess the effect of vegetable oil supplementation on *in vitro* nutrient digestibility of cattle diet. It is a semi continuous culture system which simulates the rumen in terms of pH, temperature, anaerobiosis, microbes and mixing action. Eight reaction vessels were filled with 650 ml rumen liquor and 200 ml artificial saliva and immersed in a water bath at 38°C. The feed container inside the reaction vessel consisted of two nylon bags, each containing test diet and solid rumen digesta. Saliva infusion into the reaction vessel was regulated to 800 ml/24 h and as fermentation proceeded, effluent and gas were collected in separate containers. A control diet (10 g) was supplemented with three different plant oils, namely sunflower oil (SFO), soyabean oil (SBO) and corn oil (CNO) at 6% level and incubated in duplicate. After an adaptation period of seven days, three days were allotted for sample collection. The dry matter degradability (DMD%) was 46.36 in the control group and SFO, SBO and CNO reduced ($P < 0.05$) DMD to 42.63, 40.61 and 44.16 respectively. The fiber degradability (%) was not significantly ($P > 0.01$) altered between treatments, which was 39.54, 38.06, 39.02 and 39.26 in the control, SFO, SBO and CNO groups, respectively. The current study using RUSITEC precludes surgical fistulation, associated trauma, stress and appalling appearance of the cattle. Therefore, RUSITEC can be considered as an alternative to live animal experimentation.

IV-2-551

Paddock or laboratory – What determines suitable living conditions for sheep?

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Farm animals are used in research for a variety of purposes and the associated husbandry requirements can range from being kept in a group in a paddock to intensive, indoor housing. Much research involving farm animals is undertaken in agricultural or veterinary sciences and directed towards the health and management of the species involved; specific husbandry requirements being dictated by the study purpose. But some farm animals, notably pigs and sheep, also are used in biomedical research in the study of human health and disease and, in these circumstances, most often need to be housed indoors under intensive conditions.

From both a scientific and an animal welfare perspective, the living conditions of animals need to be designed and managed to meet their specific physiological and behavioural needs; irrespective of the husbandry system the same evidence informs such decisions.

Although, when used in research, sheep are often kept under intensive conditions, to date little attention has been given to the development of science-based guidelines to benchmark acceptable living conditions. A systematic literature review highlights their need for social, visual and auditory contact, the relationship between rest and rumination, the importance of synchronisation of rest periods, the effects of space and group size on social dynamics and their response to isolation (Animal Research Review Panel, 2010). Informed by available evidence, the elements of any husbandry system that may negatively influence species-specific physiological and behavioural responses must be taken into account in the design and management of the system so as to eliminate or minimize such effects.

Reference

Animal Research Review Panel, NSW Department Industry & Investment (2010). *Guidelines for the Housing of Sheep in Scientific Institutions*.

Session IV-3: Wildlife science and the Three Rs

Session IV-3: Oral presentations

IV-3-529

A mouse in the cage is not the same as the two in the bush!

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The Three Rs (Reduce, Refine, Replace) are ethical rules for assuring humane treatment of experimental animals and thereby help ensure good science. Although field studies are subject to the same rules, complexities of field research make it difficult to apply the Three Rs in that context. Most animals used in laboratory research were bred for that purpose, and are studied in research ultimately aimed at enhancing the quality of life for humans. In contrast, animals used in field studies are usually integral parts of existing populations, species, and ecological communities, and are studied in order to assess the impacts of human activities upon these populations and communities. Laboratory and field research ask different types of questions and often use different types of research designs. These differences make it problematic to apply laboratory-appropriate rules to field studies. We will discuss how the "distress" experienced differs between wild and "domesticated" research animals and some of the resulting consequences. We will explain why Replacement rarely happens in field studies, why Reduction is problematic, and why Refinement has been primarily techniques-based. We will also explore how differences in philosophical background lead to different perspectives on, and justifications for, animal use. Finally, we will sketch the parameters of a new "ecological ethic" which could synthesize and transcend both animal and environmental ethics.

IV-3-253

Molecular tools can obviate animal killing in biosystematics studies of anurans

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The biosystematics study includes identification, taxonomy and phylogeny. For the purpose of the biosystematics study of anurans (Class Amphibia) mass collection of individuals of a species and their preservation in formalin drums followed by taxonomic categorization using keys is the general practice. We use novel digital technique and molecular tools in biosystematics studies of anurans without killing and preservation of animals. The morphological and morphometric observations of anurans can be made very precisely using high resolution digital cameras and calibration software. Osteological studies can be performed using soft x-rays without killing the individuals. Recent developments in genomics have identified many marker genes. In anurans about 26 marker genes have been identified which

may be used in biosystematics studies. The technique for identification of these marker genes in anurans is based on isolation and characterization of DNA from a few cells without killing the animals. The cells obtained from the surface of the body or from a drop of blood produce DNA copies by PCR for partial or complete sequencing using an auto-sequencer. We have sequenced three marker genes namely 12s rDNA, 16s rDNA and Histone H4 of 12 anuran species of Thar Desert of Rajasthan, India, without killing or even disturbing the individuals. About 40 of our sequences have been released by NCBI. Molecular tools along with morphological and morphometric observations can be used as humane methods in biosystematics studies.

IV-3-343

Computer simulation models as an alternative to animal-based trap testing

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Animal trapping is done in virtually every country in the world for conservation, wildlife management, pest control, and obtaining fur, skin, or meat. The Agreement on International Humane Trapping Standards (AIHTS) was signed in 1997 by the European Union, the Russian Federation and Canada to ensure a sufficient level of welfare for trapped animals. The United States signed a separate but similar bilateral agreement with the European Union. The AIHTS applies to both killing and restraining traps for 19 listed species. Only traps certified as meeting the welfare thresholds of the AIHTS are allowed for use in Canada. The Fur Institute of Canada (FIC) is responsible for coordinating the implementation of the AIHTS on behalf of Canada. Since 1995, Alberta Innovates–Technology Futures has worked with the FIC on the development of computer simulation models for evaluating traps against the requirements of the AIHTS. These models were developed from a large database of historical information and are a scientifically valid and accurate alternative to animal-based testing. Models have been built for rating killing traps for 8 species listed on the AIHTS, including beaver, fisher, lynx, marten, muskrat, otter, raccoon, and short-tailed weasel. To date, these models have reduced the number of animals required for testing by over 1,400 and have resulted in savings of over $ 4 million.

IV-3-481

Biases in bear studies: A consideration of capture effects on research results

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Recent reports from several long-term wildlife studies challenge the assumption that potential adverse effects of research methods on animal welfare are limited to the short-term by demonstrating that the effects on individuals may have enduring consequences for populations. Further, these studies underscore a serious need to reconsider biases introduced by different capture and handling procedures to avoid erroneous interpretations of results.

To illustrate this latter point, we present results from two independent studies, one concerning grizzly bears (*Ursus arctos*) and the other polar bears (*U. maritimus*), in which body condition (a measure of health) and hair cortisol concentration (a measure of long-term stress) were analyzed in response to several potential predictor variables reflecting environmental condition. With grizzly bears, we looked specifically at measurements of habitat quality and human activity. With polar bears, we considered measures of sea-ice availability. Through similar types of analyses using data from both studies, we found that failure to account for the method of capture, in the case of grizzly bears, or the number of times an individual bear has been captured can lead to different results and, as a consequence, different interpretations of the data.

These examples should challenge persons engaged in wildlife capture to evaluate their capture procedures and research results carefully – from beginning to end. Significant capture-related effects may go undetected, providing a false sense of the welfare of released animals. Further, failure to recognize and account for long-term effects of capture and handling on research results can potentially lead to flawed interpretations.

IV-3-501

Ranking the negative impacts of wildlife control methods may help to advance the Three Rs

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Systematic evaluation and ranking of the negative animal welfare impacts associated with wildlife pest control methods may allow directed application of the Three Rs to reduce welfare compromise in study animals, extension of successful mitigation strategies to field use of the methods, and selection of preferred control methods. A comprehensive literature review on vertebrate toxic agents (VTAs) used to control mammalian pests in New Zealand provided information on the following: mode of toxic action; description of effects; time to loss of consciousness/death; and details of human poisonings. This information was used to evaluate impacts in each of five domains of potential animal welfare compromise according to an established methodology. This analysis revealed the following for a range of VTAs: the level of current knowledge of negative affective experiences caused in different species; gaps in understanding of such experiences; possible ways to identify those experiences; and questions about whether their severity can be judged. At present, application of the Three Rs to VTA studies would be hindered by uncertainty regarding consciousness. In particular, information on the period from the onset of symptoms to loss of consciousness, indicative of the duration of negative experiences, is inadequate. In addition, the level of consciousness during critical events, such as convulsions and respiratory compromise, is poorly understood. Suggestions are made regarding future research directions and approaches to fill knowledge gaps that will allow more accurate evaluation of welfare impacts and enhance the application of the Three Rs.

Session IV-3: Poster presentations

IV-3-483

Why is ecological ethics necessary?

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Certain moral problems regularly faced by conservation biologists and managers are not well addressed by theories within the disciplines of animal ethics, environmental ethics, or research ethics. In this paper we explain why this gap cannot be closed merely by tweaking these three disciplines – why a fourth discipline is necessary. Animal ethics maintains that animals are intrinsically valuable and highlights moral conflicts between the interests of animals and the interests of people. Similarly, environmental ethics focuses on ecosystem vs. people conflicts, and research ethics focuses on knowledge vs. people conflicts. Unfortunately, theories of animal ethics are blocked from acknowledging the intrinsic value of ecosystems and knowledge by their Kantian and Consequentialist roots. Theories of environmental ethics are blocked from acknowledging the intrinsic value of animals and knowledge by the holistic nature and complexity of ecosystems. Similarly, theories of research ethics cannot acknowledge the intrinsic value of either animals or ecosystems. Each discipline can give only lip-service to the intrinsic values championed by the other two. Thus neither animal ethics, nor environmental ethics, nor research ethics can handle the animal vs. ecosystem vs. knowledge dilemmas faced by conservation biologists and managers. A discipline that recognizes the intrinsic value of animals, ecosystems, and knowledge – ecological ethics – is necessary.

Session IV-4: Multi-imaging modalities, telemetry and the Three Rs

Session IV-4: Oral presentations

IV-4-699

Application of radiotelemetric recording to study mouse models of gestational pathology

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Mice are valuable tools providing understanding of the physiology and pathologies of human pregnancy. Acute onset hypertension with kidney failure is the commonest (3-7%) human pregnancy complication, characterizing the syndrome pre-eclampsia, a medical emergency with immunological complications. We implanted PA-C10 radiotransmitters (DSI) into 8 week female mice. After 10 days recovery, instrumented mice were paired for mating. Upon copulation plug detection, data were collected continuously to 48 h postpartum. A fluctuating pattern of normal blood pressure was defined. The pattern, observed in randombred CD1, inbred C57BL/6J, BALB/cJ, normoglycemic NOD and immune deficient *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$/*Il2rg*$^{-/-}$, changed at specific times important for placental development. NOD *scid* and hyperglycemic NOD pregnancies differed, identifying NK cells and blood glucose values respectively as factors contributing to circulatory control over pregnancy. The precision of telemetric measurement and the concordance of findings between replicate animals made 4-6 recordings sufficient to generate publication quality data. Complications and technical failures were common in these pregnancy-based studies, requiring preparation of 10-12 instrumented mice per group. These animal numbers are much lower than needed for pregnancy time course studies requiring daily euthanasia of 3-6 replicate mice. Previous attempts to collect this information using serial, daily, tail cuff recording gave data with sufficient variability that we concluded (incorrectly) mice differed from humans because they lacked a dynamic pattern of cardiovascular regulation across pregnancy.

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IV-4-097

A novel *in vivo* approach to measure multiple organ system functions simultaneously: Combining automated sampling/delivery systems with radio telemetry (ABST)

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We describe a novel validated apparatus combining computer automated blood sampling and delivery of substances simultaneous with radio telemetry of physiological parameters in rats. For this, a unique engineering solution was created to combine the BASi Culex® automated blood sampling system with Data Sciences International Physio-Tel® Multiplus radio telemetry system. Studies were performed using Han-Wistar rats previously prepared with indwelling catheters and a radio telemetry transmitter. The system was optimized to maintain weight gain and reduce overall stress as evidence by plasma hormone biomarkers. Parameters presently measured from a single animal include: heart rate, blood pressure, body temperature, electroencephalogram, drug exposures, urine chemistries, renal biomarkers of injury, and functional measures renal plasma flow and glomerular filtration rate. Using the ABST system has enabled an ~80% reduction in animal numbers required for a single test article compared with traditional stand-alone studies for each organ system. Studies performed using the ABST system to measure multiple organ functions simultaneously *in vivo* reduce the need for multiple serial studies, allow pair-wise data comparisons, enable better temporal evaluations and provide true pharmacokinetic and pharmacodynamic analyses. Employing this model early in the drug discovery process is intended to stop progression of toxic compounds and replace testing in higher mammalian species. Studies designed to exemplify system optimization and validation will be provided.

IV-4-375

PET and MRI improve safety testing with far fewer animals (<50%)

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The new OECD guideline for an Extended One-Generation Reproduction Toxicity Study (OECD EOGRTS) aims to reduce the number of animals for REACH and other testing programs without compromising safety assessment. The EOGRTS-protocol includes reproduction, but also the developing immune and neurological systems.

Twenty rats per sex and group are required to test developmental neurotoxicology. In studies with well-known toxicants (MAM, MeHg, organotin compounds DOTC and TBTO, and ethanol) we demonstrated that this number can be reduced by about 50% using *in vivo* imaging, and prediction to humans improved when combined with micro array gene expression profiling.

Changes in brain activity (uptake patterns of [18F]FDG micro-PET imaging) were observed over time in animals with impaired behavior demonstrated by conventional means (Functional Observational Battery; Motor Activity). Changes in brain region volume (MR-Imaging) concurred with brain weight and size measured after death, but was more detailed and identified more closely predeliction areas for toxicity. Gene expression profiling of brain parts at young and adult age proved to corroborate an apparent delay in structural and/or functional development and/or persistent impairment, not only of the neurological system but also of the immune and endocrine systems.

Our results show that combined application of PET, MRI, and gene expression profiling warrants a strategy, resulting in improved prediction for man and substantial reduction and refinement of animal use. More imaging modalities are explored. With time this strategy can replace conventional, logistically complex and laborious animal testing in (regulatory) toxicology and safety pharmacology studies, saving animals on a large scale.

Session IV-4: Poster presentations

IV-4-204

Multimodal analgesia for improved recovery of dogs surgically prepared for telemetry

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Dogs are surgically implanted with DSI Physio Tel^{TM} transmitters to produce a long term telemetry model in freely moving dogs with the ability to measure left ventricular pressure, epicardial electrocardiogram and aortic blood pressure. This is achieved by a lateral thoracotomy, which is considered to be a surgical procedure with the potential for severe pain.

A standard analgesia plan has been devised for the surgery with slight modifications in response to the analgesic needs of the individual animal. Carprofen results in long acting analgesia and anti-inflammatory action. Bupivacaine is administered via an intercostal block and gives medium-length, locally-acting analgesia in the intra-operative and post-operative period. This particularly addresses the pain caused by chest wall movement. Methadone is administered strategically pre-operatively, immediately post-operatively and three hours post-operatively to coincide with the bupivacaine block wearing off. This is followed with buprenorphine, which, with carprofen, ensures good overnight analgesia. The analgesia is tailored to respond to individual pain requirements by the use of fentanyl as a short-acting analgesic with a rapid effect.

It is evident from the post-operative clinical records that it is normal for dogs to be standing and walking by 5 h, with many achieving this within 3 h. Also, the dogs are normally extremely bright and eating 24 h post- operatively.

A multimodal approach to analgesia, customised for each dog, allows us to address the individual analgesic needs of each animal resulting in smoother and more rapid recoveries from this painful procedure. This has refined welfare.

IV-4-279

Advancing technology and the 3Rs: simultaneous pharmacokinetic and multi-organ function assessment in rats

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Innovation leading to development, qualification, and implementation of new technologies produces Refinement in data quality, Reduction in animal use, improvement in clinical safety, and reduced attrition in pharmaceutical development. We demonstrate an integrated pharmacology platform capable of simultaneous acquisition of pharmacokinetic and multiple organ system function data in the same animal. This platform using conscious rats surgically implanted with radiotelemetry transmitters and externalized cannulas allows: 1) automated blood sampling; 2) simultaneous assessment of cortical electroencephalography (EEG) or electrocardiogram (ECG); 3) cardiovascular parameters (SP, DP, MAP and HR); 4) body temperature and activity; 5) renal hemodynamics (GFR and RPF) and excretory functions (electrolytes and metabolic products), 5) assessment of biomarkers of drug-induced organ system injury (DILI, DIKI, DIVI, etc.); 6) terminal histopathology. As a case example, single administration of cisplatin (15 mg/kg, i.p.) in Han Wistar rats (n=8) produced measurable plasma concentrations associated with decreased HR, temperature, GFR and RPF (-37%, -13%, -74%, -35% vs. control, respectively). Cisplatin also induced elevated DIKI biomarkers (fold increase over control: α-GST, 76; GSTYb1, 12; clusterin, 9; albumin, 27; Kim-1, 5, respectively) correlated with histopathologic renal tubular injury by day 3. In conclusion, implementation of ABST technology collected: 1) data in one study, where traditional methods required 4 or more studies; 2) data simultaneously collected in one study group rather than traditionally collected in parallel from 4 or more groups plus separate controls, and 3) a total of 8 animals compared to a minimum of 43 animals (81% reduction).

IV-4-389

Sexual dimorphism in brain function of DBA2 mice studied with functional and structural MRI – an animal refinement and reduction approach

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Gender related brain dimorphisms exist in all vertebrate species including humans and rodents. For investigations of brain structure and function mice are regularly used as models. As a novel, non-invasive technique magnetic resonance imaging (MRI) is capable of obtaining high resolution data of brain function and structure in humans and (anesthetized) mice. However, in the current literature only 1% of all MRI animal studies combine structural and functional high resolution MRI data acquisition for the very same specimen.

Because the structure-function relationship is important for detailed interpretation of MRI data, the aim of this study is to combine structural and functional MRI data from the same animals investigating gender differences in pain processing of DBA2 mice. Functional MRI data were obtained by repetitive thermal stimuli. After the functional experiment, the animals had to be sacrificed due to the ethical rules. Subsequently, high resolution MRI was performed on the dead animals.

Preliminary results of structural MRI show no gender differences in the total brain volumes, in contrast to human MRI data. Principal component analysis of functional MRI data shows gender differences in pain response and threshold.

Using non-invasive MRI allows performing functional and structural high resolution MRI experiments sequentially in the same animals. Obviously this approach reduces the number of subjects needed by 50%.

This work contributes to the goal of the 3R's by means of non-invasive imaging in anesthetized animals leading to high resolution data (refinement), as well as combining structural and functional data from the same animal (reduction).

IV-4-392

Integrative wireless monitoring for animal wellbeing

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Minipigs are recognized as an alternative non-rodent model that can improve drug safety for humans for its superior predictivity and translation to man (http://www.rethink-eu.dk). We hypothesized that this contribution to animal 3Rs could be further improved when deploying Holst Centre wireless sensor technology in this area of (mandatory) safety evaluation studies.

We explored – as a first initiative – an integrative application of the Holst Centre ECG Necklace sensor node combined with an X, Y and Z-acceleration sensor in minipig as the test subject. Primary focus was on 1) animal wellbeing during monitoring; 2) usefulness and quality of the signals; 3) relevance of the integrative simultaneous information of ECG, heart rate (HR) and acceleration (activity).

The results demonstrated that 1) the minipig could freely move during continuous or repeated monitoring and unambiguously accepted wearing the sensor (refinement). 2) The continuous ECG, Heart rate (HR) and acceleration signals were acquired and post-analysed with very good results (reduction). 3) Changes in heart rate could be explained by changes in acceleration (due to the animal's activity) and changes in orientation (X, Y, Z-position) of the animal were observed as changes in one or more of the acceleration (X, Y, Z-) signals.

It is concluded that the Holst Centre wireless sensor technology perfectly fits in to contribute to animal refinement/reduction: continuous and repeated, simultaneous monitoring with multiple sensor nodes addressing multiple organ systems seems to be within reach and so more information can be obtained from fewer animals. Moreover, decision making during drug development is stepping up.

Session IV-5: Can pain research benefit research animals?

Session IV-5: Oral presentations

IV-5-526

Assessment of pain in small laboratory animals using behaviour and facial expressions

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Considerable advances have been made in assessing pain in laboratory animals through the evaluation of behavioural and postural changes. Successful implementation of such assessments depends on establishing which behaviours and postures indicate pain and which areas of the body to focus on to observe these indicators. Failure to observe all relevant body areas will prevent successful use of behaviour-based assessment techniques.

Using eye-tracking equipment we have demonstrated that irrespective of experience observers' focus first, more frequently and for longer on the face compared to the abdomen, ears, back and hindquarters of rabbits when assessing pain following ovariohysterectomy. The general ability of the observers to identify rabbits in pain was very poor, with incorrect pain scores being positively correlated with increased observation of the face. Consequently, focusing on the face to assess abdominal pain based on behaviour is likely to reduce the effectiveness of the pain assessment.

Alternatively, if we can identify facial expressions in animals that are associated with pain as in humans then a fixation on the face may actually increase the effectiveness of pain assessment based upon such expressions. Recently Langford et al. (2010) convincingly demonstrated that mice exhibit facial expressions associated with pain and that it can be objectively measured using the Mouse Grimace Scale (MGS). We have further validated the MGS by demonstrating its effectiveness for assessing post-operative pain in mice (following vasectomy) and potentially rats (following adrenalectomy). In these studies changes in MGS paralleled those of behaviour-based indicators of pain.

Reference

Langford, D. J. et al. (2010). *Nat. Methods 7*, 447-449.

IV-5-658

Studying pain in rodents using facial expression

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Recent decades have seen an explosion in our understanding of the molecular and cellular underpinnings of pain, but virtually none of this knowledge has resulted in new clinical therapies. Many pain researchers believe that the problem may lie in the existing animal models of pain, which are of questionable clinical relevance. Most basic science studies of pain continue to rely on the measurement of reflexive, evoked hypersensitivity responses after artificial neuropathic or inflammatory injuries, whereas clinical pain in humans features mostly spontaneous pain. The *status quo* also has relevance for the veterinary pain

literature, as research into postoperative pain management is conducted using likely irrelevant assays, or measures of uncertain specificity. We recently developed the Mouse (and Rat) Grimace Scale, in which spontaneous pain is quantified from facial expression, as is commonly done in non-verbal humans. Applying this method has allowed us to reassess the true duration of spontaneous pain after surgery, and the true efficacy (or lack thereof) of commonly used analgesics.

IV-5-157

Non-invasive pain assessment in mice subjected to vasectomy

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Recognition of pain and stress is a common challenge when working with laboratory mice. The aim of this study was to identify non-invasive parameters to assess the severity and duration of possible pain and stress following vasectomy in BALB/c mice. Mice were subjected to isoflurane anesthesia and vasectomy or isoflurane anesthesia without surgery. Body weight, food and water intake, and fecal corticosterone were measured three days prior to and three days after the procedure. Behavioral observations were recorded 1, 2, 4 and 8 h after the procedure. Food and water consumption and defecation rate were significantly reduced in the vasectomized group one day post-operatively compared to the mice subjected to anesthesia only. Fecal corticosterone was elevated the first day after isoflurane anesthesia but not in the vasectomized group. Vasectomy resulted in behavioral changes that were not seen in the group subjected to isoflurane anesthesia only. In conclusion, food and water consumption and pain specific behaviors could be useful as non-invasive parameters to assess post-operative pain and stress in mice subjected to vasectomy. However, fecal corticosterone was not useful for this purpose in the present setup.

IV-5-453

Can a focus on the translatability of preclinical pain research benefit research animals?

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Neuropathic pain, a chronic and debilitating condition in humans, is often modeled in animals by inducing nerve injury. Pain-related sensory changes studied in these models include hypersensitivity to thermal stimuli (hyperalgesia), light touch (mechanical allodynia), and the injection of painful chemicals (chemogenic hypersensitivity). In modeling neuropathic pain, it is important to closely replicate the clinical condition to improve translatability. The current study explored preventive analgesia for postoperative neuropathic pain. Since human patients routinely receive postoperative morphine as part of their analgesic regime, the impact of postoperative morphine on the preventive effects of drugs being studied needed to be determined. Previous research using the spared nerve injury (SNI) model of neuropathic pain demonstrated that amitriptyline has preventive anti-hyperalgesic effects that are not significantly altered by postoperative morphine administration. In this study, propentofylline (1 h preoperatively and then daily for 7 days) alleviated long-term mechanical allodynia in the SNI model. This was not affected by morphine administration (postoperatively and daily for 3 days). When given in combination, propentofylline and amitriptyline maintain their individual long-term effects in the presence of postoperative morphine. Many nerve injury models, including the SNI model, involve extensive tissue manipulation to produce physical injury to a nerve(s), causing pain and inflammation that may not be related to the long-term sensory changes of interest. Here the exploration of postoperative morphine, to improve translatability, likely provided the rats with relief from pain which was not necessary for the study outcomes, and represents a potential refinement for further studies using this model.

IV-5-556

ATLAS: an evidence based web resource for pain control in laboratory animals

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Two types of research can benefit laboratory animals. A substantial body of pain research is performed on animals as models for humans to guide pharmaceutical development and investigate mechanisms. Clinical research specifically examines pain control for the sake of the animal itself, but the volume of this research is still small, particularly for rodents and rabbits. Believing that information to support effective mitigation of pain in laboratory animals can be gleaned from both primary and clinical literature, we have undertaken to create an evidence-based web resource for information regarding treatment of pain in laboratory animals. The concept was inspired by a well-known procedure-specific resource for guidance of physicians on pain management (http://www.postoppain.org/). Review and interpretation of the pain literature can benefit laboratory animal veterinarians and researchers by guiding them in what is currently known about efficacy, safety and other effects of pain control in laboratory animals. Use of a method to sort through previously conducted studies is also a form of Reduction, in that with this information, future studies can be targeted more specifically, using fewer animals overall. There is also a need to begin to recognize when use of certain analgesic types, or the presence of pain itself, may affect a study outcome or the success of peer review. It is therefore important to determine whether pain and analgesic criteria are being uniformly applied to animal models by examining the published literature. This presentation will describe our web resource, the ATLAS (Analgesic Therapy in Laboratory Animal Species) Database.

Session IV-5: Poster presentations

IV-5-401

Investigation about anesthesia of rodent fetuses with transplacental pentobarbital administration

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Correct anesthesia or euthanasia is an essential factor for making specimens of late stage rodent fetuses. We examined the conditions required to anesthetize rodent fetuses by pentobarbital administration to dams. Crl:CD(SD) rats were anesthetized with sodium pentobarbital intravenously 20 minutes before caesarean sections on day 21 of gestation, and Crj:CD-1(ICR) mice were treated on day 18 of gestation. The doses were 5, 10, 20, 30 or 50 mg/kg of body weight at the time of the caesarean section. Six fetuses from each dam were examined for responses to pain stimulation and their physical responses were scored 60 minutes after the caesarean section. In rat fetuses, the 10 mg/kg or more dose groups showed deep anesthesia during the 60 minutes, but fetuses of the 5 mg/kg group awoke after 40 minutes. In the 50 or 30 mg/kg groups of mice fetuses, locomotor activity and response to stimulation decreased soon after the caesarean section, and the fetuses were anesthetized for 60 minutes. In the 20 mg/kg group, response to stimulation disappeared but aroused fetuses were observed 20 minutes after the caesarean section. The mortality of fetuses at the time of the caesarean section did not increase in any dose group for both species. These results suggest that pentobarbital administration to the dam is able to anesthetize rodent fetuses for 1 hour.

Session IV-6: Broadening the application of Refinement

Session IV-6: Oral presentations

IV-6-078

Refined blood sampling of rodents

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Blood sampling is one of the most common experimental procedures performed on laboratory animals for the analysis of biochemical, metabolic, toxicological and immunological parameters or the production of antibodies.

Sampling of sufficient volumes of blood in mice and rats can be challenging due to the relatively small size of animals. All sampling methods should be evaluated and the use of the most appropriate technique for the specific purpose and species or subspecies is important. Sampling by trained and competent staff is essential for ensuring that any pain, distress or discomfort is kept to a minimum. Minimisation of such adverse effects is further important for scientific as well as ethical and legal reasons, since they can cause biological changes which may affect the blood sample, and hence the validity of the research results and the number of animals used to achieve the scientific objective.

This presentation will summarize and discuss a number of recent published papers and unpublished studies performed at Novo Nordisk or within the Danish CALAR research collaboration (www.calar.dk) combined with relevant references in order to evaluate quality of plasma sampled by different methods, e.g. amputation of the tail tip, lateral tail incision, puncture of the tail tip and periorbital puncture, for multiple blood sampling. Animal welfare implications, advantages and disadvantages, based on scientific analysis, telemetric recordings and intense observations, by using methods like submandibular blood sampling in standard and genetically modified mice and sublingual blood sampling in rats will further be presented and discussed.

IV-6-126

Good welfare – good science: Refining toxicological procedures for cynomolgus macaques (*Macaca fascicularis*) through enhanced socialisation with care-staff

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Cynomolgus macaques (*Macaca fascicularis*) are the most commonly used nonhuman primates for research and testing in Europe. They are an important in vivo model in regulatory toxicology for predicting the adverse effects of novel pharmaceuticals on clinical pathology, cardiovascular and organ parameters. Common husbandry practices, traditional capture, handling and restraint techniques, and close proximity of human care-staff may result in physiological stress responses which confound toxicological measurements. Habituating and socialising primates to human care-staff will help to avoid or reduce fear responses and facilitate handling for routine husbandry and scientific procedures, leading to improvements in scientific outcomes.

Our aim was to determine the effects of a five-week enhanced socialisation programme with animal care staff on newly acquired cynomolgus macaques in a laboratory. We used a multidimensional welfare assessment tool we have previously developed for cynomolgus macaques to compare a control (N=40) and matched group (N=40) of male and female juvenile macaques subject to a socialisation programme. As well as enhanced welfare we also found positive effects on cardiac parameters (heart rate, electrocardiogram waveforms and blood pressure) recorded at baseline by non-invasive digital electrocardiogram (ECG) and indirect high definition oscillometry blood pressure as part of the core battery of measures incorporated into regulatory toxicology. Our results provide support for the importance of positive human-macaque relationships for both primate welfare and quality of science.

IV-6-404

Ways to advance laboratory animal welfare not only when it is a regulatory mandate but whenever we recognize opportunities to enhance husbandry and/or research practices

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When William Russell and Rex Burch proposed the 3Rs (Replacement, Reduction and Refinement), they were suggesting this approach for all situations when animals are used for research purposes. Animal welfare and the 3Rs is an important focus for every good laboratory animal research program. Ethics committees must review animal welfare questions as part of a protocol review process and it is a highlight during training, semiannual facility inspections, and post-approval monitoring. The veterinary staff, animal caretakers and research staff are charged with ensuring high standards for animal welfare while carrying out their daily responsibilities for health care support, animal observations and/or research. Animal welfare concerns must be reported promptly to the veterinary and ethics committees for follow-up. But promoting animal welfare through the adoption of alternatives can sometimes fall short without enough manpower or energy directed towards the 3Rs. Abbott is building a culture of animal welfare by creating opportunities for people to develop into animal welfare leaders. Examples include Abbott CARE (Caring for Animals in the Research Environment), an Alternatives Committee, an Enrichment Committee, a Corporate Animal Welfare Committee, an animal welfare award program, a 3Rs coordinator/scientist position, and a Dog Socialization and Adoption Program. Enhancing animal welfare builds employee morale, improves institutional reputation, addresses public concerns and often minimizes variability that confounds scientific investigation. This presentation will give a broad overview of ways to promote best practices for animal welfare through the development of animal welfare leaders in a local or global program.

IV-6-418

Translating regulatory compliance into better animal welfare – are we making progress?

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The 3Rs is increasingly acknowledged as a guiding principle also in legal documents such as the recent European Directive 2010/63. To assess how animal welfare and refinement have been considered in biomedical research, and how they relate to regulatory compliance, we reviewed published research over a 10-year period in two biomedical fields in which animal research is central: experimental infection and hereditary neurodegenerative diseases. We performed a systematic review of papers on murine models of tuberculosis (TB) (1997-2007, N=244) and Huntington's disease (HD) (1999-2009, N=233). A 4-level severity scale was devised for each case, based on disease stage (from asymptomatic to moribund stage / spontaneous death), invasiveness of experimental procedures and any refinement applied. Overall, reports of regulatory compliance increased significantly ($p<0,01$ for TB, $p<0,01$ for HD) across the ten-year period analysed. Moreover, we found a significant ($p<0,05$) relationship between regulatory compliance and application of humane endpoints in lethal TB studies. However, the proportion of the most severe studies did not change significantly (43% in TB, 36% in HD, overall) over time. Of these most severe studies, 49% of the TB studies and 79% of the HD studies reported ethical approval. The application of humane endpoints and other refinement measures increased, but in a considerable proportion of studies animals were allowed to reach severe levels of distress. This may be scientifically required or may be the result of insufficient refinement, as will be further discussed in the presentation.

Session IV-6: Poster presentations

IV-6-042

The appropriateness of the use of therapeutic drugs on regulatory toxicology studies and ethical considerations

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Historically there has been reticence to administer therapeutic drugs to animals on toxicology studies for fear of adversely affecting the outcome of the study. However, there are occasions where drugs to alleviate pain, inflammation or to treat general symptoms of disease should be administered as study animals should be alert, active and healthy. All logistics behind animal health ensures that only animals in good health are allocated to study. It is also clear that during a toxicology study incidents occur, such as damage to extremities, lesions and general ill health, not related to test article toxicity that require intervention.

Our objective is to explore the issues surrounding the humane treatment of animals to ensure that they are treated with appropriate therapies when the unforeseen occurs. These include the necessity of not administering drugs needlessly, trying the least invasive methods first and appropriately researching the likelihood of drug interactions with the test article.

The clinical veterinarian (CV), technical staff, study director and sponsor play pivotal roles in this process. There must be interaction between each of the members of the group to ensure appropriate actions are taken when the need arises.

The CV's inherent responsibility includes monitoring and providing recommendations concerning the following: preoperative procedures, surgical techniques, qualification of institutional staff performing surgery and providing postoperative care. Examples of classical vs. conservative treatments are listed together with the logistics of the process and the information flow between scientific personal and the CV.

The cornerstone of the whole processes is to ensure reliable data from the studies whilst respecting animal welfare.

IV-6-156

Automated blood sampling – refinement of repeated blood sampling techniques in permanently catheterized mice

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Refinement of blood sampling techniques in laboratory rodents is important to improve animal welfare and reduce variation in the experimental results. Manual blood sampling is known to be associated with a stress response, which besides being a serious welfare concern is a well-known source of experimental bias. The Accusampler® (Dilab, Lund, Sweden) can sample blood automatically, theoretically without disturbing the animal at the time of withdrawal. Furthermore, samples of 5-50 μl can be collected, hence more samples can be taken per animal and fewer animals would be required in a study.

Automated blood sampling concurs with two of the 3Rs by potentially reducing the number of animals used when sampling blood and refining the method of repeated blood sampling. The method has been thoroughly investigated in rats. However, it is currently unknown to what extent automated blood sampling causes stress in mice.

In a recent study, male BALB/c mice were subjected to micro surgery with catheterization of the common carotid artery and connected to the Accusampler®. Corticosterone levels in blood and feces were quantified to evaluate the stress response associated with this method. Based on both results and technical difficulties it is concluded that the method is stressful to some extent, but the technique has the potential to refine the process of repeated blood sampling.

This presentation will address technical difficulties that are associated with the method, both regarding animal welfare issues and data collection, and how these difficulties are best resolved.

IV-6-251

Group housing of male mice in long term toxicity studies

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Due to their naturally aggressive behavior, male mice are often housed individually in long-term toxicity studies, and group housing of male mice has often been described as a stressful condition. However, several studies advocate group housing of mice to enable normal social behavior and interactions between the animals. A handling procedure that makes group housing of male CD1 mice possible in long-term toxicity studies has been developed at Safety Assessment within AstraZeneca. The key factors of the procedure include allocation into groups before sexual maturation (4-5 weeks of age; 2-4 animals/cage) and thereafter a one week acclimatization period. At cage cleaning, used nesting material should be transferred from the used to the clean cages. External changes should be avoided as far as possible, e.g. the cages should be kept at the same place in the room throughout the study. Observations on the effect on aggression/fighting in group-housed male mice following different procedures performed in toxicity studies have shown that temporary removal of animals from the group for blood or urine sampling does not affect the group dynamics. However, temporary removal of animals for mating leads to fighting if the animals are taken back to the original group. In addition, treatment with test compound might affect the general condition of the animals and the social hierarchy could be changed. In such cases, aggression/fighting might occur and the animals have to be separated. Our experience clearly indicates that group housing of male mice in long-term studies leads to less stressed and more easily handled animals, as compared to individually housed mice.

IV-6-312

Novel canine housing in the United Kingdom – a welfare perspective

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AstraZeneca's original dog facility in the UK was built in the 1970s, prior to the introduction of the Animals (Scientific Procedures) Act 1986. Despite alterations in the 1990s, it became clear that the facility was not fit for purpose considering Home Office recommendations and new thinking around animal husbandry and best practice.

A decision was made by AstraZeneca in 2004 to design a new dog facility for regulatory studies to incorporate the latest thinking in animal welfare and science, engineering and Health and Safety. The layout of a 36-pen unit was changed from a corridor effect to an open plan design. Individual pen size was increased from 2.1 m^2 to 4.5 m^2. Pen dividers were manufactured from 10 mm glass to enable dogs to sit on benches within their home pen and see across an entire room. Specific indoor and outdoor exercise areas were included. Natural light was a significant feature of the new building. From a scientific perspective, the physical building and working practices were specifically designed with Good Laboratory Practice requirements in mind.

Since opening in 2008, a consistent observation has been that the dogs have been significantly quieter and easier to handle in their new surroundings in comparison to the old facility. This may be due to specific design features and working practices.

IV-6-349

Combination of a rapid screening PK method and lateral marginal vein sampling technique to generate pharmacokinetic and pharmacodynamic data from *M. tuberculosis* infected mice

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Mice are commonly used disease models for tuberculosis. Obtaining PK data from the TB infected animals provides tremendous value in terms of PK/PD analysis. However, collection of multiple blood samples for pharmacokinetics studies from TB infected animals in a Bio-safety level 3 laboratory (BSL3) environment remains a major challenge. Hence, we used a combination of a lateral marginal vein blood sampling technique with a rapid screening PK method and sampling lungs for bacterial burden to obtain both PK and PD data from the same infected mouse. Animals were sampled after multiple days of dosing to obtain PK profiles and subsequently lungs were collected for bacterial enumeration. The PK parameters obtained from the improved technique and from the conventional method were highly comparable. The single mouse PK from TB infected animals allowed not only high quality PKPD data but also reduced animal usage by almost nine to tenfold. Thus, this rapid screening method achieved significant 3R (Replace, Reduce, and Refinement) benefit.

IV-6-370

Environmental enrichment in laboratory mice: its effects upon reproductive physiology

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Although a growing body of evidence indicates that environmental enrichment (EE) facilitates normal development and behaviour in laboratory mice, few studies have been conducted to demonstrate its impact upon male and female reproductive physiology. Our study investigated the effects of EE on the development and reproductive success of Albino Swiss mice, using PVC tubular devices and shredded paper as physical enrichment.

Animals were allocated in regular housing cages in groups of five individuals and treated as non-enriched (control, C) or enriched from weaning to adulthood (E). To evaluate the EE effects upon pups' development, a third group of females were enriched from the time the male was introduced to achieve pregnancy (EP). In males, evaluated parameters were body, testicular and accessory glands weight, sperm functional activity (motility, viability, acrosome and membrane integrity), testosterone concentration, *in vivo* fertilization rates, litter size, and embryo degeneration percentages. In females, evaluated parameters included body, uterine and ovary weight, spontaneous ovulation, estradiol concentration, *in vivo* fertilization rates, litter size, pups neurobiological (cliff avoidance, negative geotaxis, surface righting reflex), physical (body weight at d 1, 7, 14 and 21, bilateral pinna detachment, low incisor eruption and eyes opening) and reproductive development (testicular descent, balanoprepucial opening and vaginal opening).

No differences were evident in any of the parameters reflecting the basic reproductive physiology of males or females. However, a significantly higher number of pups were born from enriched mothers (C: 9.5 ±0.6, n=4; E: 10.7 ±0.2, n=5; EP: 12.2 ±0.7, n=5; p=0.03). A strong tendency was detected towards a faster development of the physical and reproductive parameters of pups born from both enriched groups, yet significant differences were only observed for testicular descent (day 19, C: 0 ±0%, n=16; E: 62.5 ±12.5%, n=17; EP: 21.6 ±9.7%, n=21; p=0.002). Although EE showed limited effects on reproductive physiology in this strain of laboratory mice, a faster pup development appears to be favored by an increased physical complexity in the environment from birth.

IV-6-512

Double decker caging for ferrets

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There are no USDA or NIH Guide housing space requirements specifically mandated for ferrets beyond allowing for "normal postural adjustments." Ferrets are usually housed in cage types such as large tubs or modified rabbit cages, which cannot be used in our infectious disease research work. We identified a new caging type, initially designed for rats, and worked with the vendor to adapt it to provide an enhanced ABSL2 compatible housing environment for ferrets.

In 2008, we noticed a new caging prototype at a vendor show which had been developed to allow rats to have greater cage complexity while also held in an Internally Ventilated Caging (IVC) system. This caging system was called the "Double Decker" and contained a solid shelf that fit half the cage width. We had the idea that this caging might be just the thing we needed for our ferrets – IVC caging that was double the normal height and with the added benefit of also providing more complexity (the deck) and the vertical space for the ferrets to stand completely upright.

We tested a sample cage. The ferrets were unable to use the shelf since the solid plastic was too slippery for them to "get a grip" on and pull themselves onto effectively. However, the height was perfect! The other issue was feeder development and placement within the cage. We worked with the vendor to find solutions for both issues and have been successfully using this caging in our program now for over a year.

IV-6-709

Primate life in two American laboratories

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We reviewed long-term daily records of 146 rhesus macaques and 37 chacma baboons from two American research facilities. The study aim was to obtain a longitudinal perspective of laboratory life for primates, and to assess potential problems regarding events that cause pain and/or distress and which affect psychological well-being. We systematically tabulated data under the following major headings: demographic, housing, experiments, manipulations, chemical agents, illness and injury. On average, macaques survived 7 years in the lab, and baboons survived nearly 9 years; most were killed as part of or died during an experiment (55% macaques, 61% baboons). On average, macaques were moved 22 times, spent slightly over half their lives (53%) caged alone, and were handled (e.g., for blood collection, injection, physical exam, or surgery) every three days. Baboons were moved 31 times, spent 41% of their lives caged alone, and were handled nearly once per week. Forty-one different parasites and microbes infected the macaque population, and nearly half suffered chronic diarrhea. Twenty macaques suffered at least one amputation (usually fingers) or avulsion, most of which were self-inflicted. Post-surgical pain relief given to macaques (0-2 days) contrasted sharply with what is provided to human patients following similar procedures (1-3 weeks). Environmental enrichment records covered, on average, 30% of a macaque's time in the lab, and 31% of a baboon's time. We discuss these and other findings in the context of legislation designed to minimize pain, distress and suffering in laboratory primates.

IV-6-719

The use of mouse inhalation insert's: in house study to investigate welfare improvements to mice while restrained for inhalation studies

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Due to unforeseen complications regarding the use of mouse restraining tubes in a large inhalation study, it was discovered that when mice were placed into restraining tubes the metal bars situated at the front section of the nose cone caused skin abrasions around the snouts of a percentage of the mice. To avoid any possible issues around the possible completion of the study due to animal welfare, an alternative solution was investigated.

The aim of this study is to produce a working solution to combat the damage caused by the current restraining tubes used in mouse inhalation studies. This study was run in 2 parts. Firstly approximately 50 inserts were produced and used to see if moving an animal from bar tubes to an insert tube will reduce any damage caused. Secondly, providing an entire study with inserts to see if they are a good replacement for the bar tubes. If this study is successful then all mouse inhalation studies will use the new restraint tubes and inserts.

IV-6-720

Covance Animal Environmental Enrichment Program

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At Covance we ensure the well-being and needs of the animals in our care as a priority. Over the years we have tried to provide the most up to date and naturally beneficial enrichment all animals we work with to help mimic their natural environment. We currently source items and products from companies that are experienced in this field and work closely with them to produce new products that have the needs of the animals at the front of their thoughts. As time goes on, new and more advanced products are introduced into the market which look like the next phase of improving the environment of the animals, but is it? At Covance we have a system in place that takes new items and puts them to the test. This poster shows some of the items we currently use and the process we follow.

IV-6-721

Comparative analysis of blood sampling techniques in the rat

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Blood sampling is one of the most common procedures performed on laboratory animals as part of scientific research. As an on-going commitment to quality and welfare, Covance (Harrogate), identified a need to improve the current blood sampling method used for rats.

The aim of the study was to investigate and evaluate sampling from the jugular vein (JV) as a new blood sampling technique at Covance and compare it to alternative methods, including the sublingual vein (SV), the orbital sinus vein (OS) and the lateral caudal vein (LCV), being the preferred method used at Covance.

A 22-day study was conducted comparing the LCV, JV, SV and OS as sites of blood sampling. Blood samples were taken from animals on several occasions and a number of parameters evaluated throughout the study.

The most pertinent findings noted included localised tissue damage of the tail, increased food consumption, body weight and water consumption in animals sampled from the LCV. Animals sampled from the OS had lens opacities after blood sampling. The JV route was the only route that produced no clotted EDTA or trisodium citrate samples in week 4. When compared to alternative blood sampling methods on welfare grounds, sampling from the JV does not require the use of anaesthesia or a warming device. A major benefit of this is that blood can be taken within one minute of the animal being dosed due to the manual restraint method used, consequently reducing the stress which could potentially affect the physiological state of animals and measured blood parameters.

In conclusion, the comparative study indicated that from an animal welfare and sample quality perspective, the JV was the preferred site of blood sampling for rats. In addition, the implementation of JV blood sampling method has successfully focused on the 3R's, specifically on reduction and refinement.

Theme V
Replacement and Reduction in Basic Research

Session V-1: Novel methodologies and their potential *in vitro* application for drug development and safety assessment

Session V-1: Oral presentations

V-1-353

A novel platform for automated production and screening of scaffold-free, organotypic microtissues

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Physiological tissue-like culture systems have shown to improve the relevance of *in vitro* testing of substances. Although the advantages of organotypic 3D-cell-culture models to increase the performance of *in vitro* compound assessment have been known for years, complex production and readout processes impeded its industrial implementation. Here, we present a novel automation-compatible 96-well platform to produce organotypic microtissues and its implementation in an automated screening process. This technology allows the production of tumor and primary microtissues in a scaffold-free hanging-drop culture by liquid top-loading similar to standard 96-well monolayer plates. This is achieved by a microfluidic channel connecting an inlet funnel at the top and an outlet funnel at the bottom of the plate, where a hanging drop is formed by a combination of capillary and surface-tension forces in which the tissue is formed. We present an implementation on a robotic liquid-handling device with a 96-multichannel pipette head, showing the same volumetric precision as in standard multi-well plates. This results in an excellent size uniformity of <10% for the microtissues. Finally, a whole screening process using four reference compounds was performed in comparison to classical monolayer cultures, underlining the different behavior of both cell-culture models. Based on the high flexibility of the microtissue production technology using either animal or human primary cells and cell lines, an efficient and automation-compatible technology will further accelerate the use of *in vitro* models in drug development.

V-1-400

The application of pattern recognition data mining and knowledge discovery for systems to replace rodent models in fundamental research

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In spite of the greater awareness of the 3Rs, reliance on rodent models in fundamental biomedical research has increased, largely due to technologies that allow the precise genetic engineering of inbred strains. In competitive research environments, many researchers accept that control of whole organism genotype and broader experimental conditions (e.g. virus or compound dose, diet) are reasons to rely upon laboratory rodents. Through these genetic technologies and control, there is a rodent "system" in place that serves the researcher's needs for whole organism experimentation.

Another system that has developed rapidly is bioinformatics. Within this field the sub-discipline of pattern recognition (or knowledge discovery) has emerged as an efficient strategy with which to analyse enormous databases. This strategy includes advanced statistical analysis, as well as machine learning algorithms developed by computer scientists, and has been successfully applied to a variety of knowledge domains. Recursive partitioning and Support Vector Machines (SVMs) are two examples of machine learning techniques currently being applied to biomedical data as a component of fundamental research in infectious disease and immunology. These and other bioinformatics techniques allow the modelling of multi-dimensional data and the identification of "patterns" associated with a disease outcome. Such patterns, or profiles, can then be summarised as a set of "rules" that allow the clustering of human data associations with an outcome. With these rules, human genetic information can be exploited to assist with the identification of disease genes and pathways after appropriate biological validation of *in silico* models.

V-1-430

Plants as animal alternatives in the production of antibodies and other therapeutic agents

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Antibodies recognize and bind to target antigens with great specificity, a property which allows them to be used for a wide variety of application. Traumatized animals and tissue culture have been used for the production of some of these immunoglobulins. However, fermentation and tissue culture systems are not ideal for synthesizing mammalian therapeutic proteins and injecting an animal with hybridoma in order to induce ascite production in its abdomen is very painful and traumatic. Recently, molecular farming has opened up the opportunity of using plants for the production of antibodies and other proteins in plants. Plants are safe, extremely cost-effective and can carry out post translational modification. New plant expression systems, strategies in the control of post-translational maturation and purification of these recombinant proteins are currently under development to improve the yield and quality of plant-made proteins. Therefore, plants could and should serve as excellent replacement for animals in the preparation of diagnostic and therapeutic proteins thereby avoiding unnecessary suffering experienced by animals.

V-1-584

Organotypic *in vitro* human epithelial models (EpiAirway, EpiDerm-FT) with engineered toxicological reporter functions

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In vitro human 3D (NHu-3D) epithelial models including skin (e.g. EpiDerm, EpiDerm-FT) and airway (e.g. EpiAirway, EpiAirway-FT) are important advances over traditional monolayer cell culture models. For toxicology applications, these animal alternative models provide more realistic, *in vivo*-like structure, barrier properties, metabolic functions and dosing capabilities. Here we describe NHu-3D epithelial models with the added feature of engineered toxicological reporter functions. Early passage normal human epidermal keratinocytes, dermal fibroblasts, tracheobronchial epithelial cells and fibroblasts were transduced with lentiviral vectors containing NFκB reporters linked to either GFP or luciferase. Stably transduced cells were selected by puromycin resistance, expanded several passages and cryopreserved to produce large pools of reporter-expressing cells. Reporter-expressing cells were then utilized to produce NHu-3D skin and airway epithelial models. Organotypic structure and barrier properties of models produced from reporter-expressing cells were found to be similar to models produced from untransduced cells, as determined by histological assessment, barrier assessment by transepithelial electrical resistance and/or resistance to TX-100 penetration. NFκB reporters linked to either GFP or luciferase were found to be activated about 5-fold above background by treatment of the organotypic models with TNFα. GFP was detected in formalin fixed paraffin sections by epifluorescence microscopy. Luciferase activity in tissue extracts was quantified with a microplate luminometer. Production of models containing other reporters of toxicological significance (e.g. for DNA damage, oxidative stress, heavy metal stress, ER stress, etc.) by the same process will provide a suite of human epithelial reporter models that can be utilized to provide mechanistic toxicity screening assays.

V-1-617

Optimized 1- and 3-dimensional isolation and expansion of multipotent human adipose tissue-derived stem cells: evaluation of their multipotency

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In the present study, a modified isolation and expansion procedure for human adipose tissue-derived stem cells (hADSC) in terms of differentiation efficacy and up-scalability is described. The trilineage differentiation potential was investigated upon exposure to various (non)commercially available differentiation media. hADSC, differently isolated, displayed a distinct predestined cell fate. Indeed, cells isolated by means of (i) Ficoll gradient centrifugation and subsequent cultivation as monolayers were identified as having a preference for neuro-ectodermal differentiation. On the contrary, cells with hepatic potency were isolated by (ii) red blood cell lysis treatment and subsequent cultivation as spheres, followed by monolayer culture. Another critical factor affecting differentiation efficacy was the use of selective culture media. Commercially available "cocktail" media, claiming induction of keratinocyte differentiation, mainly led to heterogeneous cell populations with mixed phenotypes. Unidirectional hepatic differentiation, however, was obtained upon exposure to different sequential media, reflecting hepatogenesis *in vivo*. Exposure to the commercially available NPMM®/NPDM® media resulted in the formation of neural structures, whereas oligodendrocyte-like cells were produced using NeuroCult®/NeuroCult Diff® media. In summary, our data show that hADSC-subpopulations harbour as well neuro-ectodermal as hepatic predestined cells. The isolation/purification methods and differentiation culture media used are essential factors for successful unidirectional differentiation.

V-1-372

A mechanistic rationale for the prediction of skin irritancy effects implemented in a workflow process

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An automated workflow algorithm was developed to predict a molecule's potential for skin irritancy (SI) based on *in vivo* legacy data. A key requirement from safety evaluators was the possibility to distinguish irritants from non-irritants but also to predict the severity of effects. The algorithm was developed for diverse chemical classes using a set of chemicals representative of cosmetics. It adopts the combination of rule-based and regression modeling on structural features and physicochemical descriptors relevant to the underlying mechanisms of action (e.g. bioavailability, reactivity, interactions with cell membranes).

Due to their importance in cosmetic formulations, special emphasis on the measurement and calculation of properties associated with surfactants is drawn in this work. Nonionic surfactants containing polyethoxylated chains are of interest due to their potential to induce SI via interactions with the cell membrane's phospholipid components. One representative structural rule is based on the ratio of EO-chains to alkyl chain length, i.e. HLB. The extent to which these molecules partition into lipid membranes is quantitatively taken into account by descriptors such as the packing indicator (total volume/volume defined by head group), and molecular shape. New surfactant properties related to the packing indicator and HLB values have also been developed. Experiments with model phospholipid membranes were devised to study mechanisms and derive new descriptors. Preliminary results indicate that the extent to which the surfactants penetrate into a lipid membrane correlates with a higher potential to induce SI.

Overall this predictive scheme improves the reliability of SI estimations. A pipeline workflow process has been developed to provide a user-friendly implementation for toxicologists.

Session V-1: Poster presentations

V-1-044

Abattoir-sourced isolated ileum from *Gallus gallus domesticus* as an experimental tool

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A study was undertaken to determine the longevity of active muscarinic receptors on abattoir-sourced isolated ileum preparations from *Gallus gallus domesticus*, with a view to using the tissue as an experimental tool for functional response assays in laboratory experiments. A concentration-response curve for acetylcholine (1-256 μM) was plotted, in the presence and absence of 1, 3 and 6 nM atropine. In a second experiment, unknown concentrations of acetylcholine samples were determined by using an interpolation method. In this experiment, four sample concentrations were used and the calculated values were found to be almost equal to the actual values. Finally, an experiment was carried out to elucidate the effects of post-sacrifice time on the contractile response of the tissue. The results showed that the tissue maintained considerable contractile response at the 6-hour post-sacrifice time-point. Competitive antagonistic activity was observed between acetylcholine and atropine on the chicken ileum, and the pA2 value was calculated to be 9.21 by using an Arunlakshana-Schild plot. The results suggest that isolated ileum preparations of *Gallus gallus domesticus*, obtained from a meat abattoir, can be used as a basic experimental tool for bioassays in routine laboratory experiments. However, its potential as a research tool still needs to be confirmed.

The work has been already published in *ATLA 38*, 361-366, 2010 and is reproduced after obtaining necessary copyright permission

V-1-065

Connexin43 signalling contributes to spontaneous apoptosis in cultures of primary hepatocytes

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Primary hepatocyte cultures are still considered the "gold standard" in the field of liver-based *in vitro* modelling. However, these experimental tools undergo progressive dedifferentiation and the subsequent onset of spontaneous cell death. The current study was set up to investigate dedifferentiation in cultures of adult primary hepatocytes at the level of gap junctions, which consist of two hemichannels of adjacent cells that are composed of connexin32. For this purpose, freshly isolated adult rat hepatocytes were cultivated under conventional conditions for four days with daily monitoring of connexin expression, connexin localization and gap junction functionality. The latter was low after hepatocyte isolation and increased to reach a plateau during cultivation. This was accompanied by gradually decreasing connexin32 protein amounts and the concomitant appearance of the fetal hepatocellular connexin43 species, as observed both at the transcriptional and translational level. Connexin32 was hereby mainly located at the plasma membrane surface, while connexin43 was preferentially associated with intracellular membrane structures. To elucidate the relevance of these findings, three connexin43 inhibitor strategies were applied, all of which resulted in downregulated connexin43 hemichannel functionality and overall gap junction activity. This was paralleled by decreased expression and activity of caspase 3 as well as by reduced production of Bid. Collectively, these data show that connexin43 signalling actively contributes to the occurrence of hepatocellular dedifferentiation *in vitro* by facilitating spontaneous apoptosis. In turn, this finding suggests that counteracting connexin43 channel functionality might be a potential strategy to increase cell survival in cultures of primary hepatocytes.

V-1-079

Reducing animal use: Validating the ovine psoas muscle from the abattoir as a replacement model for testing in extirpated human uteri using the Gynecare VersaPoint II Electrosurgical System

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Gynecare VersaPoint™ II Electrosurgical System is indicated for the removal of benign intrauterine lesions. A 4 mm Bipolar Resecting Loop Electrode was developed for this system to enable the resection of larger tissue volumes than the 2.5 mm Bipolar Resecting Loop Electrode.

The objective of this study was to compare the 4 mm and 2.5 mm bipolar loop electrode performance in ovine psoas muscle from an abattoir to that in freshly extirpated human uteri. The following characteristics were evaluated: (1) maximum depth of thermal injury, (2) gas volume produced, and (3) tissue removal rate. This study was performed to develop a preclinical tissue model that provides clinically predictive data that is similar to that generated in human uteri and to eliminate the need for dedicated research animals.

Both electrodes were tested in human uteri and fresh ovine psoas muscle. The tissues were placed in a saline treatment tank. The electrodes were mounted on a pivot and controlled by a linear actuator that provided a consistent rate of speed across the tissue. The electrodes were operated in the cut and coagulate modes. The tissue volumes resected, gas generated, and tissue necrosis depths (TTC and NBT viability staining) were measured.

The maximum thermal injury depth was similar for both electrodes in ovine muscle and extirpated human uteri. The 4 mm

electrode provided a greater rate of tissue resection and generated a lower volume of gas than the 2.5 mm electrode.

Thus, freshly harvested ovine tissue from the abattoir provides a predictive preclinical model to assess uterine thermal injury and reduces the need to use dedicated research animals. The tissue removal rate for the 4 mm loop electrode was greater than the resection rate of the 2.5 mm loop electrode without an additional safety risk to the patient.

V-1-080

Development of an *in vitro* assay for the assessment of photosensitizers

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Photoactivation and binding of photoactive chemicals to proteins is a known prerequisite for the formation of immunogenic photoantigens and the induction of photoallergy. Up to now, no adequate *in vitro* alternatives for photoallergenic hazard identification were available. Dendritic cells play a pivotal role in the induction of photoallergic dermatitis. Human peripheral blood monocyte derived dendritic cells (PBMDC) were thus perceived as an obvious choice for the development of a novel *in vitro* photosensitization assay using the modulation of cell surface protein expression in response to photosensitizing agents.

CD1a-/CD14+ monocytes are positively selected from human peripheral blood and differentiated by IL-4 and GM-CSF supplementation for 5 days. Known chemicals with photosensitizing, allergenic or non-allergenic potential were pre-incubated with PBMDCs prior to UVA irradiation (1 J/cm^2). Following 48 h incubation, the expression of CD86, HLA-DR and CD83 were measured by FACS.

All tested photosensitizers induced a significant and dose-dependent increase of CD86 expression after irradiation compared to non-irradiated controls. Moreover, the phototoxicity of the chemicals could also be determined. In contrast, (i) CD86 expression was not affected by the chosen irradiation conditions, (ii) increased CD86 expression induced by allergens was independent of irradiation and (iii) no PBMDC activation was observed with the non-allergenic control. The assay proposed here for the evaluation of the photoallergenic potential of chemicals includes the assessment of their allergenic, phototoxic and toxic potential in a single and robust test system and is filling a gap in the *in vitro* photoallergenicity test battery.

V-1-081

In vitro detection of contact allergens: Development of an optimized protocol and performance of an international ring study using human peripheral blood monocyte-derived dendritic cells

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Allergic contact dermatitis is a delayed T-cell mediated allergic response associated with relevant social and economic impacts. Until now animal experiments (e.g. the local lymph node assay) were supplying most of the data used to assess the sensitization potential of new chemicals. However, the 7th Amendment to the EU Cosmetic Directive will introduce a testing ban for cosmetic ingredients after 2013. *In vitro* alternative methods are thus being actively developed. Although promising results have been obtained with cell lines, their reduced functionality and inherent genomic instability led us to reinvestigate the use of peripheral blood monocyte derived dendritic cells (PBMDCs) for the establishment of a reliable *in vitro* sensitization test. To solve the issues associated with the use of primary cells, the culture and exposure conditions (cytokine concentrations, incubation time, readout, pooled vs. single donors and cytotoxicity) were re-assessed and optimized. Here we propose a stable and reproducible protocol based on PBMDCs. Wider acceptance and feasibility of PBMDCs for the reliable detection of human skin sensitizers were tested in a international ring study. First results will be presented.

V-1-085

Evaluation of multiple drug interactions

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In an attempt to reduce the number of mammals used in drug research, we have been examining the use of chick embryos and found that they may be superior for predicting the effects of drugs. There are not very many reports of multiple drug interactions. Almost all of the studies involve just two drugs. At the present study, we investigated the multiple drug interaction in chick embryos.

Fertilized eggs of White Leghorns were incubated and amitriptyline, fluconazole and disopyramide were injected into the fertilized eggs. Electrocardiograms were recorded after the injection, and heart rate was determined.

In this study, the heart rate was significantly decreased by a combination of amitriptyline, fluconazole and disopyramide. In addition, arrhythmia was produced by a combination of amitriptyline, fluconazole and disopyramide.

We have reported that toxic interactions involving just two drugs were demonstrated in chick embryos. The combination with disopyramide modified the pharmacological effects of amitriptyline or fluconazole in chick embryos and led to an arrhythmia detected in the ECGs. These findings indicate that the multiple drug interaction of amitriptyline, fluconazole and disopyramide has a marked influence on the heart rate in chick embryos. Although the exact mechanism underlying the influence of the multiple drug interaction on the pharmacological effects of the drug remains to be clarified, the multiple drug interaction seems to enhance the toxicity of the drug in chick embryos.

In conclusion, our recording system for ECG of chick embryos may be useful for investigating multiple drug interactions.

V-1-092

The Slug Mucosal Irritation (SMI) assay: A tool to predict ocular and nasal stinging, itching and burning sensations

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The aim of this study was to investigate whether the Slug Mucosal Irritation (SMI) test could demonstrate a relation between an increased mucus production (MP) in slugs and an elevated incidence of clinical ocular and nasal stinging, itching and burning sensations.

The stinging potency of an artificial tear (ArtTear), 5 shampoos (A-E), and 6 nasal formulations (F-K) was evaluated with the SMI-test. Additionally, a human eye and nose irritation test (HEIT and HNIT) was set up (24 participants in each study). Evaluation of the test items was performed by participants and an ophthalmologist (only HEIT) at several time points.

For the study with the shampoos, analyses reveal that (1) a significant positive association existed between immediate stinging reaction reported by the participants and the mean total mucus produced by the slugs (MTMP) (Spearman's Rank correlation = 0.986, $p < 0.001$); (2) ArtTear was best tolerated in both tests; (3) moreover, all shampoos induced higher reactions than ArtTear and water; (4) Shampoo B was the best tolerated shampoo in both tests, while C, D and E resulted in more pronounced reactions; (4) Shampoo A induced the highest MTMP and received higher scores for immediate discomfort; (6) lacrimation might not be a valuable parameter to evaluate the general tolerance of a product. The clinical trial with the nasal formulations is ongoing.

The SMI assay shows promise as an evaluation method for discomfort in the human eye and nose. Screening prototype formulations allows formula optimization prior to investment in a clinical trial.

V-1-093

The cytotoxic and inflammatory response of bronchial epithelial cells exposed to cigarette whole smoke and vapour at the air-liquid interface

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Cigarette smoke contains >5000 chemicals distributed in the particulate and vapour phases (VP). The VP contributes over 90% of whole cigarette smoke (WS), therefore, traditional submerged cultures exposed to cigarette smoke lack a vast proportion of WS as they utilise only elements of the particulate phase. We have developed a whole smoke system enabling exposure of cell cultures to WS at the air-liquid interface (ALI).

Bronchial epithelial cells (NCI-H292) were grown in submerged culture to confluence on semi-permeable Transwell® membranes and raised to the ALI immediately prior to exposure. The cells were exposed to 3R4F reference cigarette WS at a range of dilutions (1:400 to 1:5 smoke:air, v:v) for 30 min. In parallel, cells were exposed to the VP (particulate fraction was removed using an inline Cambridge filter pad). Following 24 h recovery, cytotoxicity and secreted mediators of inflammation were measured using the neutral red assay, ELISA and Meso Scale Discovery platform. Spectrofluorometry was used to quantify particulate depositing onto Transwell® membranes during the whole smoke exposure.

Doses of particulate were calculated, using a standard curve of 3R4F total particulate matter and ranged from 0.67 to 58.53 $\mu g/cm^2$ for a 1:400 to 1:5 smoke dilution. Results demonstrate that the VP is significantly cytotoxic in our system and constitutes 82% of the total cytotoxicity derived from WS exposures. Subtoxic dilutions of WS (>1:250) increased the concentrations of inflammatory mediators compared to cells exposed to equivalently diluted VP. Further studies are underway to determine in-line real-time dosimetry analysis of particle deposition during smoke exposure.

V-1-094

The development of an anchorage-independent growth assay for the assessment of the carcinogenicity of whole mainstream cigarette smoke

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Exposure to tobacco smoke has been shown to induce malignant transformation in cells. In general, previous studies employed cigarette smoke in the form of total particulate matter (TPM) or aqueous extract solution (CSEaq). Whilst this has provided a wealth of information, TPM and CSEaq do not include all the components of cigarette smoke.

Recent technical advances have enabled the exposure of cells to whole mainstream smoke (WS) at the air-liquid interface (ALI), allowing for a more physiologically relevant route of exposure. Here we describe the use of this approach, using an exposure chamber developed in BAT (Patent publication No. WO 03/100417 A1) in conjunction with the anchorage-independent growth (AIG) assay to assess the carcinogenic potential of WS.

BEAS-2B cells, a human bronchial epithelial cell line, were seeded into Transwell® inserts and left to incubate for 24 h. The inserts were transferred to the exposure chamber and exposed to varying dilutions of WS generated by a Borgwaldt RM20S smoking machine under ISO conditions (35 ml puff drawn over 2 s every min) for 15 min. Following a 48 h recovery period, cytotoxicity was assessed using the CellTiter-Glo® assay. In parallel, exposed cells were plated in Noble agar and incubated at 37°C. Colonies were scored after 21 days using the GelCount™ colony counter.

Initial results demonstrate that WS exposure induces colony formation in BEAS-2B cells, with colony sizes directly proportional to the smoke concentration. This model may provide a valuable tool in the assessment of the carcinogenicity of WS and other aerosols.

V-1-096

Involvement of acetylcholine and response to reduction in phosphorylated connexin43 in drug development research for ischemic heart disease

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Pathological sympathetic overactivation and vagal withdrawal are thought to reduce the survival rate after acute myocardial infarction (AMI). Previously, we tested if aortic depressor nerve stimulation improved the survival rate after AMI in rats. AMI was induced by ligating the left coronary artery. After the ligation, 10 Hz stimulation of the intact ADN was started and continued for 30 min in the treatment group, whereas no stimulation was performed in the control group. The survival rate at 60 min after AMI was only 6.6% in the control group, whereas it increased to 76.5% in the treatment group. With regard to life-threatening arrhythmias in acute ischemia, the effect of vagal nerve stimulation has been reported to prevent ventricular fibrillation in animals. Therefore, we investigated the effect of acetylcholine (ACh), a parasympathetic nerve system neurotransmitter, on the gap junction component Cx43 using H9c2 cells.

H9c2 cells, which are spontaneously immortalized ventricular myoblasts from rat embryos, were used due to their conserved electrical and signal transduction characteristics. The cells were cultured in DMEM supplemented with 10% FBS and antibiotics. H9c2 cells were pretreated with 1 mM ACh for 8 h, followed by 1 h of hypoxia. When cells were subjected to hypoxia, the total Cx43 protein level was decreased. The hypoxia group showed a marked reduction in the amount of phosphorylated Cx43, whereas the hypoxia-ACh group showed only a slight reduction compared with the hypoxia group. These results suggest that ACh is responsible for restoring the decrease in the Cx43 protein level, resulting in functional activation of gap junctions.

V-1-105

Novel culture configuration accelerating and enhancing hepatocyte polarization

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We present a novel approach to accelerate and enhance hepatocyte polarization by spatially patterning hepatocytes in a collagen gel on an oxygen-permeable membrane. We fabricated circular microcavities made of collagen gel having 100 μm depth on an oxygen-permeable membrane, and aligned primary hepatocytes within the microcavities. Aligned hepatocytes were overlayed with collagen gel. As a result, the hepatocyte clusters were constantly made in the microcavities ranging from 40-120 μm in diameter. We show that pattern size induced the embedding of some hepatocytes, thus allowing control of the number of hepatocytes used to induce hepatocyte polarity. These hepatocytes in clusters were more rapidly polarized compared with conventional non-patterned hepatocyte collagen sandwich culture and formed functional bile canaliculi at 2 days of seeding. Furthermore, these hepatocyte clusters had wider areas of bile canaliculi compared with those of fully polarized hepatocytes in conventional sandwich culture. We also demonstrated that width of Mrp2-positive bile canaliculi and the cell layer of each patterned hepatocyte cluster were thicker compared with those of conventional non-patterned hepatocyte collagen sandwich culture. Especially, the formation of bile pools at the center of the cluster could be controlled by varying diameter of circular cavity. The culture configuration of our approach would reduce time and culture size for the estimation of drug metabolism including biliary excretion without animals.

V-1-119

Endocrine disruption and steroidogenesis: integrated evaluation from gonadal steroidogenic enzymes *in vitro* and *in vivo* to hormonal balance and fertility assessment *in vivo*

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In the present study, we propose an integrated evaluation of the effects of some endocrine disruptors *in vivo* in male and female adult rats, and *in vitro* in several models. We focused our work on the last step of steroidogenesis and the aim was to determine to what extent *in vitro* models could be predictive of *in vivo* alterations.

We have evaluated the selected chemicals for their ability to (i) modulate the expression and activity of steroidogenic enzymes *in vitro* and *in vivo*, to (ii) disrupt hormonal balance, and to (iii) disrupt certain fertility parameters, during a sub-acute exposure. Gene expression profiles were assessed by RT-qPCR, *in vitro* and *ex vivo* aromatase activity was assessed by the method of tritiated water, blood and gonadal steroid concentrations were evaluated with LC-MS/MS and ELISA.

Our results showed that all treatments induced disturbance of hormonal balance, which was related to gene expression disorders in some cases. For example, rats treated with atrazine presented high levels of estradiol and low levels of testosterone. This observation may be related to aromatase induction observed in testes and ovaries as well as in gonadal primary cultures and cell lines. A decrease of testosterone level is also observed in male rats treated with methoxychlor, which can be linked not only to aromatase induction, but also to HSD17B3 down-regulation.

Taken together, these results contribute to identify toxic endpoints as well as relevant biomarkers of endocrine disruption. Further studies should determine if, on the basis of these data, a new predictive tool could be developed for endocrine disrupting chemicals assessment.

V-1-122

The Doerenkamp-Zbinden Foundation's Chairs on alternatives to animal experimentation: Projects

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The DZF is a Swiss foundation that has dedicated its activities and support to the development and promotion of alternatives to animal experimentation in biomedical research and education according to the well-known 3R principle (replacement, reduction and refinement) in the field of biomedicine. Lately the foundation's strategy has been focused on establishment and support of university chairs on alternatives to animal testing worldwide.

Up to now six endowed chairs have been established worldwide:

1 The Doerenkamp Professorship for Innovations in Animal and Consumer Protection, University of Erlangen, Germany
2 The Doerenkamp-Zbinden Chair of *in vitro* toxicology and biomedicine, University of Konstanz, Germany
3 The Doerenkamp-Zbinden Professorship for Alternative Methods in Toxicology, Utrecht University, The Netherlands
4 The Doerenkamp-Naef-Zbinden Professorship on Alternative Methods to Animal Experimentation, University of Geneva, Switzerland
5 The Doerenkamp-Zbinden Endowed Chair for Evidence-based Toxicology, Johns Hopkins University, Baltimore, USA
6 The Mahatma Gandhi Doerenkamp Center for Alternatives to the Use of Animals in Life Science Education – Gandhi-Gruber-Doerenkamp Chair, Bharathidsan University, Tiruchirapalli, India

V-1-124

Development of human T cell priming assay using PBLs depleted in regulatory cells for *in vitro* screening of weak contact allergens

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Allergic contact dermatitis (ACD) is mediated by IFNγ-producing hapten-specific $CD8^+$ T cells and down-regulated by different subsets of suppressive cells, i.e. $CD4^+25^+$ Tregs and $CD56^+$ NK cells (Vocanson et al., 2009; 2010). Based on these observations we have developed an *in vitro* human T cell priming assay (hTCPA) based on a classical autologous mixed DC-lymphocyte reaction (MDLR) in the presence of contact sensitizers. Priming of T cells is measured at day 6 by cell proliferation and IFNγ production after *in vitro* restimulation with submitogenic doses of PHA. We have shown that the sensitivity of hTCPA to detect the sensitizing properties of haptens is directly and negatively correlated to the presence of regulatory cells in the assay: i) hTCPA using whole PBLs can only detect strong haptens; ii) when PBLs are depleted in $CD25^+$ cells (depletion of $CD4^+$Tregs), hTCPA can detect strong and moderate haptens; iii) when both $CD25^+$ (Tregs) and $CD56^+$ (NK) cells are depleted, hTCPA can detect strong, moderate and weak haptens. Positive results were obtained with TNBS, pPD, TMD, MGN, Oxazolone, Eugenol, Isoeugenol. The specificity of the hTCPA is excellent, since no significant priming was obtained using non-sensitizer compounds (Glycerol, Salicylic Acid or Lactic Acid). Collectively our results provide strong evidence that hTCPA is a sensitive method to test for the immunogenic properties of contact allergens, especially those endowed with weak sensitizing properties.

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V-1-132

Modification of an existing mouse model of human skin infection to reduce animal use

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There are many murine models of human skin and soft tissue infections in the literature. The majority of these models (90%) involve a single infection lesion per animal. We investigated the impact on bacterial load, lesion size, and body weight of a second local skin infection in the same animal.

Mice were infected subcutaneously in one or both flanks of the animal with *Pseudomonas aeruginosa* suspended in dextrin beads. Animals were weighed and treated twice-daily for two days with doripenem (DOR), a parenteral carbapenem antibiotic, starting one hour after infection. Forty-eight hours post-infection, the animals were euthanized, weighed, skin lesions measured, collected and the number of bacteria in the skin was determined.

Untreated control animals had bacterial loads of 8.7 and 8.8 log10 CFU/g skin tissue present in animals with single- or double-sided infections, respectively, 48 h after infection. Treatment with DOR (1.6-100 mg/kg/day) resulted in similar, dose-dependent reductions in bacterial load and lesion size in animals having either single- or double-sided infections. No significant statistical difference in bacterial load, lesion volume, or % body weight loss was observed in animals with single- vs. double-sided infections. This improved method resulted in reducing animal usage by 50%, while increasing the throughput of compounds tested in this model.

V-1-133

pH cycling models for evaluating the efficacy of fluoridated mouth rinses for caries control

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New anticaries fluoride formulations in the USA are required to meet animal caries testing requirements as defined by the US FDA anticaries final monograph (21 CFR 355.70). The final monograph allows for the use of alternate models of "equivalent accuracy" to the animal caries model. pH cycling models are *in vitro* protocols that involve exposure of dental substrate (either enamel or dentin) to a series of demineralizing and remineralizing conditions to mimic the dynamics of mineral loss and gain involved in caries formation. pH cycling models have been shown to demonstrate fluoride dose responses equivalent to those observed in the animal caries model. Therefore, several pH cycling studies with varied protocols were carried out to study enamel fluoride uptake and remineralization efficacy of fluoridated mouth rinses to provide additional support for the anticaries potential of marketed products: Listerine® Total Care® (LTC) and Listerine® Smart Rinse™ (LSR), marketed by Johnson and Johnson Consumer Companies Inc.

Using pH cycling studies and combinations of response variables, such as surface micro hardness (SMH), cross-sectional micro hardness (CSH) and fluoride content (from various enamel depths), we evaluated different mouth rinse formulations with varying fluoride concentration as well as different treatment regimens. These studies provided an understanding of various factors and processes that determine fluoride efficacy in mouth rinses and also allowed us to successfully demonstrate the remineralization efficacy of LTC and LSR. Controlled pH cycling models that have been optimized with respect to the substrate used, lesion depth, and measurement and analysis tools are reproducible and cost effective in evaluating anticaries potential of fluoridated mouth rinses. These models have effectively mitigated the need for animal caries testing or human clinical studies to support efficacy claims. Further improvements to the pH cycling protocols are being implemented to improve their predictive value by including environments that mimic complex salivary components and plaque fluid, ionic concentration, etc.

V-1-147

Alginate based 3D hydrogels as an *in vitro* co-culture model platform for the toxicity screening of new chemical entities

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Accurate prediction of human response to potential therapeutic drugs and vaccines is done by conventional methods of *in vitro* cell culture assays and expensive *in vivo* animal testing. Alternatives to expensive animal testing require sophisticated *in vitro* model systems that replicate *in vivo*-like function for reliable testing applications. Advancements in biomaterials have enabled the development of three-dimensional (3D) cell encapsulated hydrogels as systems to mimic *in vivo*-like function for *in vitro* models used in drug screening. In this study, we have developed a platform to enable 3D high density (~10^7 cells/ml) culture of liver cells combined with a monolayer growth of two-dimensional (2D) target breast cancer cell line (MCF-7) in static culture environments for *in vitro* drug toxicity testing. A test platform that incorporates a porous poly-carbonate disc is integrated within standard cell culture plates to enable the co-culture of multiple cell types. Alginate hydrogels encapsulated with serial cell densities of HepG2 cells (10^6-10^8 cells/ml) were supported by the disc platform and co-cultured with MCF-7 breast cancer cells during a 3 day study period. The clearance rates of drug transformation by HepG2 cells were measured using the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) metabolized to HFC (7-hydroxy-4-trifluoromethyl coumarin). The platform was used to test for HepG2 toxicity using commercially available known drugs such as acetaminophen, diclofenac, rifampin and quinidine. The cytotoxicity 50% value of candidate drugs derived from dose-dependent testing using our platform correlate well with the reported *in vivo* LD_{50} values. The developed test platform allowed us to evaluate drug dose concentrations to predict hepatotoxicity and its effect on the breast cancer cell line. The *in vitro* 3D co-culture platform provides a scalable, reusable and flexible approach to test multiple-cell types in a hybrid setting within standard cell culture plates and therefore opens up novel, relatively inexpensive techniques to screen NCE compounds.

V-1-154

An image processing analysis of corneal alteration induced by chemicals

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Sodium fluorescein is widely used for the evaluation of ocular surface integrity. The fluorescent property of this molecule allows the corneal defects to be directly examined and visualized by the intensity of the ocular surface staining. For ophthalmological practitioners, slit lamp biomicroscope examination and the assessment of the type, depth and area of corneal injury are performed after sodium fluorescein instillation and observation using a cobalt blue filter. This routine and useful exam permits the discrimination and classification of an ocular irritant based on the extent of the corneal defects. Several *in vitro* ocular models currently exist to predict moderate and severe/corrosive ocular irritation; nevertheless these models lack accuracy to discriminate between mild/slight/very slight ocular irritants. Even if these methodologies are highly informative, they do not offer at the same time a rapid and direct assessment of the area and the depth of the corneal injury. The aim of this study was to propose and investigate the reliability and validity of a method based on the Principal Component Analysis of injured cornea images for objective and quantitative measurement of surface and depth of corneal injury induced by chemicals. Such methodology could be a useful tool for cosmetic industries for the safety assessment of ingredients and finished cosmetic products.

V-1-162

Evaluation of the individual and synergistic value of the HET-CAMVT and the dynamic solubility model in order to predict the potential of new IV formulations to cause injection site reactions

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Prior to administering an intravenous (IV) drug formulation in humans, it is necessary to evaluate its vascular irritation potential so that clinical injection site reactions (ISRs) and, as a consequence, a delay in the clinical development of a compound, can be prevented.

Two *in vitro* models that have shown promise for improving the screening process and reducing the need to evaluate formulations *in vivo* are a dynamic solubility model developed by Yalkowski et al. (2003) and the HET-CAMVT (Vascular Toxicity Hen's Egg Test-Chorioallantoic Membrane). While in the dynamic solubility model, the precipitation of the solution infused in a tube is monitored, in the HET-CAMVT the irritation potential of the formulation is evaluated after topical application on to the vasculature (coagulation, hemorrhage and lysis).

The aim of this study was to evaluate the individual and synergistic value of both models in order to predict ISRs. Different IV formulations with known *in vivo* ISR were tested in a dose dependent manner both in the HET-CAMVT and the dynamic injection model.

It was shown that the results of the HET-CAMVT model correspond well with the observed *in vivo* ISR. In the dynamic solubility model a clear dose response was observed, although at other concentrations compared to HET-CAMVT. By testing more compounds that cause irritation by different mechanisms and by evaluating the importance of different parameters in the dynamic solubility model, both models can help us to guide whether a compound structural change or a formulation change is needed to avoid clinical ISRs.

V-1-193

Development of an integrative approach for the prediction of systemic toxicity: Combination of cell toxicity, pharmacological and physical chemical properties

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Ethical, scientific and economic constraints have motivated the scientific community to develop alternatives to animal testing. Developing alternatives for acute/chronic systemic toxicity testing represents a challenge because of the complex biological processes implied. A realistic approach could rely on the combination of data generated for multiple endpoints. The Ctox panel®, which is a multiparameter cell-based *in vitro* system for predicting rat acute systemic toxicity, is a typical example. Preliminary studies conducted in a blinded manner showed a good sensitivity and specificity (91% and 78%), while defining a LD_{50} threshold at 2000 mg/kg. However, the model failed to accurately predict very toxic chemicals displaying LD_{50} below 300 mg/kg. Further to an in-depth analysis of the misclassified chemicals, we concluded that both pharmacological data (for the reduction of false negatives) and physical-chemical properties (for the reduction of false positives) had to be considered. The modified approach was applied to 76 non-proprietary compounds previously tested with the standard method. A significant improvement in the prediction of the GHS categories was observed. Indeed, 75% of the chemicals pertaining to GHS 1, 2 and 3 were correctly classified, compared to 50% with the standard model. In addition, at an arbitrarily defined LD_{50} threshold of 500 mg/kg, the sensitivity and specificity were 85% and 89% with the new model against 71% and 83% with the standard model. Future directions will consist of challenging the newly built model with a new set of chemicals and foreseeing the application of such a strategy for repeated dose-toxicity.

V-1-199

Development of an integrative approach for the prediction of systemic toxicity: Combination of cell toxicity and metabolism data

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Increasing societal concerns for animal welfare and current legislation constraints have made the industry enter a new phase in its innovation and R&D processes. For acute and chronic toxicity testing, which consumes a large number of laboratory animals, no alternative is available, one of the reasons being the complexity of the biological processes involved. As such, many research programs have been launched with the aim of developing integrative approaches that would accurately predict such an endpoint. The purpose of the study presented herein was to develop an integrated testing strategy on the basis of the data previously generated with The Ctox panel®, a multiparameter, cell-based *in vitro* system and the Solidus® DataChip/MetaChip platform, developed to assess metabolism-mediated toxicity. The set used consisted of 63 proprietary chemicals, categorized as Toxic (25 compounds) and Non Toxic (38 compounds) on the basis of a LD_{50} threshold of 500 mg/kg in rat following oral administration. A statistical analysis of the data led to the construction of several integrative models. The most predictive model (discriminant analysis) required the consideration of a total of 22 parameters. On the basis of a LD_{50} threshold of 500 mg/kg, the sensitivity and specificity of the prediction was 92% and 87%. The next steps will consist of challenging the model with a set of diverse chemical classes. It is noteworthy that the number of parameters considered for the model correlates well with the complexity of the endpoint mentioned above.

V-1-211

Cardiovascular model for cardiac toxicity testing

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The heart has been shown to be particularly prone to toxic effects of cardiac and noncardiac drugs. Adverse cardiac drug reactions are typically serious and even fatal. The aim of this study was to develop a cardiovascular model for *in vitro* testing of pharmacological and toxicological potential of compounds. The model was constructed by plating neonatal rat cardiomyocytes on the top of the co-culture of human fibroblasts and endothelial cells forming a network of tubular structures. As controls, cardiomyocyte monoculture and tubular network co-culture were established. The specific goal was to investigate whether the presence of tubular structures has an influence on survival and functionality of primary cardiomyocytes.

The results showed that by culturing cardiomyocytes with tubular structures the survival and functionality of neonatal rat cardiomyocytes was improved. Cardiac cells contracted synchronously for at least 14 days a cardiomyocyte monoculture maintained contractile function only for 7 days. The number of cardiomyocytes and the composition of stimulation media were found to be critical. Immunocytochemical staining demonstrated that the cardiomyocytes were oriented in line with the formed tubular structures, and the typical morphology of mature cardiomyocyte was observed.

In conclusion, interaction between tubular structures and cardiomyocytes seems to be important in promoting neonatal cardiomyocyte cell survival and their functionality. The developed model has great potential to be further developed to a routine test for pharmaceutical and toxicological industry. Further studies aim at accomplishing a completely human cell based cardiovascular model by using human pluripotent stem cell derived cardiomyocytes.

V-1-216

An organotypic microliver platform for high throughput drug testing

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Predictive toxicology testing is a major challenge in regulatory toxicology and drug discovery. Traditionally, many toxicity tests are performed in animals. However, the need for testing thousands of compounds in a high-throughput manner would require millions of animals for toxicity testing. Therefore, novel models for predictive toxicity testing *in vitro* are urgently required. So far, mainly monolayer cultures of primary hepatocytes were used to study drug metabolism, enzyme induction and compound hepatotoxicity *in vitro*. While 2-dimensional sandwich cultures improve maintenance of hepatocyte function, these systems are technically challenging, of little use for chronic toxicity testing and only fairly suited for higher throughput. In order to preserve liver-specific functions of hepatocytes over extended time periods and to provide a versatile platform for toxicology testing, we developed a novel scaffold-free, organotypic production technology for a 3-dimensional culture of rat liver microtissues in a standard 96-well microtiter plate format. The GravityPLUS system allows the production of regular-sized microtissues with a morphology and architecture close to native liver tissue when co-cultured with non-parenchymal liver cells. Stainings of paraffin-sections indicate tight cell-cell contacts, extensive glycogen storage and formation of bile canaliculi between hepatocytes. The rat liver microtissues show stable cell survival and CYP3A induction over 5 weeks in culture. Our results demonstrate the validity and suitability of the GravityPLUS hanging drop platform for liver microtissue culture, offering a highly functional and reliable *in vitro* toxicology testing system which can easily be implemented in standard lab automation processes.

V-1-221

A pertinent screening tool to measure permeability coefficient: Episkin® reconstructed human skin model

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According to their similarities to native human tissue in terms of morphology, lipid composition and biochemical markers, reconstructed human epidermis (RhE) has been identified as a useful tool for the *in vitro* testing of phototoxicity, corrosivity and irritancy. These last years, some papers claim that RhE is an appropriate alternative to human skin for the assessment of skin permeation and penetration *in vitro*.

Among all RhE models commercially available, Episkin® from SkinEthic is particularly adapted for testing. Indeed, its design allows to measure penetration directly in the insert without mounting the tissue in a diffusion cell. These results lead to the development of reliable protocols for the upstream ranking assessment of the skin penetration of cosmetic ingredients under their conditions of cosmetic use.

Permeability coefficient (Kp) measurement requires sampling as a function of time. It could be done using a flow through diffusion cell (as PermeGear cell) or using the insert directly with total or partial replacement of a given volume of receptor fluid at given time gaps. Both approaches have been tested with caffeine as reference compound. With sink condition and infinite dose, flux as a function of time should reach a constant value corresponding to the steady state. Results show that steady state is not reached with the PermeGear cell contrary to the insert.

Comparison with human skin data reinforces previous studies' conclusions on RhE model as a relevant alternative to human skin for *in vitro* penetration studies.

V-1-225

Alternative approach to maximum flux for TTC applied to safety evaluation of cosmetic ingredients

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REACH legislation will increase the toxicology evaluation drastically. To meet this challenge, relevant and accurate prediction of dermal uptake/exposure of topically applied chemicals is essential for risk assessment. This could be obtained without the need for an experimental measurement, avoiding any problems with ethical issues, recruiting volunteers or housing animals, using QSAR models.

Typically, QSAR model predicting permeability coefficients (i.e. kp) are used. Many models were developed; all of them led to the same conclusion: small lipophilic chemicals have the greatest skin permeability. This analysis often causes confusion. The dataset used to build up this relation considered percutaneous transport from aqueous solution. Whereas, kp increases with log P, aqueous solubility decreases with lipophilicity. The resulting flux and effective absorbed amount of chemical are then balanced between two competitive factors (permeability and solubility).

The concept of maximum flux means that a chemical cannot cross the skin faster than the flux measured at steady state with a chemical applied on the surface in saturated solution (or in neat chemical form). It allows assessing the maximum absorbed dose. This concept was recently used in a TTC approach for cosmetic ingredients. A classification of potential of cutaneous chemical absorption was proposed on the basis of the substances' physicochemical properties. Unfortunately, the proposed classification does not cover the full range of molecular weight and log P. Moreover, some physicochemical properties known to affect cutaneous absorption (i.e. ionisation, volatility) are not considered. At least, the default proposed values greatly overestimate the absorption obtained experimentally.

An alternative approach was developed by L'Oréal to overcome these limitations.

V-1-227

Adaptation of the validated SkinEthic RHE skin corrosion test method to 0.5 cm^2 tissue samples

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In vitro human reconstructed epidermal models have been used to develop protocols able to discriminate corrosives from non-corrosives. The SkinEthic RHE test method, using 0.63 cm^2 inserts, was validated in 2006. Due to manufacturing constraints, the model is now produced in 0.5 cm^2 instead of 0.63 cm^2 sample sizes.

This study demonstrates that the RHE skin corrosion protocol could be adapted from 0.63 cm^2 to 0.5 cm^2 RHE samples. For such purpose, the protocol size adaptation was performed using 25 chemicals including the 12 OECD TG431 reference chemicals. To test the robustness and relevance of the test method, particular attention was given to choosing chemicals that are correctly classified (non-corrosive, corrosive) but also chemicals known to be misclassified (false positives/negatives). Results obtained with the 0.5 cm^2 skin corrosion test method showed that all corrosives were correctly classified, and 11 out of 13 chemicals were identified as non-corrosives. The overall accuracy over the 25 chemicals was 92%. The specificity and the sensitivity of the OECD chemicals were 100%.

In addition, the quality of RHE tissues was not only maintained but also improved. The quality control, performed on 136 (0.63 cm^2) and 262 (0.5 cm^2) RHE batches, showed a mean of 0.99 and 1.17, respectively. This similarity over years demonstrates the high quality production of the tissues using both viability and morphology parameters.

In conclusion, the quality of 0.5 cm^2 SkinEthic RHE tissues was thoroughly maintained over 9 years and the performance of the skin corrosion test method fully met OECD and ECVAM requirements.

V-1-248

Development of an alternative to the oral mucosa irritation test by modified STE test

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In recent years, the economy of urban areas in China has been greatly developed, and many oral care products are imported from overseas. However, the classification of oral care products used to be unclear in China. Since 2008 they have been classified as cosmetics, although oral mucosa irritation has rarely been evaluated. The Shanghai CIQ is responsible for safety management of domestic and import cosmetics, and Japan and China collaborated to develop an alternative method for evaluation of oral mucosa irritation. An oral mucosa irritation study was performed in Hartley guinea pigs with reference to CTFA Guidelines and obtained the irritation score. The alternative method employed was a modified STE method (mSTE), where the cell viability was evaluated after exposing the study article (concentrations: 5.0%, 0.5%, 0.05%) for a short time to SIRC cells or 3T3 cells. Eighteen raw materials for oral care products and fifteen products (eleven marketed products) were tested, and the results were compared with those of animal studies. CPC and SLS showed potent irritation of the oral mucosa, although glycerin and sorbitol did not show any irritation. In addition, all of marketed products showed slight irritation. These results corresponded well with those obtained from the mSTE test, and the high concordance rate was seen when SIRC cells were used and the exposure concentration was 0.5%. Based on these results, it was suggested that the mSTE could possibly be used as an alternative method for an irritation study in the oral mucosa.

V-1-249

Improving human vascularized mucosa/intestine models to study substance adsorption phenomena

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Robust and reliable dynamic bioreactors for long-term maintenance of various tissues at ml-scale within a vascularized matrix, i.e. BioVaSc®, have been developed at the Fraunhofer IGB in Stuttgart, Germany. Once seeded with Caco-2 cells, such vascularized matrix bioreactors support self-assembly of an intestinal microenvironment with the typical histological appearance of villi structure and morphology (Schanz et al., 2010). This indicates that in dynamic bioreactors microcapillary vasculature and proper architecture support organoid self-assembly. To further improve the equivalence of the system to human *in vivo* performance we have replaced the Caco-2 cell seeding by seeding of human primary intestinal tissue. An improved bioreactor hardware operating at least four identical dynamic bioreactors simultaneously has been engineered. The hardware supports long-term bioreactor operation over weeks and months. Process parameters, such as nutrient perfusion rate, medium composition in the inner vascular space and in the inner gut lumen, as well as culture time/duration, have been optimized to qualify the system for repeated dose testing of orally administered drug candidates on adsorption properties. Daily intravascular and inner lumen samples have been analyzed to monitor metabolic activity of the tissue culture. Histo- and immunostaining of cryopreserved or paraffin-embedded tissue slices have been analyzed to compare self-assembled organoid tissue structures with their corresponding *in vivo* counterparts. Evidence is provided for the use of the system for reliable evaluation of adsorption properties of drugs at different dosages over long periods.

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V-1-268

Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes

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Technological developments are driving *in vitro* methods towards integrated "omic" strategies. However, there is still an over reliance on classical viability assays for dose range finding. Such assays require termination of the experiment, which makes it difficult to monitor temporal alterations. To this end, we investigated the use of lactate production as a marker of cell stress in long term repeat dose *in vitro* experiments.

We conducted daily exposures to 8 compounds at 5 concentrations for 14 days on human renal proximal tubular cells (RPTEC/TERT1), human hepatoma cells (HepaRG) and mouse fibroblast (BALB-3T3) cells. Compounds were chosen from a training set used in the 7th EU Framework project Predict-IV. At days 1, 3, 7 and 14, lactate was measured in the supernatant medium. At day 14, cells were assayed for resazurin reduction capability and subsequently lysed in methanol for ATP determination.

Compound-induced loss of viability was comparable across all cell lines. In all cell types, lactate production was induced prior to a decrease in viability. In some situations, lactate also fell below control values, indicating cell death. Thus, temporal alterations in supernatant lactate alone gave information on the time and concentration of stress induction and the time and concentration of loss in viability.

Supernatant lactate production is a simple, cheap and non-invasive parameter. Since many molecular pathways converge on the glycolytic pathway, enhanced lactate production may be considered as a global marker of sub-lethal injury and thus an ideal marker for investigating temporal alterations in long-term repeat dose testing *in vitro* regimes.

V-1-285

Correlation of *in vivo* and *in vitro* degradation profiles for bio-absorbable polymer implants

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A key goal in characterization of bio- absorbable polymer is a good understanding of its *in vitro* and *in vivo* degradation behavior. One of the challenges of absorbable polymer research is to develop a tool that correlates the *in vitro* degradation strength profile from several formulations (different buffer and temperature settings) to the *in vivo* degradation strength profile. Developing a predictable *in vitro* tool not only saves animals used for preclinical studies but also shortens the product development cycle. Our poster showcases examples of correlation studies conducted for bio-absorbable polymer implants that can be used as guidance for future correlation work.

In vivo degradation is performed by implantation in Long Evan rats' posterior dorsal subcutis (between the skin and skeletal muscle). Animals are sacrificed at specific time points and test materials are explanted. *In vitro* degradation is conducted by submerging the samples in phosphate buffered solution of pH between 7.27 and 8.8 and the temperatures of the bath can vary between 37-55°C depending on the polymer. The samples are tested using a material testing machine. Testing method is specific to each material (tensile, shear, etc.).

Example 1: Poly(p-dioxanone) (PDS) anchor attached to PDS suture. *In vitro* conditions: pH 7.27 maintained at 37°C temperature. Both *in vitro* and *in vivo* testing were conducted on a weekly basis for 10 weeks. Results from the *in vitro* and *in vivo* studies indicate that the strength profiles have similar trends with a drop in strength after 7 weeks. Statistical analysis was conducted using Minitab software to correlate the *in vitro* and *in vivo* degradation profiles.

In vitro strength (lbs) = bo+ co* *in vivo* implant strength (lbs), where bo & co are constants.

Example 2: PALG anchor. *In vitro* conditions: pH 7.27 maintained at 37°C temperature. Tests were conducted on up to 91 days; *in vitro* and *in vivo* curves for shear and tensile tests.

Shear strength *in vivo* = b1 * *in vitro*, where b1 is a constant.

Tensile strength *in vivo* = c1* *in vitro*, where c1 is a constant.

These studies indicate that good *in vitro* models can be developed to predict *in vitro* polymer behavior.

V-1-303

Development of cell-based endocrine disruptor screens using automated image analysis to quantitate receptor binding and dynamics

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Endocrine disruptors are compounds that alter steroid activity, thereby perturbing endocrine system functionality. Environmental endocrine disruptors have been linked to numerous adverse health effects and reproductive problems in both humans and wildlife. The advent of complex chemical library production in addition to a large catalog of existing compounds necessitates an automated procedure to assess a chemical's *in vitro* endocrine activity prior to investigating potential organismal and environmental impact. Utilization of *in vitro* screens for potential endocrine active chemicals (EACs) reduces animal testing by categorizing and prioritizing chemicals based on their ability to alter endocrine receptor activity.

We have designed a cell-based workflow to efficiently test chemicals for their ability to activate redistribution GFP-tagged steroid receptors estrogen receptor alpha (ERα) and androgen receptor (AR). These GFP-tagged receptors form nuclear foci in response to stimulation that can be easily imaged and quantitated by high content analysis, thereby establishing an automated *in vitro* assay for EACs. Redistribution ERα and AR cell lines are assayed under similar conditions, simplifying EAC screen-

ing. The dose response for each test chemical is compared to a vehicle control, a weak response control, and a positive control dose response via user-defined thresholds to visualize both the potency and magnitude of the EAC response. Additional outputs provide insight into the dynamics of receptor activation while simultaneously monitoring cell cycle perturbations and cytotoxicity. Together these assays provide a detailed *in vitro* mechanism of endocrine receptor activation resulting in a more thorough assessment of the chemical's potential *in vivo* endocrine disruption.

V-1-305

Replacing the use of animals in antibody production

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Monoclonal antibodies (mAbs) are ubiquitous in research and medicine. They have emerged as effective therapeutic treatments for cancer, various auto-immune disorders, and other illnesses. In molecular biological research, mAbs are reagents often used to detect and measure protein and drug levels in biological fluids and to register changes in cellular proteins after exposure to a chemical agent.

The mouse ascites method of mAb production is now widely discouraged due to the pain and distress it causes the animals used and the wide availability of *in vitro* alternatives. However, the misnamed "*in vitro*" method continues to rely on mice for initial generation of the antibody and presents serious animal welfare concerns as well as methodological limitations.

Fortunately, there are non-animal alternatives available for the generation of antibodies. Aptamers and recombinant antibodies (rAbs) can be created without the use animals or animal tissues and can be used in the same ways as traditional monoclonal antibodies and can be engineered to recognize a wider range of epitopes than traditional mAbs. We explore the proven applications of both rAbs and aptamers while illustrating the efficiency and efficacy introduced when these fully *in vitro* methods are employed.

V-1-317

Ex vivo tape striped human skin model: an alternative method to animal testing for skin pharmacology studies and the pharmacological evaluation of cosmetic ingredients and finished products

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Topically applied products need to be evaluated with regard to safety and clinical efficacy. Mostly the efficacy of cosmetic products is delivered by active ingredients in the formulation. Previously the efficacy of the ingredients or formulated product was determined by testing in animal models before conducting human clinical studies. For pharmacological efficacy, few non-animal models existed which were as physiologically similar to human skin. We therefore established a model involving skin obtained from non-invasive tape-striping, studying effects on skin cells attached to the tape and quantifying skin physiological responses. The tape striped human skin model is an effective way to quantify biomarkers from the skin of subjects in clinical studies of various skin conditions, such as diaper rash, aging, and the measurement of antimicrobial peptides present on facial skin as related to acne. This quantification has been validated in freshly acquired clinical samples as well as after prolonged storage at subzero temperatures. Since these noninvasive methods can also be used to evaluate skin *in vivo* as a function of age, regional site variations, and external challenges, this technique is ideal for clinical assessment and as an investigative tool for skin biomarker studies as it is simple, non- invasive and versatile. To summarize, this model is a sensitive, non-invasive technique to assess skin inflammation, oxidative stress, skin differentiation and biomarker expression in clinically acquired samples in lieu of animal testing.

V-1-331

In vitro BBB modeling: From research to high-throughput screening

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Since the early 90's, our goal is to focus on the development of relevant *in vitro* Blood-Brain Barrier (BBB) models adapted for all stages of R&D and for all users. The BBB is a physical and metabolic barrier located at the level of brain capillaries, which regulates exchanges between blood and brain, maintains brain homeostasis and provides defense against blood-borne toxins or infective agents.

Our most famous BBB model consisting of a co-culture of bovine brain capillary endothelial cells (BBCE) together with rat glial cells has been successfully used for medium-throughput screening and mechanistic purposes in a number of major pharmaceutical companies for more than a decade. By modifying this highly predictive model, a procedure has been developed to obtain a differentiated endothelial cell monolayer after only 4 days and without using primary glial cells, which substantially reduces the use of animals. This model represents the first *in vitro* BBB model suitable for High-Throughput Screening (HTS) of compounds (drugs, chemicals, cosmetics and consumer products). Furthermore, a new generation BBB *in vitro* model that is easy to use, quick and suitable for HTS, is now available in frozen ready-to-use format and consists of BBCE frozen onto collagen-coated 24-well cell culture insert plates. This model considerably reduces the technical needs to obtain after only 4 days a functional *in vitro* BBB model.

These models answer growing needs to identify compounds that have the lowest risk for toxicity and highest probability of success in accordance with the concept of the 3Rs.

V-1-336

New tools for discovery: stem cells, adipose tissue engineering and small animal imaging

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Tissue engineering represents a key technology that will ultimately enable the replacement and reduction of animal use. Our tissue engineering strategy is based on adipose-derived stem cells (ASCs). These accessible and abundant cells can differentiate into a variety of cell types, including adipocytes. We have used human ASCs to recreate *in vitro* reconstructed tissues very similar to their native counterparts. Using the self-assembly approach of tissue engineering, tridimensional adipose tissues, devoid of exogenous materials but featuring functional adipocytes, were reconstructed. They secreted a variety of bioactive molecules (leptin, VEGF, Ang-1) in addition to their capacity to mediate lipolysis following stimulation by various agonists. When endothelial cells were incorporated, they assembled into a newly formed network of capillaries throughout the adipose construct. The structure of this network was modulated by use of TNFα. Therefore, reconstructed adipose tissues represent innovative tools to test cosmetic/pharmacological products and study their impact on metabolic functions of the adipocyte/vasculature, which play central roles in obesity and related complications. Finally, translational magnetic resonance imaging (MRI) was performed in order to image and quantify the volume of the reconstructed adipose tissues after implantation onto nude mice. MRI allowed the non-invasive assessment of the grafted tissues on the same animal over weeks, reducing by at least 80% the number of animals required. In summary, the joint evolution of stem cell and tissue engineering research, coupled with advanced imaging modalities, will contribute to the reduction of animal use in both basic and applied research.

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V-1-337

Utility of the *in vitro* mutagenicity assay in Muta™Mouse FE1 cells for regulatory assessment of genotoxicity

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In vitro assays for mutagenicity assessment provide a rapid and effective means for evaluating various types of chemical products. However, mammalian cell assays that are commonly used for regulatory decision-making show high sensitivity, but low specificity for *in vivo* genotoxicity and carcinogenicity. In other words, these assays elicit a high frequency of "false" or "irrelevant" positives for *in vivo* effect. The *in vitro* assay based on pulmonary epithelial cells (i.e., FE1 cells) from Muta™Mouse has proven useful for mutagenicity assessment of a variety of chemical agents including PAHs, aromatic amines, nitroarenes, extracts of complex environmental matrices, and nanoparticulate materials. The performance of the FE1 mutagenicity assay was evaluated by examining the response (i.e., frequency of lacZ mutations) to 9 non-DNA-reactive (i.e., Ames test-negative) chemicals that have been reported to elicit irrelevant positives in regulatory *in vitro* assays (i.e., chromosomal aberrations and micronuclei in various cell lines, and/or tk mutations in mouse lymphoma cells). When tested up to the 10 mM/5 mg/plate limit, 8 compounds were negative in the FE1 Muta™Mouse assay, and one compound (eugenol) was positive at the highest sub-toxic doses. Analysis of true positives and true negatives confirmed satisfactory assay performance (i.e., ~20-fold greater than control and no significant response, respectively). All compounds are currently being re-tested in the presence of an exogenous S9 metabolic activation mixture to confirm the negative findings. In addition, detailed cytotoxicity assessment is underway. Collectively, these results will contribute to the continuing evaluation of the *in vitro* mutagenicity assessment system based on Muta™Mouse FE1 cells.

V-1-350

Development of a multicolor luciferase assay system for *in vitro* chemical risk analysis

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The reporter assay system using luciferase is widely used as a conventional and powerful tool for quantitative monitoring of gene expression. Recent advances in luciferase technology, involving improvements in both the luciferase and the detection system and a newly cloned luciferase gene, allow us to monitor the expression of multiple genes simultaneously when luciferase are used that induce differently colored emission spectra in the catalysis of a common substrate. Recently, we have developed a multicolor luciferase assay system in which three gene expressions can be simultaneously monitored using green- (SLG, λ_{max}=550 nm), orange- (SLO, λ_{max}=580 nm), and red-(SLR, λ_{max}=630 nm) emitting luciferases. To develop a rapid- and high reliance-chemical *in vitro* risk analysis assay system, we applied the multicolor luciferase assay system to immunotoxicity tests. We generated a multicolor stable cell line by introducing three reporter vectors (IL-2-SLG, IFNγ-SLO and G3PDH-SLR) into Jurkat cells. To verify the accuracy of the measurement system, the stable cell lines were seeded on a 96-well plate and respective luciferase activities were measured. We successfully measured expression of two marker genes and one internal control gene (total 288 gene expressions) within 20 min, with a coefficient of variation of less than 15%. In addition, we confirmed that induction of IL-2 and IFNγ expression stimulated by PMA/ionomycin were stably maintained during eight weeks after start of culture. These results suggest that combined use of the measurement system and the stable cell line established in this study are useful tools for chemical risk analysis.

V-1-357

Quantitative proteomic profiling for drug toxicity prediction in human organ model systems

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The objective of the European Union funded project Predict-IV is to implement an effective drug test system using specific markers to predict toxicity in an early development stage and reduce costs and time. Therefore, primary cells and cell model systems for kidney, liver and CNS are treated with organ specific reference toxins and different omic approaches are used for biomarker identification.

Human renal proximal tubular epithelial cells (RPTEC/TERT1) for nephrotoxicity profiling were treated with reference toxins. Processed cells and controls were harvested after 1, 3 and 14 days in 3 biological replicates. After tryptic digestion and isobaric labeling using iTRAQ, peptides were separated by capillary high performance liquid chromatography (HPLC) followed by LTQ-Orbitrap mass spectrometry (MS). We used the open source software OpenMS which allows the combination of different search engines to increase the number of identifications and the quantification of labeled peptides over several iTRAQ-experiments.

HPLC-MS analysis of 27 samples comparing untreated cells with cells that have been exposed to the immunosuppressant drug Cyclosporin A led to the identification of more than 2500 proteins. Differential quantification is based on the observation of iTRAQ reporter ions representing the changes in protein abundances induced by drug stimulation. The obtained dataset serves as a basis for systematic pathway analysis using bioinformatic tools in order to reveal proteins that are indicative of toxic effects in target tissues. Putative biomarkers will be further corroborated by analyzing the effects of other reference compounds and comparison with transcriptomic and metabolomic data.

V-1-358

Development of a new reconstructed human epidermis (RhE)-based screening assay for contact allergens

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Recent changes in regulatory restrictions and social views against animal testing called for the development of reliable *in vitro* tests for predicting skin sensitizing potential of a broad range of cosmetic raw materials. Also, many of these raw materials include lipophilic compounds and complex mixtures, which could be out of the applicability domain for known cell-based assays and peptide reactivity assays. To address both the political and technical issues, we employed commercially available reconstructed human epidermis (EpiDerm), on which test samples can be applied at high concentrations in the same manner as in animal testing. We first determined gene expression profiles in EpiDerm after application of the known allergens DNFB and oxazolone (OXA). A unique set of genes encoding redox regulatory enzymes was upregulated significantly by DNFB and/or OXA, but not by a non-allergen, benzalkonium chloride. Real-time PCR analysis demonstrated that expression of the selected target gene(s) was robustly (>3-fold) induced by hexyl cinnamic aldehyde, abietic acid, and ethyleneglycol dimethacrylate, which are categorized as weak allergens in LLNA, but not by non-allergens, methylsalicylate and lactic acid. Furthermore, lipophilic sensitizing compounds, such as beta-propiolactone and palmitoyl chloride, induced marked expression of target genes without affecting cell viability, but not so a lipophilic non-allergen, hexane. Not only do our results demonstrate the predictivity of our new assay platform, which we called Epidermal Sensitization Assay (EpiSensA), they also highlight the much broader applicability domain against raw materials including highly-lipophilic compounds and complex mixtures.

V-1-377

Development of a non-injuring cell test to measure acetylcholinesterase activity for neurotoxicological high-throughput alternative methods

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Acetylcholinesterase (AChE) is a membrane-bound enzyme with its active site on the cell outside. It is an important enzyme studied in neurotoxicology and neuropathology, since it is involved in neuron system development and neurophysiological processes.

The aim of this study was to develop a modified Ellman's method for AChE activity assay without cell damage, allowing its use on *in vitro* neurotoxicological test batteries, reducing costs, experimental times, procedures, and contributing to future replacement of animal testing in neurotoxicology.

150 μl of phosphate buffered saline (PBS) or Dulbecco's modified Eagle's medium (DMEM) high glucose, with or without 10% fetal bovine serum (FBS), were added with 1 mM acetylthiocholine (AceScol) and either 0.150 mM or 0.050 mM of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) on a 96 well microplate, following OD_{412} for 50 minutes. The reaction medium with lower interference (background) was subsequently tested in the presence of undifferentiated neuroblastoma (SH-SY5Y) cells, seeded 24 h before ($4x10^4$ cells/well).

FBS induced a fast degradation of AceScol, possibly due to butyrylcholinesterase. PBS media presented the lowest background of AceScol degradation (0.012 OD_{412}/h). In the presence of cells, it was possible to detect enzyme activities using 1 mM of AceScol and 0.05 of DTNB in PBS, allowing up to 0.6 OD_{412}/h of activity.

In summary, changes in the pattern of DTNB reaction on microplate wells with and without neuroblastoma cells indicate a possible method to measure AChE activity of these cells.

V-1-378

A performance evaluation of Simcyp Dog – a fully mechanistic physiologically-based pharmacokinetic dog model – based upon a variety of theophylline i.v. and oral formulations

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The purpose of this study was to evaluate the performance of Simcyp Dog – a physiologically-based pharmacokinetic (PB-PK) dog model – to predict the pharmacokinetics of theophylline (THP) following intravenous (IV) and oral (immediate release (IR) and sustained release (SR) formulations) dosing.

Simcyp Dog is a 10 kg virtual beagle dog, which uniquely includes both inter-individual variability of major oral absorption-related parameters and a fully mechanistic gut wall permeability model. Simulations were performed to predict THP plasma concentration-time (Cp-t) profiles following the administration of 4 IV, 3 IR and 3 SR formulations (fed vs. fasted); the latter characterised by *in vitro* dissolution profiles. Simulation results were compared against published results (Tse and Szeto, 1982; Shiu et al., 1989; Mongozzi et al., 1998; Ochoa et al., 2010) to evaluate model performance.

Simulated THP Cp-t profiles for 3 of 4 IV doses were in good agreement with published literature. Preliminary simulations with IR formulations indicated a slight under-prediction of Cp-t profiles for 2 of 3 formulations tested whereas SR formulation results were in good agreement with the published data.

Thus, the Simcyp virtual Beagle model is reasonably successful in predicting THP Cp-t profiles after IV and SR formulations. The slight under-prediction of Cp for IR formulations may be attributed to factors like gastric emptying time and *in vitro* and *in vivo* dissolution rate differences. This study demonstrates promising potential for virtual veterinary drug development.

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V-1-396

Evaluation of neurotoxicity using automated image analysis

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There is increasing belief that environmental exposure to industrial chemicals, certain food additives, or substances used as therapeutic agents can contribute to development of a variety of neurodegenerative and pathophysiological conditions, such as Alzheimer's and Parkinson's disease. It is within these abnormalities that changes in neuronal cell morphology, overall cell death, neurotransmitter activity, and outgrowth, elongation, and branching of neurites are observed. Automating an *in vitro* process to screen chemicals for neurotoxic effects would allow further insight to mechanisms at the cellular level to help prioritize further selection for animal studies, while offering ease of manipulation, and increased scalability.

For this study, a select panel of neurotoxins (including retinoic acid, mercuric chloride, K252a, and okadaic acid) were tested on Neuroscreen^{TM}-1 (NS-1) cells, an established *in vitro* model system for neuronal physiology and toxicity. Neuronal viability and general morphology were evaluated in conjunction with neurite outgrowth following NGF treatment with a dose-response of various neurotoxins over 96 hours. Cells were then fixed and fluorescently stained to identify the nucleus, cell body, and neurites. Samples were analyzed using automated image analysis tools to evaluate which compounds showed general decreases in viability, as well as those that affected neurite elongation and/or branching. Multiplexing these features with automated image analysis can aid in pre-screening potential neurotoxicants and help decrease the overall need for animal testing.

V-1-411

Prevalidation of the Cultex® system assessing the inhalation toxicity of nanoparticles by direct exposure of cells at the air-liquid interface

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Over the last decade nanochemistry has developed with a tremendous pace, not without realising that the safety and risk assessment of nanoparticles will challenge current toxicological approaches. As for many nanoparticles the respiratory tract is a main portal to the human body, thus inhalative exposure route requires special attention. The Cultex® RFS system has specifically been designed to model inhalation *in vitro*, also addressing issues such as atmosphere generation, particle distribution and deposition, in order to optimise and standardise the *in vitro* exposure of cells at the air-liquid interface.

The usefulness of this system for the assessment of the acute inhalative toxicity of nanoparticles is currently explored in a prevalidation study. In this A579 cells are exposed to several well-defined nanoparticles, which have been selected primarily based on the availability of toxicological data and substances, measuring cytotoxicity as the main endpoint. In a classical approach with three laboratories, the method including all test protocols and the technical equipment was successfully transferred by the lead laboratory to the two "naïve" laboratories. Within- and between-laboratory reproducibility are being investigated by testing up to twelve different nanoparticles in independent experiments. Data are reported and analysed by the fourth project partner, who will also develop a preliminary algorithm that relates the *in vitro* results to *in vivo* reference results.

V-1-437

Need and perspectives for the implementation of relevant *in vitro* methods in the field of inhalation toxicology

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The EU REACH legislative for chemicals of 2006 represents one of the largest challenges for toxicological testing, because 68,000 to 101,000 chemicals have to be investigated according to the newest estimates. In the field of acute toxicology, internationally accepted methods are available with regard to oral toxicity. Comparable validated approaches for inhalation toxicology are not available at the moment, probably due to the difficulties in exposing cells of the respiratory tract directly to inhalable substances in a way comparable to the *in vivo* situation. In the last ten years, the optimization of the biphasic cell culture and exposure techniques as well as the availability of human cell lines for such studies offer promising possibilities to integrate this type of *in vitro* study into the research strategies for inhalable chemical compounds. Particularly, prevalidation studies are under investigation for analysing the biological activity of gaseous and particulate matter using special *in vitro* exposure systems like the CULTEX® RFS module for exposing cultivated cells at the air-liquid interface (ALI). Special attention is placed on issues like controlled generation, distribution and deposition of the test atmosphere in order to optimise and standardise the *in vitro* exposure of cells at the air-liquid interface.

V-1-474

The Bionas Discovery™ 2500 system – applications for *in vitro* alternative tests to identify eye irritants

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Advanced *in vitro* methods help to identify pharmacodynamic properties of substances and the prediction of compound toxicity. Recently, the cytosensor microphysiometer, measuring the extracellular acidification rate of cells by using a pH sensor, was recommended for the use as part of a tiered testing strategy for regulatory classification in a Bottom-up/Top-down approach to identify ocular corrosives/severe irritants. So far, this device is the only validated *in vitro* test method recommended to identify chemicals not classified as eye irritants.

The Bionas Discovery™ 2500 system measures extracellular pH in combination with two additional parameters in a very similar way. Here the suitability of this similar instrument was shown in experiments performed according to the Test Guidelines of the ECVAM, using the INVITTOX protocol 102 as a standard. L929 fibroblasts were grown on silicon sensor chips hosting the sensors for pH, oxygen and impedance measurement. The cells were exposed to increasing concentrations of test chemicals and the metabolic rates i.e. the change in pH, were determined. In addition, a potential regeneration from the toxic insults was investigated. Toxic effects induced by the test chemicals were indicated as MRD50, a value which is calculated from the concentration response curve to provide a measure of the ocular irritancy potential.

The use of the Bionas Discovery™ 2500 system serves as an alternative method for animal experiments. Regarding the similar principle of measurement, the system provides a high potential to investigate the toxicity of chemicals for eye corrosion.

V-1-544

A strategy combining high-content screening and zebrafish larvae to predict human drug-induced hepatotoxicity

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Drug-induced liver injury (DILI) represents a major challenge for pharmaceutical preclinical development as it signifies a major reason for pre-and clinical compound attrition and market withdrawal. High-content cytotoxicity screening (HCS) on human HepG2 cells has been proposed as a valuable early screening model for the prediction of DILI in humans (O'Brien et al., 2004). With a specificity of 100%, the sensitivity of detection for HCS is 67% for the prediction of strongly hepatotoxic compounds and 53% when all hepatotoxicants are included. To further improve the prediction of DILI, we propose a combined strategy including HCS and a whole-liver system. Although several 3D-liver models are currently under development, at this time no *in vitro* model is capable of mimicking the complexity of a whole liver system; zebrafish larvae nonetheless represent an attractive *in vivo* alternative. While the simplicity of HepG2-based HCS allows high-throughput screening, the liver of zebrafish larvae enables detection of more complex mechanisms of hepatotoxicity at a later stage of the discovery phase. Our evaluation of zebrafish larvae as an additional model to predict human hepatotoxicity shows that higher sensitivity indices are achieved, indicating that the whole-liver system of the zebrafish is capable of detecting more hepatotoxicants in comparison with HCS. However, the new complex model system of zebrafish larvae also requires thorough investigation of uptake and metabolism, and evidence of similarity of the most important mechanisms of drug-induced hepatotoxicity between zebrafish and humans needs to be investigated before zebrafish results are applied in decision-making processes in the drug discovery phase.

Reference

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V-1-547

Evaluation of an integrated testing strategy: comparison of *in silico* predictions with *in vivo* toxicity

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This research was conducted within a larger program to investigate possibilities and limitations of the use of non-animal data (*in silico* and mammalian *in vitro* data on kinetics, modeling and toxicity) for predicting human risk. To this aim a set of 17 widely varying chemicals was selected based on the availability of *in vivo* toxicity data. In the absence of animal data, a first step could be the use of *in silico* predictions to select *in vitro* test systems. Here we compare the output of QSAR and statistically based software with the observed *in vivo* toxicity.

Structural alerts were investigated using the knowledge-based systems DEREK (Lhasa Ltd, UK), the OECD Toolbox, and the quantitative structure-activity relation (QSAR)-based TOPKAT®. *In vivo* toxicity data for rodents after oral exposure were obtained from North American and European risk assessment agencies.

Several, but not all, *in vivo* effects are predicted *in silico*. Critical effects were generally targeted in the predictions, although this information was in several cases included in the software. For carcinogenicity, skin toxicity and hepatotoxicity false positives were predicted (respectively 7, 7, and 2 out of 17 chemicals). More problematic for human chemical safety, false negatives were also observed, in particular in the predictions for developmental toxicity, hepatotoxicity, nephrotoxicity, and neurotoxicity (respectively 4, 4, 3, and 8 out of 17 chemicals).

Formation of metabolites is not yet included in the *in silico* predictions, while this is in several cases critical for *in vivo* toxicity. This emphasizes the importance of the inclusion of metabolite formation *in silico* (followed by classifying the metabolites by structural similarities and feeding into toxicity prediction software).

V-1-560

Ex vivo tumour sphere approach as a potential alternative method for tumour xenografts in preclinical drug efficacy assays

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We previously demonstrated that 3D colospheres, directly derived from mechanically dissociated colorectal cancer tissue, are a short term culture model formed exclusively by cancer cells and associated with tumour aggressiveness (Weiswald et al., 2009). Similar approach gives successfully mastospheres from breast cancer xenografts. Cancer preclinical assays involve both *in vitro* and *in vivo* testing. Consequently, spheres as potential alternative method to reduce mouse numbers in drug screening assays was investigated.

We used human breast and colon cancer xenografts established in *Nude* mice directly from patient samples. Their mechanical dissociation gave rise to numerous spheres in only 3 days. Spheres and xenografts were analysed for gene expression profiles and anticancer drug responses.

Clustering analysis of gene expression clearly showed that spheres matched with their parent xenografts, demonstrating 1) the lack of *ex vivo* culture artifacts, and 2) the confirmed relevance of spheres for mimicking *in vitro* tumour cells.

Design of colosphere collecting method, culture conditions and cell viability/toxicity assays allowed us to obtaining reproducible results. Besides, preliminary results show that the *in vivo* chemosensitivity responses of xenografts are reproduced in derived spheres.

As one xenograft is able to give rise to abundant spheres which closely reproduce parental tumours, spheres deserve further investigation to determine if they are relevant alternative method for drug screening, leading to decreased number of mice used in anticancer drug pipeline.

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V-1-571

Application of Upcyte® technology to primary cells for developing alternatives to current *in vitro* ADME-Tox models

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We have developed a new technology to allow the proliferation of differentiated primary cells without inducing permanent immortalization, uncontrolled cell growth, or loss of phenotype. The Upcyte® ("upregulated") technology involves a viral gene transfer system to introduce a unique combination of genes that induce and maintain cell proliferation until the cells reach confluence. This allows the primary cells to be passaged many times and the generation of billions of cells. Upcyte® technology has been applied to different cell types, including keratinocytes, endothelial cells, proximal tubular kidney cells and hepatocytes. We summarise some of the comparisons we have made between primary cells and their Upcyte® equivalents. The flexibility of the application of Upcyte® cells to different cellular-based assays, together with their abundant availability from different donors for routine testing, means models are now available with sustained quality and in sufficient quantities to allow for reproducible and reliable *in vitro* ADMET studies.

V-1-578

Micro electrode chip assay (MEA) as method to detect neurotoxicity *in vitro*

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Detection and characterization of chemical-induced toxic effects in the nervous system represent a major challenge for registration and assessment of chemicals. So far, no *in vitro* method for evaluating the neurotoxic hazard has yet been validated and accepted for regulatory purposes. *In vivo*, neurotoxicological assessments exploit the fact that activity of neurons in the central and peripheral nervous system has functional consequences.

The microelectrode array (MEA) assay consists of a culture chamber with an integrated array of microelectrodes, capable of measuring electrophysiology (spikes and bursts) from electroactive tissues, such as primary cultures of nervous system tissue. Recordings from such an *in vitro* cultured system are non-invasive, label-free evaluations and provide a higher throughput than conventional electrophysiological techniques.

In this first study 21 blinded substances (12 known to be neurotoxic, 2 non-neuroactive and non-toxic and 7 non-neuroactive but toxic) were tested in a dose-response curve on embryonic rat cortical neuronal networks on a MEA for their toxicity. The experimental procedure consisted in the evaluation of the firing activity (spiking rate) and modification/reduction in response to the chemical administration. Native/reference activity, 30 minutes of activity recording per dilution, plus the reversibility/recovery points (after 24 h) were recorded. The IC_{50} values were calculated using Hill Equation Fitting tool of the averaged data. The preliminary data show a good predictivity (sensitivity: 0.93; specificity: 0.57; predictivity: 0.81). Thus, the MEA with a neuronal network could potentially become a powerful tool to evaluate neurotoxicity *in vitro*.

V-1-602

Predicting dermal toxicity using the OECD TG 404 integrated testing strategy: an evaluation of the SkinEthic RHE test methods

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The use of testing strategies based on alternative methods was adopted by the OECD test guidelines TG404 for predicting dermal toxicity. To date the test methods using the SkinEthic Reconstructed Human Epidermis (RHE) were independently validated for dermal skin corrosion and skin irritation.

The aim of the present study was to develop a testing strategy combining both SkinEthic RHE skin corrosion and skin irritation test methods to support the ongoing revision of OECD TG404 and TG431.

For this purpose, about 40 chemicals (from the ECVAM validation studies) were evaluated in both skin corrosion and skin irritation. The results showed that 15 *in vivo* irritant chemicals were identified *in vitro* as non-corrosives but correctly classified as irritants using the SkinEthic RHE skin irritation test method. In addition 15 *in vivo* non-irritants and non-corrosives were correctly predicted *in vitro* using both skin corrosion and irritation test methods. Finally 12 corrosive chemicals identified by NICEATM/ICCVAM as incorrectly predicted *in vitro* were evaluated. The results showed that corrosive chemicals misclassified in the *in vitro* corrosion test were identified as irritants – with the exception of one substance – using the SkinEthic skin irritation test method. So, when applying the OECD TG404 testing strategy on the substances identified by NICEATM/ICCVAM as potentially false negative corrosives, all these substances were correctly identified.

In conclusion, when the determination of corrosivity or irritation cannot be made using a weight-of-the-evidence analysis, a preferred sequential testing strategy (skin irritation/corrosion), which includes the performance of accepted *in vitro* SkinEthic RHE tests should be considered.

V-1-613

Comparative sensitivity of tumor and non-tumor cell lines to cytotoxicity of anionic lysine-based surfactants

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Surfactants are applied as additives in topical pharmaceuticals, cosmetic products, and recently in intracellular drug delivery systems. In this context, amino acid-based surfactants deserve particular attention and could be a promising application in these fields. Here we have compared the toxicological effects of five anionic lysine-based surfactants with heavy (tris and lysine salts) and light (sodium, lithium and potassium salts) counter-ions in their structure by *in vitro* cytotoxicity assays (MTT and NRU endpoints) using two tumor and four non-tumor cell lines.

Cytotoxicity was assessed in mouse fibroblasts (3T3 and 3T6), human keratinocytes (HaCaT and NCTC 2544), human breast cancer (MCF-7) and human cervical cancer (HeLa) cell lines. Uptake of vital dye neutral red (NRU) and MTT reduction assays was used as an endpoint to evaluate cell viability. Cytotoxicity of each surfactant was expressed in terms of IC_{50} (50% inhibitory concentration) and individual values were analysed by ANOVA followed by Tukey *posthoc* test.

Following 24 h exposure, surfactants showed varied levels of cytotoxicity to cell lines. IC_{50} values revealed that the cytotoxicity of surfactants with light counter-ions was in general more pronounced than that of surfactants with heavy counterions. Moreover, cytotoxic effects were greater in the MTT assay than in the NRU assay. Comparative analysis of cell lines showed that the NCTC 2544 and 3T6 cells were the most sensitive, while tumor cells were markedly less sensitive to the surfactant with lithium counter-ion. These differences between cells may be due to specific mechanisms underlying toxic response, and highlight the choice of cell type as very important in toxicity studies.

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V-1-635

Validation of a 3D skin model for cosmetic, chemical and medical device phototoxicity testing (EPARS)

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We have enhanced our validated *in vitro* phototoxicity test using human skin models by exploring inflammatory mediator and gene expression endpoints. The Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS) is based upon a 3D skin model that closely parallels human skin morphology. Major advantages of this test system are that test substances can be applied topically, avoiding the problems of (1) difficulty in solubilizing test materials, and (2) indirect application of test materials to cell monolayers via culture media. In addition, the tissues are composed of differentiated layers of primary human keratinocytes, a more relevant model than mouse tumor fibroblasts. Phototoxic effects are determined by measuring the viability of UV irradiated vs. non-irradiated exposed tissues. In order to increase the sensitivity and specificity of the test, we have measured the release of cytokines into the culture media via ELISA. The release of the inflammatory factor PGE2 was shown to be an early predictor of the toxic effects demonstrated in the viability assay. When compared to human phototoxicity test results and the 3T3 NRU PT validation test material set, EPARS had 100% accuracy, sensitivity and specificity. Microarray analysis of gene expression showed that chlorpromazine treatment with UVA irradiation caused changes in gene expression over time that were not observed without UVA irradiation. These genes include those for keratins, collagens and fibronectins. EPARS is an accurate and sensitive test for detecting phototoxic substances at doses representative of those that cause actual human skin reactions. Thus, EPARS is a highly predictive phototoxicity assay, with endpoints of inflammatory mediator and gene expression that allow for investigation into the mechanisms of photosensitivity in a wide variety of consumer products.

V-1-636

In vitro phototoxicity test methods compared: 3T3 NRU PT vs. phototoxicity assay in reconstituted skin

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Currently, only the 3T3 Neutral Red Uptake Phototoxicity Test (3T3-NRU PT) has been approved as a non-animal phototoxicity test by governmental regulatory agencies. However, the 3T3-NRU PT has serious drawbacks as a model for human skin, specifically: it uses a monolayer cell culture consisting of mouse tumor cells, it is overly sensitive resulting in many false-positives, and test substances must be soluble in tissue culture media. Thus, we have developed a more relevant *in vitro* system based on three-dimensional, differentiated human keratinocyte cultures, which can accommodate a wide range of vehicles and allow direct topical application of test substances, a "Phototoxicity Assay in Reconstructed Skin (PARS)". We present a side-by-side comparison between the 3T3-NRU PT and the PARS test systems using the same Solar Simulated Light (SSL) source and the 8 reference standard chemicals used for validation of the 3T3-NRU PT. The PARS test correctly predicts with 100% accuracy the phototoxic potential of all reference test substances. The concentrations of test agents needed to induce cytotoxicity in reconstituted skin, when compared to the Neutral Red assay in 3T3 fibroblasts, is one to two orders of magnitude higher, reflecting the thickness and complexity of a three-dimensional tissue structure. This better approximates the exposure levels of chemicals needed to induce a phototoxic effect in animal tests and actual human skin. In addition, the most important practical advantage gained over the 3T3 NRU PT is that test substances can be applied topically, overcoming both pH and solubility problems encountered when dosing via the culture media.

V-1-637

Gene expression profiling of an *in vitro* human skin model after psoralen plus ultraviolet light-induced phototoxicity

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To reduce the number of animals for safety screening of potential irritating chemicals and phototoxins, efforts have been made to develop more predictive *in vitro* assays. One model in more common use is reconstituted human epidermis (MatTek Epiderm) resembling *in vivo* human skin. In this investigation, these human skin models were exposed to a known skin irritant, 8-methoxypsoralen (8MOP), at a dose comparable to the EC10. The samples were also either kept in the dark or exposed to solar simulated light (SSL). Gene activity was analyzed with mRNA microarrays at 1, 6, and 20 h to determine potential cellular and molecular mechanisms of action. Two levels of biological control samples were used: a) samples not treated with 8MOP and kept in the dark or exposed to UV light and b) samples treated with sodium dodecyl sulfate (SDS) [positive control]. Purified, labeled, and fractionated cRNA isolated from each of the biological samples were hybridized onto whole human genome mRNA expression microarrays, each containing 41,000 unique probes. Data analysis was done by a tiered approach. Coefficients of variation (CV) from all the probes passing quality measures or a total of 11,335 probes for each biological sample within the treatment groups ranged from 18.5-33.1%. The least variability was observed with the principal components analysis (PCA) for the negative control samples (those not exposed to 8MOP) and the samples exposed to 8MOP under dark conditions. The most activity was seen with 8MOP and SSL exposures at 6 and 20 h as well as exposures to SDS, the positive control. Several genes in common between treatments with SDS and 8MOP were CXCL14, fibrillin2, tropomyosin alpha 1, CYP26B1, HSP70B and VEGF-A.

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V-1-638

Oxidative stress and hypoxia as factors in phototoxic damage to a reconstituted human skin model: Gene expression profiling evidence

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Currently there is worldwide emphasis on more predictive *in vitro* assays for phototoxicity, especially in screening dermatological and pharmaceutical products. Reconstituted human skin tissues modeling human skin *in vivo* were exposed to a known phototoxin, chlorpromazine (CPZ) with and without ultraviolet light, at a dose correlating with the EC10. The goal was to understand what cellular and genetic mechanism(s) contribute to the phototoxic response. The skin models were treated with CPZ in the dark as well as exposed to solar simulated light (SSL) and gene activity was measured with mRNA expression microarrays at 1, 6, and 20 h post-exposure. For comparison, biological control tissues were concurrently treated with vehicle alone or left untreated, and kept either in the dark or exposed to UV light. Purified, labeled, and fractionated cRNA isolated from each of the tissue samples were hybridized onto whole human genome mRNA expression microarrays. The microarrays contained 41,000 unique probes corresponding to the full complement of sequenced human genes. The data was analyzed using a tiered approach. Coefficients of variation (CV) from all the probes passing quality measures or a total of 10,299 probes for each tissue sample within the treatment groups ranged from 5.3 to 19.2%. The least variability was observed with the principal components analysis (PCA) for the skin model samples not treated with CPZ under either dark or light conditions. The profiles for each treatment group were more similar by time point rather than by light/dark exposure. The numbers of down-regulated genes ranged from 10 to 52 and the numbers of up-regulated genes ranged from 96 to 236. Noteworthy genes which were down-regulated included genes involved in differentiation and normally expressed in epithelial tissue, such as CXCL14 and DAPL1. Several genes (e.g. HSPA6, ERO1L and ANGPTL4) related to oxidative stress and hypoxias were up-regulated.

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V-1-640

Decellularized liver matrix, a remarkable tool in the bio-engineering of three-dimensional *in vitro* liver systems

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Simplified liver-based models are gaining ground as *in vitro* tools to evaluate drug metabolism, efficacy and safety. Yet, a major drawback is the progressive loss of hepatic functionality, limiting their applicability over time. As cell-extracellular matrix and cell-cell contacts partially overcome this problem, the development of a three-dimensional *in vitro* model, closely resembling the *in vivo* liver architecture, seems of major importance. Here we describe the development of a three-dimensional, naturally derived liver scaffold with an intact microvascular system and capable of withstanding fluid flows in the three hepatic circular systems. Whole rat livers were subsequently perfused with a selection of mild detergents to specifically remove cellular components, while preserving all major extracellular matrix components, including laminin, collagen I, collagen IV and fibronectin and extracellular matrix bound growth factor islets. This unique scaffold is available within 60 minutes and represents a remarkable tool in the bio-engineering of complex three-dimensional *in vitro* systems for pharmaco-toxicological purposes.

V-1-641

Acceptance criteria: the challenge in the development of stem cell based toxicity tests

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Currently, a big effort is directed towards the optimization of *in vitro* cell models for reliable and human-based toxicity testing. However, current human cell models are either based on primary tissues that are difficult to standardize or rely on cell cultures with carcinogenic origin which have often unwanted/unknown characteristics that might impact the cellular response to xenobiotics. The availability of human pluripotent stem cells and their derivatives opens up a new avenue to overcome these shortcomings and offers the opportunity to convert stem cell based models into test systems that can support new ways of safety assessments based on a mode of action paradigm. However, stably culturing pluripotent stem cell lines and obtaining homogenous differentiated cell cultures are still challenging. The quality of the initial undifferentiated stem cell culture can affect the differentiation process, the phenotype or functionality of the differentiated cells. Thus, it is pivotal to optimize current cell culturing methods for appropriately growing undifferentiated stem cells and to establish efficient differentiation protocols in order to get toxicologically relevant cell derivatives. To do so, it is mandatory to standardise the quality control assays serving as acceptance criteria to judge the suitability of a stem cell line and their derivatives for toxicity testing. The poster describes the establishment of a set of quality criteria standards for undifferentiated cells and their derivatives which are needed for pluripotent stem cells-based toxicology studies as well as the endeavour to encourage a consensus with the use of pluripotent stem cells based *in vitro* systems.

V-1-646

Stem cells for relevant, efficient, extended and normalized toxicology

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In the development of products for use by humans it is vital to identify compounds with toxic properties at an early stage of their development, to avoid spending time and resources on unsuitable and potentially unsafe candidate products. Human pluripotent stem cell lines offer a unique opportunity to develop a wide variety of human cell-based test systems because they may be expanded indefinitely and triggered to differentiate into any cell type. SCR&Tox aims at making use of these two attributes to provide *in vitro* assays for predicting toxicity of pharmaceutical compounds and cosmetic ingredients. In order to demonstrate the value of pluripotent stem cells for toxicology, the consortium will focus on four complementary aspects:

– Relevance, i.e. establishing and maintaining discrete cell phenotypes over long-term cultures; providing large versatility to adapt to assays of specific pathways.
– Efficiency, i.e. i) automated cell production and differentiation, ii) cell engineering for differentiation and selection, and iii) multi-parametric toxicology using functional genomic, proteomic and bioelectronics.
– Extension, i.e. i) scalability through production of cells and technologies for industrial-scale assays and ii) diversity of phenotypes (5 different tissues) and of genotypes (over 30 different donors).
– Normalization, i.e. validation and demonstration of reproducibility and robustness of cell-based assays on industrial-scale platforms to allow for secondary development in the pharmaceutical and cosmetic industry.

SCR&Tox is part of a Research Initiative of the European Commission and the European Cosmetic Association in which 7 projects are aiming to contribute towards the replacement of *in vivo* repeated-dose systemic toxicity testing.

Session V-2: Systematic reviews of animal experiments

Session V-2: Oral presentations

V-2-543

Systematic reviews of animal studies: a necessary step to take

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Within evidence-based medicine, Systematic Reviews (SR) are routinely performed for clinical studies. The phases when performing a SR are: Phrasing the specific research question/objective of the study; doing a systematic search for original papers in at least 2 databases; selecting relevant papers; doing a quality assessment; extracting data and performing meta-analyses; synthesizing the data, interpreting results and writing the paper. Animal studies are used as a preparation and risk assessment for clinical studies, however, SRs of animal studies are not yet routinely performed, even though there are very good reasons for doing so. SRs of animal studies will lead to (1) better quality science (Kilkenny et al., 2009; Sena et al., 2010), (2) improved patient safety (Pound et al., 2004), and (3) the prevention of unnecessary duplication of animal studies and 3R implementation (Hooijans et al., 2010a).

We have therefore developed practical guidelines for research and teaching and training. The Gold Standard Publication Checklist has been developed as a checklist for optimal planning, design, executing and reporting of animal studies in order to make sure that all necessary elements are covered (Hooijmans et al., 2010a). To do a more effective literature search for animal publications, our group has developed two validated search filters for the databases Pubmed (Hooijmans et al., 2010b) and Embase (de Vries et al., submitted). A search guide, describing which steps to take, is currently under development (Leenaars et al., in preparation). Several SRs on the choice of animal models and translational validity are currently being executed. Over the last 2 years we have already incorporated education and training on SRs of animal studies together with a workshop on a more effective literature search in our FELASA category C courses for researchers.

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V-2-355

Study quality and publication bias in experimental studies of neurological diseases

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The poor conduct and reporting of animal experiments, due to compromised internal and external validity and the presence of publication bias, have been implicated in the discrepancies between the results of animal and human studies. Systematic review and meta-analysis have proven to be useful tools in quantitatively estimating the impact of study quality on the outcome of animal studies.

Assessment of publication bias in 499 focal ischaemia publications using 1300 animals identified that 1 in 6 experiments remain unpublished, which leads to an overstatement of efficacy of at least 30%. Furthermore, only 3% of studies report performing a sample size calculation, and about a third of studies report random allocation to group and blinded assessment of outcome – both associated with overstatements in reported efficacy. These findings are not unique to experimental stroke. In publications reporting the use of transgenic mouse models of Alzheimer's disease only 16% report random allocation to group, 22% report blinded assessment of outcome and no publications performed a sample size calculation. In publications of experimental autoimmune encephalitis (a model of multiple sclerosis) efficacy was substantially overstated in those reporting measures to avoid bias (random allocation to group: 20.6% [95% CI 11.4-29.7] versus 41.6% [36.7-46.5] and blinded assessment of outcome: 29.8% [19.8-39.8] versus 41.0% [36.2-45.8]).

Quantitatively estimating the impact of potential sources of bias has allowed us to develop good laboratory practice guidelines but also to highlight the impact of not disseminating data and the value of reviewing evidence before embarking on clinical trials.

V-2-163

The ARRIVE guidelines to improve the retrospective analysis of animal studies

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Systematic reviews and meta-analyses are commonly used in clinical trials. There is also increasing awareness about their utility for analysing preclinical data, for example to assess the evidence supporting the effect of a treatment or to evaluate and improve the predictability of animal models. Two examples of systematic review and meta-analysis of animal data will be presented: first investigating the effect of anti-emetic drugs in a ferret model to identify opportunities to refine the model of chemotherapy-induced emesis, and second comparing the self-administration of opioids in rats and non-human primates to provide science-based evidence for the choice of species in models of abuse potential.

One of the hurdles to carrying out meta-analyses of animal studies is the poor quality of animal data published and it is therefore essential to improve the reporting of animal studies. To address this, the NC3Rs, in consultation with scientists, statisticians, journal editors and research funders, developed the ARRIVE guidelines (Animal Research: Reporting *In vivo* experiments). The guidelines consist of a check list of 20 items describing the minimum information that all scientific publications reporting *in vivo* research should include, in order to maximise the availability and information gained from animals and allow in-depth critique by scientific peers. Their goal is to guide authors and reviewers during the publication process to ensure completeness and transparency. They also enable an objective assessment of the quality of studies included in a systematic review.

The ARRIVE guidelines were recently endorsed by many high quality journals and major bioscience funding bodies in the UK; international dissemination will ensure a wider impact and improve the quality and comprehensiveness of scientific reporting.

Session V-2: Poster presentations

V-2-077

Harnessing opportunities in non-animal asthma research for a 21st century science

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The asthma field has relied heavily on animal use to model this human disease. Yet despite decades of intensive funding and animal experimentation, the incidence of asthma continues to increase, and only two new classes of asthma drug have progressed from the laboratory to the clinic in the last 50 years. Some fundamental research questions are still the mainstay in laboratories, and there is growing recognition of the need to more fully incorporate the "Three Rs" principle of Replacement, Reduction and Refinement in this area of research. At the same time, progress in research techniques with the potential to reduce, or in some cases replace, the use of animals is reaching a level where commitment and integration are necessary. Asthma research could benefit from a "21st century" targeted strategy incorporating multidisciplinary research from computational modeling to three-dimensional *in vitro* systems. There is growing consensus that progress in this field rests on the linking of disciplines to make research directly translatable from the bench to the clinic. With this in mind, the current research status of asthma will be critically examined, with a focus on the animal models currently employed, together with a look to the future, and to methodologies which have already shown their value and could be incorporated into a robust, and potentially more human-relevant research strategy.

V-2-166

Meta-analysis of the application of weight of evidence (WoE) and read-across for the assessment of repeat-dose systemic toxicity

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Animal welfare considerations, as laid down in the 2013 deadline for a ban on animal testing according to the 7th Amendment of the European Cosmetics Directive, demand the use of non-animal alternatives to evaluate repeat-dose systemic toxicity. A meta-analysis of open literature and databases was initiated by the COLIPA (European Cosmetics Association) Safety Assessment Task Force to identify non-animal approaches that are currently available and already applied in risk or safety assessment in various sectors, the focus being on examination of the practical application of WoE and read-across in the assessment of hazard and risk for toxicological effects usually assessed by repeat-dose toxicity studies. The analysis included subacute, subchronic and chronic toxicity, toxicity to reproduction and carcinogenicity. The search included PubMed/Medline, Toxline, OECD dossiers, HERA risk assessments, CIR reports, SCCNFP/SCCP/SCCS opinions and RIFM group assessment reports. Overall trends and examples from this meta-analysis are presented, which is considered a complementary exercise to other research projects in this area like the SEURAT-1 cluster co-funded by the European Commission and COLIPA under the 7th Framework Programme. The aim of this set of related projects is to establish testing and assessment strategies in order to finally replace animal testing in this area.

V-2-244

Fewer animals – more quality data with process improvement and engagement in Refinement, Reduction and Replacement (3Rs) culture

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In vivo studies are necessary to answer questions about poorly known targets and to develop safe and efficacious medicines for unmet needs in chronic pain. At our institution, we have implemented a working model that combines efficient processes, refined scientific methodologies, guiding principles and an engaged culture to attain best 3Rs practices. A stringent discovery phase screening cascade reduces the number of compounds from several thousands to tens of most advantageous ones prior to *in vivo* testing. Annual 3Rs improvements are incorporated in site/department goals and cascaded as individual objectives for scientists and animal care staff. Our animal use protocols undergo critical 3Rs evaluation by the animal care committee (ACC). Sectional representatives were included in the ACC to facilitate protocol review and ensure the close integration between science and ethics. Statistical expertise is provided to researchers to strengthen the science whilst optimising study designs to minimize animal numbers. A new animal management system linked to the protocol system ensures rodents can be efficiently used to obtain maximal data and to track animal use for protocols. Finally, different microsampling methods and study designs are used routinely to reduce animals for pharmacokinetics (up to 70%).

V-2-247

The ECVAM search guide – Good search practice on animal alternatives

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The "ECVAM Search Guide" is aimed at untrained database users and will be most relevant, where comprehensive searches are required, as part of authorisation processes for animal experiments and where regulatory requirements mandate the application of the 3Rs.

A major challenge to locate relevant high quality information about a proposed field of scientific investigation is the exponential increase of scientific publications in the recent past. Over the last years the electronic resources, originally developed to offer a potential solution to this problem, have shown a similar proliferation. The question arises: how best to search for information specifically on the 3Rs (replacement, reduction, refinement of animal use) in the World Wide Web that is heterogenic, constantly changing and growing?

The ECVAM Search Guide provides a systematic step-by-step search procedure and user guidance to facilitate the location of the desired information on 3Rs alternatives in addition to an inventory of relevant resources providing an answer to the question: What can I find where?

The project has been initiated and sponsored by ECVAM and represents the outcome of a close collaboration with the National German Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) and an international project advisory team composed of scientists and representatives of ethical and regulatory authorities in support. Its publication as a handbook and on the Internet by ECVAM is expected for this year.

V-2-267

Reduction of animal use in toxicity studies in the pharmaceutical industry: fact or fiction?

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A systematic animal welfare culture, based on the principles of the 3Rs, has been implemented over recent years within Safety Assessment at AstraZeneca. In order to review the actual outcome of different projects/activities aimed at reduction, refinement or replacement (and combinations of these), all 3R improvements implemented since 2006 have been registered within the unit. The most prominent finding was the large reduction in the number of animals used, which is the focus of the data presented. "Reduction" projects/activities (40) were listed and categorized, and numbers of animals spared in toxicity studies, including regulatory studies, were estimated. Study directors, animal technicians, molecular toxicologists, genetic toxicologists, safety pharmacologists, reproductive toxicologists, pathologists, clinical pathologists and veterinarians all contributed to these changes resulting in diminished animal use. The implemented reduction activities were a result of improvements in study design (18 activities), method development (12) and collaboration between departments (10). All these activities together resulted in reductions in use of rats (51%), mice (34%) dogs (19%) and rabbits (8%). Importantly, concomitant refinement improvements were made in some cases as well. These data clearly indicate that reduction of use of animals in toxicity studies, including regulatory studies, is achievable by systematic use of 3R principles in study designs and that building a strong 3Rs culture in everyday work at all levels of the organization is essential to achieve this. The data show that systematic implementation of 3R principles in the Safety Assessment paradigm enhances ethical use of animals in research, without compromising the scientific quality of the study.

V-2-284

Reduction in the number of species required for design verification studies

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Design verification studies for Ethicon Endo-Surgery, Inc.'s HARMONIC® energy devices are performed to evaluate vessel sealing performance. The HARMONIC® instruments must be able to ligate and transect vessels within a specific diameter range (<5 mm). Historically, verification studies utilized multiple animals and two species (porcine & caprine): one species for large diameter vessels (3-5 mm) and another for small diameter vessels (<2 mm). Each animal provided a maximum of four data points and two blood pressure challenges. Internal studies indicated similarities between vascular physiological functions, biochemical properties, histological healing parameters and the absence of differences in evaluative measures for vessel sealing between the two species. Therefore, it was concluded that each species could be used interchangeably in evaluation of efficacy in blood vessel cutting and coagulation. The goal of this study was to validate the swine model for verification studies.

An exploratory procedure was performed to identify vessels in the small (<2 mm) and large (3-5 mm) diameter range. Surgery was performed in 4 pigs. Four pigs implied 32 vessels <5 mm (8 vessels per pig) were targeted for vessel sealing.

Eight vessels were identified, 4 small and 4 large. One blood pressure challenge was utilized to test the durability of the seals. Vessel sealing performance was evaluated and compared favorably to historical norms. In conclusion, one species, porcine, can be utilized in place of two species for verification studies.

V-2-495

Is the baboon model appropriate for endometriosis studies?

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Endometriosis, characterized by the growth of endometrial tissue outside the uterine cavity, is a common gynecological disorder affecting 10% of reproductive-aged women. The baboon is commonly used as a model for the study of human reproductivity. Previous studies have shown successful endometriosis induction after injection of autologous menstrual effluent into the pelvic cavity of baboons, resulting in the formation of endometriotic lesions with gross morphological characteristics similar to those seen in women. The aim of our study was to determine (i) the prevalence of spontaneous endometriosis and (ii) the incidence of induced endometriosis after transcervical resection of the endocervix in baboons. Between February 2009 and July 2010, a total of 41 baboons underwent diagnostic laparoscopy. In a first step, 30 subsequently underwent transcervical resection of the endocervix. In a second step, 20 of them underwent uterine horn resection. Two out of 41 baboons were diagnosed with spontaneous endometriosis (4.8%). Twelve months after the surgical procedure to induce endometriosis, 8/29 animals (one died) presented with endometriotic lesions diagnosed by laparoscopy and confirmed by histology. The cumulative incidence of induced endometriosis in our model was thus 27.6%. In two baboons, endometriosis disappeared over time, resulting in a final rate of 20.7% (6/29). In conclusion, our data lead us to doubt that the baboon is a relevant model for endometriosis, since our observations suggest that baboons develop extensive and effective mechanisms, lost by women in the course of evolution, to cleanse and renew their peritoneum.

V-2-513

Open-access journals and the increased availability of animal alternatives information

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Online publication of full-text journal articles has greatly simplified and increased access to scientific literature, including literature concerning animal alternatives and animal welfare. However, the open-access publishers and journals are those responsible for making access truly equally accessible. Without requiring paid subscriptions, open-access journals may be read by anyone with access to the internet. This poster will describe a few options for authors interested in locating an open-access publisher, as well as identify those open-access journals most likely to be of relevance to Congress attendees.

PubMed Central, directly related to the NIH (National Institutes of Health) Public Access Policy, is a free digital archive of biomedical and life sciences journal literature. BioMed Central and PLoS (Public Library of Science) publish peer-reviewed scientific and medical research literature freely and available as public resources. These are just a few examples of the options available to the scientist, for both research and publishing.

V-2-515

People making information matter

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Within the United States, there are requirements to address alternatives in the context of the 3Rs. This has been a regulatory requirement since the 1985 amendments to the Animal Welfare Act. To this day most people within the regulated community still have difficulty in "addressing alternatives." Over time the research community has come to understand that they must search the literature in order to address unnecessary duplication and to determine if alternatives are available. With the advent of the internet, Google, online databases, and generalized information access at your fingertips, much of the regulated research community believed that they could adequately address alternatives by plugging in a few keywords to get all they need. The error in this thinking is that they are only working with computer language and not with a true understanding of information access. When it comes to addressing alternatives, it is vital that people (those trained in information access-information providers) be involved. Utilizing the knowledge of "Information Providers" is a must in gaining the most relevant information. Only with the knowledge of people can the information results "truly matter" to the research community. Examples of computer vs. information provider will be provided.

V-2-562

How the 3Rs can benefit from systematic reviews

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Searching for available 3R methods is not an easy task. Survey results indicate that existing 3R possibilities are regularly not found nor implemented (Leenaars et al., 2009; Van Luijk et al., submitted). Relevant 3R information is scattered over various sources such as: databases, persons and text books. Conducting systematic reviews (SR) will not be the ultimate answer for finding all existing 3R possibilities; however it does have great potential for maximizing 3R implementation (Hooijmans et al. 2010).

When planning and designing a new animal experiment, a systematic search for relevant literature can answer questions on the animal model or the novelty of the experiment. This systematic search is an important step in doing SRs. Therefore SRs can contribute to a more evidence-based use of animal models and will prevent unnecessary duplication of animal experiments. SRs will also contribute to more transparency in the animal experiment designing process and the choices made. Assessing the legitimacy of chosen methods is not an easy task according to Animal Welfare Officers and members of Animal Ethics Committees (AEC) (Van Luijk et al., submitted). Systematic Review steps such as systematic search for literature and quality assessment of primary studies will provide important insights into the decision process and provide a better basis for the ethical evaluation.

During the presentation I will elaborate further on how SRs contribute significantly to the quality of animal experiments, and on how SRs can be of benefit to society, policy makers and animal welfare.

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Session V-3: Cell culture and tissue engineering

Session V-3: Oral presentations

V-3-121

Human organs-on-chip: 3D human tissue engineering as a technological innovation, an intelligent replacement alternative to animal testing

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Three-dimensional (3D) *in vitro* living organs that can mimic organ and tissue structure and function *in vivo* will be of great benefit for a variety of biological applications from basic biology to toxicity testing and drug discovery. There have been several attempts to generate 3D tissue models but most of these models require costly equipment, and the most serious disadvantage in them is that they are too far from the human organs *in vivo*. Because of these problems research and development in drug discovery, toxicity testing and biotech industries are highly expensive, involve sacrifice of countless animals and it takes several years to bring a single drug/product to the market or to find the toxicity or otherwise of chemical entities. Our group has been actively working on several alternative models by merging biomaterials science, nanotechnology and biological principles to generate 3D *in vitro* living organs, to be called "Human Organs-on-Chip," to mimic natural organ/tissues in order to reduce animal testing and clinical trials. We have fabricated a novel type of mechanically and biologically bio-mimicking collagen-based hydrogel that would provide for interconnected mini-wells in which 3D cell/organ culture of human samples in a manner similar to human organs with extracellular matrix (ECM) molecules would be possible. These products mimic the physical, chemical, and biological properties of natural organs and tissues at different scales. The presentation will review the outcome of our experiments so far in this direction and the future perspectives.

V-3-269

Temporal transcriptomic alterations during renal epithelial monolayer formation

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The ability of the kidney to concentrate, metabolise and secrete compounds underlies its susceptibility to xenobiotics. The proximal tubule epithelium is one of the most susceptible regions of the nephron. Even minor disturbances in proximal tubule function can have serious consequences for homeostasis. Injury to the epithelial cells releases cell to cell contacts and promotes proliferation and tissue repair. In order to better understand these processes, we analysed temporal transcriptomic alterations of human renal proximal tubule cultures during monolayer formation.

Primary human proximal tubule cells and the recently developed human RPTEC/TERT1 cell line were seeded at ~30% confluence. Cell cultures were maintained in hormonally defined DMEM/F12 and fed every day for 16 days. At day 1 and then every third day, cultures were harvested for RNA isolation and cell cycle analysis. In addition, supernatants were collected to analyse glycolysis rates. Using Illumina HT-12 whole-genome expression arrays 1390 temporally differently expressed probes were identified.

The time of culture had a large impact on the gene expression, stabilising around day 13 after seeding, concomitant with retardation in G1/0 cell cycle phase. Temporal increases in cell adhesion (CDH-1 and 16) and tight junction proteins (ZO-3, CLDN2, CLDN3, CLDN10) were observed. Additionally, subunits of Na,K-ATPase were up-regulated (ATP1A1, ATP1B1, FXYD2), whereas FXYD5, TGFbeta 1, PCNA and cyclin D1 were down-regulated during monolayer formation. Downgraded glycolysis was accompanied by alterations in energy metabolism genes (e.g. LDHB, HKDC1, PFKB3/4, PDHA1, NDUFB5, PRKAB1, ACACB, FASN, ACOT7, ACSM3, ACSS1, ACAD10). The generated data set will be useful to identify mechanistically linked injury biomarkers of epithelial cell injury processes.

V-3-419

Direct oxygen supply to liver-derived cells using oxygen permeable membranes

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A study in the 1960s pointed out the fact that a confluent adult hepatocyte monolayer cultured in conventional tissue-culture-treated polystyrene (TCPS) is usually in an extremely anaerobic condition. To completely overcome this problem in static cultures using microplates, we prepared a special plate that has bottom surfaces made from polydimethylsiloxane (PDMS), an oxygen-permeable material. This plate enables direct oxygen supply to the cell layers cultured beneath the culture medium layers, thus meeting the cellular oxygen consumption at well-controlled exposure oxygen concentrations determined by its atmospheric concentrations. Using this plate, various liver-derived cells, such as human hepatocarcinoma Hep G2 cells and adult/fetal rat hepatocytes, could be cultured in thick pseudo-3D tissues comprised of several cell layers. In addition, glucose consumption and lactate production revealed that these cells utilized mainly their aerobic energy production as opposed to when they are in conventional polystyrene plates, leading to remarkably enhanced protein synthesis. Control of actual oxygen concentrations at the cells was also important in growing fetal hepatocytes; initially low (5%) but later high (21%) oxygen concentrations showed remarkable self-organization of fetal rat hepatocytes into heterogenic thick liver tissues. These results demonstrate the high future feasibility of this simple plate system in the culture of various liver-derived cells for cell-based assays.

V-3-424

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

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Most of the *in vitro* cell models for long term testing of chemicals suffer from at least two shortcomings: 1) The failure of reproducing the *in vivo* physiological characteristics of the corresponding tissues. 2) A limited shelf-life. Herein is reported the use of a standardized 3D air-liquid interface *in vitro* cell model of the human airway epithelium (MucilAir™), which is free of these limitations.

MucilAir™ is morphologically and functionally differentiated and it can be maintained in a homeostatic state for more than one year. The typical ultra-structures of the human airway epithelium, such as tight junctions, cilia, mucus, basal/goblet/ciliated cells can be observed. Classical airway transporters, ion channels and CypP450s are expressed and are functional up to one year. The epithelia react to pro-inflammatory mediators in a physiological manner. The epithelia can be stimulated regularly with inflammatory substances to simulate chronic inflammatory reactions for up to several months. A large panel of cytokines/chemokines/metalloproteinases has been detected in MucilAir™.

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/chronic effects of drugs/chemicals on the respiratory tract *in vitro*. Late effects of chemicals/mixtures (several weeks after exposure) can be observed. Several applications of MucilAir™ will be presented:

– Acute, long-term and chronic toxicity testing (first *in vitro* transposition of OECD TG412 for 28 days repeated dose study will be presented)
– Inflammatory effect assessment
– Assessment of reversible vs. irreversible toxic effects
– Recent advances in the detection of respiratory sensitizers and irritants

V-3-417

A human tissue-engineered vascular substitute with a functional vasa vasorum

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Due to the paucity of available healthy human blood vessel samples and the intrinsic variability they can display, animal models are often necessary. However, we have previously demonstrated the capacity of tissue-engineered human vascular media and adventitia to respond *in vitro* to classic vasoactive agents such as endothelin and sodium nitroprusside. Recently, we were able to add to our model a vasa vasorum, the network of capillaries supplying the external layer of arteries and veins with nutrients and oxygen. We have demonstrated that the addition of a vasa vasorum to our model significantly benefited the inosculation of grafted tissues. However, the functionality of this tissue-engineered vasa vasorum and its contribution to the vascular tone still remain to be established. Four different constructs were made, comprised of an adventitia with or without a vasa vasorum and in the presence or not of an endothelium. As expected, immunohistochemical localisation of the endothelial nitric oxide synthase (eNOS) confirmed its expression by the endothelial cells of the vasa vasorum. To induce the production of nitric oxide, rings from the constructs underwent stimulation with various concentrations of histamine and acetylcholine. Our results indicate that the vasa vasorum is capable of inducing a significant nitric oxide-mediated vasodilation in our constructs. In conclusion, a functional tissue-engineered vascular substitute could represent a very useful model permitting the *in vitro* study of human vascular pharmacology.

Session V-3: Poster presentations

V-3-063

Tissue engineered human bronchial models to study asthma

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The isolation of human bronchial epithelial and fibroblastic cells from biopsies of asthmatic and non-asthmatic volunteers provided unique cellular materials to be used for the production of tissue-engineered bronchial models *in vitro*. The epithelial cells are grown on a mesenchymal layer seeded with fibroblasts. The reconstructed bronchi can be maintained for at least 15 days in culture. Under the culture conditions established, epithelial cells undergo differentiation into ciliated and goblet cells, within a pseudostratified organization comparable to human bronchi. Comparative histologic and functional analyses of non-asthmatic and asthmatic bronchial models were performed. Our data indicated the maintenance of the asthmatic phenotype of the cells isolated from asthmatic bronchial samples. Desquamation, scarce distribution of cilia and excessive mucus secretion are some of the features observed with the asthmatic bronchial models. These models appear to be powerful alternatives to animal use for the study of the mechanisms involved in asthma *in vitro*.

V-3-171

Replacement of xenobiotic components applied in the culture medium for maintenance of human keratinocytes by human equivalents

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Confluent epithelia of autologous keratinocytes cultivated according to standardized methodology by Rheinwald & Green in 1975, have been used as grafts in different clinical situations. However, the presence of xenobiotic components of the culture medium applied by this method implies the possibility of transmission of zoonoses, prions, and viruses to patients, besides involving ethical issues related to the use of animals to obtain the components. Such concerns have driven researchers to seek alternatives that overcome this deadlock, as the formulations obtained so far are not completely satisfactory. Thus, our proposal in this study was to omit or replace the xenobiotic components traditionally used in the medium for keratinocyte culture with human equivalents. As a result, we have standardized a culture medium whereby we omitted the use of cholera toxin, replaced fetal bovine serum with human platelet lysate at a 2.5% or 5% concentration, and bovine insulin was replaced by recombinant human insulin at the same concentration as the original method (5 μg/ml). With the results obtained we conclude that the method is viable to cultivate human keratinocytes kept in culture medium free of xenobiotic components.

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V-3-174

Expression and induction of xenobiotic metabolism genes in the StrataTest® human skin model

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Human *in vitro* skin models have shown broad utility for toxicological testing due to their similarity to the *in vivo* state and ability to evaluate compounds as dosed in actual use or exposure. Numerous studies have confirmed the expression and induction of phase I and phase II metabolic enzymes in keratinocytes and skin models. In the present study, the StrataTest human skin model was evaluated for expression and induction of genes involved in xenobiotic metabolism. The StrataTest full-thickness human skin model, containing both epidermal and dermal components, faithfully recapitulates many of the biological characteristics of human skin. The model is generated using NIKS® keratinocytes, a clinically-tested and consistent source of non-tumorigenic, pathogen-free human keratinocyte progenitors. To confirm that NIKS keratinocytes possess metabolic capacity, gene expression profiles of phase I and phase II enzymes from NIKS and primary keratinocytes were compared. Differences in expression of two-fold or greater between NIKS and primary keratinocytes were considered significant. Concordance for expression of 51 phase I and phase II metabolic enzymes in NIKS and primary human keratinocytes was 98%. In the three-dimensional skin model, baseline gene expression and induction after exposure to 3-methylcholanthrene (3MC) was also examined. Cytochrome p450 1A1 (CYP1A1) and CYP1B1 were weakly expressed, while N-acetyltransferase 1 displayed more robust constitutive expression. Upon exposure to 3MC, CYP1A1 and CYP1B1 expression was strongly induced. Similar to other *in vitro* skin models, StrataTest skin tissues express genes critical to xenobiotic metabolism, further demonstrating the utility of this model for toxicological testing applications.

V-3-246

Standardization of the culture of human fibroblasts in medium enriched with platelet lysate

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Fibroblasts originate from mesenchymal stem cells and are responsible for the synthesis of extracellular matrix proteins and the secretion of growth factors which control the proliferation and differentiation of epidermal cells. The human fibroblasts can be cultured in the laboratory and used in the manufacture of dermo-epidermal substitute for the treatment of burns and chronic ulcers. However, there is a recent concern about the possibility of transmitting prions and animals viruses to transplanted patients, considering that fibroblasts are cultured in medium supplemented with fetal bovine serum. Based on this premise, the present work aims to cultivate human fibroblasts in a medium enriched with human platelet lysate. For this purpose the lysis of platelet lysate was standardized and tested in human fibroblasts under several concentrations. The results revealed that 10% platelets lysate improved cell adhesion and proliferation of human fibroblasts if compared to fetal bovine serum. Therefore, it was possible to standardize the human fibroblast culture to use these cells for clinical purposes, as well as to eliminate the xenobiotic component.

We are thankful to CNEN (Comissão Nacional de Energia Nuclear) and FAPESP (Fundação de Amparo á Pesquisa do Estado de São Paulo) for the financial support.

V-3-278

A novel approach to assess irritant or respiratory allergenic potential of chemicals *in vitro*

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There are currently no validated methods for the identification of chemical respiratory allergens, neither *in vivo* nor *in vitro*. Due to increasing health concerns associated with occupational or chemically-induced asthma, and impending directives on the regulation of respiratory sensitizers, standardized test methods are urgently required to identify respiratory allergens.

To establish an *in vitro* test system for the identification of chemical respiratory sensitizers, an immunocompetent, 3D triple cell co-culture system, representing the proximal alveolar region of the human lung, was developed. It is comprised of immature dendritic cells, derived from human peripheral blood monocytes, human lung alveolar epithelial-like cells (A549 cells) and macrophages differentiated from U937 cells.

Employing the Vitrocell® system, two well-known respiratory allergens, toluene-2,4,diisocyanate (TDI) and trimellitic anhydride (TMA), as well as two irritants, acrylic acid and acetaldehyde, were applied to the model at the air/liquid interface, mimicking the *in vivo* situation in the lung. Since this system allows for the delivery of test materials in their native form, liquid aerosols (TDI, acrylic acid, acetaldehyde) or particle aerosols (TMA) were generated to mimic a real exposure scenario. Aerosol particle sizes and the amount of test substance applied were quantified.

After a 4 hour exposure scenario, cellular viability was evaluated in dose response studies using flow cytometric evaluation of cell cycle analysis. Cytokine release and dendritic cell maturation was investigated to identify whether chemical exposure induces specific inflammatory mediators and/or cellular changes, in order to identify predictive endpoints and biomarkers that may be indicative of potential respiratory allergens *in vivo*.

V-3-341

HepaRG cells: A novel human model for the study of drug hepatotoxicity

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In vitro models have reduced the number of animals required for preclinical drug testing. Unfortunately, the usefulness of these models for toxicology is limited due to poor cytochrome-P450 (CYP) enzyme expression in hepatoma lines, as these enzymes are required for conversion of many drugs into toxic metabolites. HepaRG cells, recently isolated, have been shown to express P450s at near physiological levels. For this reason, we tested the usefulness of these cells for the study of acetaminophen (APAP) hepatotoxicity. APAP toxicity begins with formation of a reactive intermediate which depletes glutathione (GSH) and adducts proteins. Mitochondrial protein binding initiates mitochondrial dysfunction and cell death. Treatment with 20 mM APAP (6, 12, and 24 h) depletes GSH levels in HepaRG cells (80, 57, and 26% of control, respectively) and the JC-1 assay revealed loss of mitochondrial membrane potential (93, 45, and 20% of control, respectively). At later time points, release of lactate dehydrogenase (LDH) was detected (29 and 62% of total LDH at 24 and 48 h, respectively, vs. 5% with control), indicating cell death. A clear dose-response relationship was observed for all parameters. Dihydrorhodamine fluorescence demonstrated increased oxidative stress after APAP treatment. Parallel experiments using HepG2 cells, which lack CYP expression, did not show any cell injury. Therefore, HepaRG cells are a valuable alternative to animal models for studies of xenobiotics requiring metabolic activation. Importantly, the mechanism of APAP toxicity in these cells resembles what is known from rodent studies, while the kinetics of injury are similar to what has been reported in humans.

V-3-347

Use of liver microsome S9 fraction-containing microcapsules to include liver biotransformation processes in cytotoxicity tests

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Liver microsome S9 fraction obtained from the livers of rats and humans have often been used to include biotransformation processes of the liver in various cytotoxicity tests, but the fraction sometimes causes strong toxicities to the relevant cells due to the self-production of lipid peroxides. Therefore, the final concentration of S9 fraction in cytotoxicity tests should be optimized for each particular test both in terms of its biotransformation effects and original toxicity. To solve this problem, we immobilized rat liver microsome S9 fraction in Ca-Alginate microcapsules whose surfaces were post-coated with poly-D-lysine to retain detoxification enzymes and lipid peroxides produced, while allowing small chemicals and their metabolites to be transported between the capsules and outer culture medium. Although the initial metabolic rate in terms of CYP 1A1/2 capacity of the S9-encapsulated gel microcapsules was about 70-80% that of the bare S9, such encapsulation completely avoids the original toxicity of S9. Using conventional membrane culture inserts and their accompanying multi-well plates, we cultured NIH 3T3 cells (in the lower compartment) with the microcapsules (in the upper compartment) and observed enhanced cytotoxicity of an indirect mutagen, cyclophosphamide, in almost the same manner as observed in the presence of bare S9 fractions added to the culture. These results clearly show that use of microcapsules containing liver microsome S9 fractions is simple, general and thus practical to include liver biotransformation processes in various cytotoxicity tests.

V-3-365

Immunohistochemical characterization of a pituitary derived cell line from adult Atlantic salmon, *Salmo salar*

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Atlantic salmon, *Salmo salar*, are highly valued fish for both fisheries and aquaculture. Growth and reproduction in this species have been widely studied and factors regulating their life cycle, including somatic growth, sexual maturation and reproduction, spawning, smoltification, have been extensively investigated. However, the molecular mechanisms controlling expression of the hormones involved in these processes are largely unknown. Pituitary cell cultures could be valuable for elucidating these mechanisms, and a cell line derived from this teleost's pituitary could make significant impacts in understanding growth and hormonal regulation as well as endocrine disruption by environmental contaminants. Here we report on the development and histochemical characterization of a continuous cell line derived from adult Atlantic salmon pituitary dubbed ASP309. The cells display a fibroblastic morphology and have been grown for almost 2 years in L15 media supplemented with 10% FBS at 18-20°C. DNA barcoding confirmed the cells as derived from *Salmo salar*.

V-3-415

The development of human skin explant cultures as an *in vitro* alternative to animal testing for translational research in skin care

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The development of *in vitro* model systems for translational research and skin care applications is of increasing priority, following the increased awareness of the 3Rs concepts and the European Community ban on animal testing for cosmetics. The goals of our studies were: 1) To develop an *in vitro* model system that best represents the physiological complexity of human skin, with metabolically active and structurally intact epidermis, dermis, and subcutaneous fat layers. 2) To create a portfolio of assays for evaluating dermatological and skin care agents for their biological activities, that would enable the prediction of clinical efficacy and expanding new biological insights without animal studies.

We established a human skin explant system using full-thickness human skin biopsies, obtained from healthy donors undergoing abdominal surgeries with informed consent. We optimized culture conditions that enable maintaining structural integrity and metabolic activity of the epidermis, dermis and subcutaneous fat layers of the skin explant. In addition, biomarkers were identified and a portfolio of assays was developed for examining the epidermal, dermal and subcutaneous adipose functions, and for the evaluation of biological effects of dermatological and skin care agents on pigmentation, skin aging, and subcutaneous lipid metabolism. The skin explants system was validated using TGF-β (an experimental positive control) and several known cosmetic, skin care actives, such as retinol. In summary, a human skin explant system that is viable and metabolically active was established, for better understanding of skin biology, for mechanistic understanding and for predicting efficacy of agents

V-3-486

Evaluation of microbicide toxicity and efficacy using a novel three-dimensional human organotypic co-culture vaginal model

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Recent advances in technology and immunology together with a lack of successful vaccines and microbicides against sexually transmitted viruses (STV), demonstrates a need for alternative investigative models. Human Immunodeficiency Virus (HIV) and Herpes Simplex Virus-2 (HSV-2) are examples of human vaginal mucosal STV that infect a substantial portion of the world's population, with no currently approved interventions. The lack of interventions could be a consequence of utilizing non-physiological relevant animal models, explants, and monolayer cell cultures in preclinical studies. Here we utilize 3D vaginal epithelial cells cultured in a rotating-wall-vessel (RWV) bioreactor to investigate the toxicity and efficacy of various microbicides. We have shown previously that our RWV-derived vaginal model recapitulates *in vivo* structural and functional properties. In this study, we expand our monotypic 3D vaginal model to more closely model the human vaginal tract with the incorporation of human innate immune cells. Using this co-culture model, we screened a panel of well-characterized microbicides to test their toxicity by measuring mucosal toxicity biomarkers (including TRAIL and IL-1RA). In addition, we evaluated microbicide efficacy by quantifying their impact on HSV-2 replication in our 3D vaginal models. Data generated from our 3D human monotypic and co-culture models are similar to observations in human cervical explants, demonstrating how our *in vivo*-like human models can serve as robust, predictive models for screening for microbicide toxicity and efficacy. Overall, our work supports and illustrates the numerous advantages of utilizing physiologically relevant human models as alternatives to animal-derived models.

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V-3-489

Functional studies of proteins: towards a human knock-down model using tissue-engineered skin substitutes

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Knock-out mice and RNA interference in cell culture are useful tools for functional studies of proteins. However, those methods may be less appropriate to study proteins expressed in specific contexts such as in human epidermal differentiation. Indeed, the differentiation of epidermal cells is incomplete in monolayer culture and many differences exist between mouse and human skin. The Dual Leucine zipper-bearing kinase (DLK), expressed in normal human skin, has been reported as an inducer of terminal differentiation of human epidermal cells into corneocytes, which form the protective layer of the epidermis: the cornified layer. To better understand the role of DLK in the cornified layer formation, we developed a Skin Substitute (SS) model underexpressing DLK. SS were transduced at day 0 of air-liquid interface culture with lentivirus vectors containing a short hairpin RNA sequence against DLK. Biopsies were harvested 14 days later. Immunoperoxidase staining showed a reduction of DLK expression. Immunofluorescence staining revealed reduction of filaggrin and transglutaminase 1, two proteins of the cornified envelope. The decrease of DLK expression was associated with cell detachments and the reduction of desmocollin expression, suggesting defects in desmosome assembly. Finally, microarray data indicate that DLK may contribute to the cornified envelope assembly by regulating expression of the Tazarotene Induced Gene 3, a potential inducer of transglutaminase 1. The development of this kind of tissue-engineered model should provide an interesting alternative to animal testing and contribute to a better comprehension of processes involved in human tissues.

V-3-531

Cutaneous wound healing in the EpiDerm-FT™ full-thickness *in vitro* human skin model: Role of serum growth factors

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Cutaneous wound healing involves interactions between dermal fibroblasts and epidermal keratinocytes, as well as cell and extracellular matrix interactions. This poster describes new progress in wound healing experiments conducted with a full-thickness *in vitro* human skin model (EpiDerm-FT™). Normal human epidermal keratinocytes (KC) and dermal fibroblasts (FB) are cultured to produce the highly differentiated full-thickness skin model. Small wounds were induced in the epithelial model by means of a battery operated cauterizer or a dermal biopsy punch. The wounded EpiDerm-FT™ cultures were fixed at various times and H&E stained sections were prepared to evaluate the wound healing process. The effect of human serum (HS) on the rate of wound healing was evaluated over a time course of 9 days. KC migrated over the wounded area to regenerate the epidermis within several days. FB proliferation and dermal matrix repair were also observed. HS produced a concentration dependent increase in the rate of cutaneous healing. Gene expression profiling and immunohistochemical staining of the wounded area showed temporally regulated increases in expression of basement membrane components, collagens and genes involved in extracellular matrix remodeling. Increased FB proliferation in dermal areas directly adjacent to migrating KC was observed. FB proliferation and epidermal healing were severely impaired in the presence of an EGFR tyrosine kinase inhibitor or a TGFα neutralizing antibody. These results demonstrate that EpiDerm-FT™ is a useful animal alternative skin model for investigating dermal-epidermal interactions during wound healing, and for development of new dermal wound healing therapeutics.

Session V-4: Replacement and Reduction in the use of genetically-engineered animals

Session V-4: Oral presentations

V-4-671

Gene supplementation and editing in livestock for biomedical and agricultural applications

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The conservation of gene-function and physiology between people and livestock species advocates for their utility in modeling human disease. Furthermore, agricultural animal production will play a pivotal role in providing high-quality protein to advance human health in the face of a burgeoning global population. Both the development of animal models and the improvement of livestock for food can be greatly facilitated by genetic engineering. Historically, genetic modification has largely been restricted to mice due to technological and logistical challenges. Recent developments have essentially eliminated the technological barriers to livestock genetic modification. Transposons provide an efficient, non-viral method for gene addition in livestock, without the use of an antibiotic resistance gene that could confound regulatory acceptance. The facile use of engineered nucleases to inactivate genes and to stimulate gene conversion in livestock will also be described. Finally, the loci to be engineered, and the species-dependent and geopolitical constraints on implementing genome engineering into animal production paradigms will be discussed.

V-4-109

Optimizing fluorescent protein choice for transgenic embryonic medaka models

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Transgenic medaka (*Oryzias latipes*) have proven to be pertinent models for a variety of ecotoxicological and research applications. Very early stages of aquatic vertebrates, just after hatching of the eggs, using non-feeding embryonic fish, represent an alternative non-compliant with the European regulatory definition of a laboratory animal. However, a number of technical issues needed to be solved, such as limiting the impact of autofluorescence at different embryonic stages, which could inhibit

the read out of biomarkers *in vivo*. To overcome this problem, we have determined the spectrum of emission wavelengths of different developmental stages of medaka submitted to a broad range of excitation wavelengths and various experimental conditions.

For each developmental stage tested, ten medaka embryos were individualized in a 384 well plate and each well was subjected to excitation wavelengths from 350-670 nm in 5 nm increments. For each excitation wavelength, emitted light was quantified from 20 nm above the excitation wavelength to 700 nm in 5 nm increments. The results show a fairly homogenous level of autofluorescence across the spectrum prior to hatching, with almost undetectable levels at 6 days post fertilization. After hatching, higher levels of autofluorescence were observed in specific regions of the spectrum from blue to red (375-700 nm) emission with emission wavelengths close to the excitation wavelength.

This information will allow selection of optimal reporter genetic constructs, providing high signal to noise ratio for the quantification of fluorescence. Furthermore, this will pave the way to combining multiple biomarkers with different fluorescent proteins to detect various signals within the same organism.

V-4-067

The importance of genetic background in mouse and rat models

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It is increasingly recognized that the genetic background (i.e. all genomic sequences other than the gene(s) of interest) can have profound influences on the phenotype of an animal model. It has been shown that mutations (spontaneous and induced), transgenes, and targeted alleles (knock-outs and knock-ins) that are "moved" onto a different background can show a change in phenotype. One of the first cases involved the classical diabetes (Leprdb) mutation that presented transient diabetes on a C57BL/6 background but overt diabetes on C57BLKS. In order to highlight the importance of this issue, I will present a selection of recently published articles showing the influence of genetic background on different mouse and rat models. I will also discuss some of the problems arising from the use of genetically engineered mice, like mixed backgrounds after breeding chimeras, the genetic variability among 129 sub-strains (ES cells), and the "flanking genes" concern. Finally, I will present different ways to avoid or resolve these drawbacks, including the development of congenic strains by marker-assisted backcrossing and the use of newly available ES cells from strains other than 129. In order to stay away from confounding or unreliable experimental results, particularly with the increasing number of mouse and rat strains, attention to the genetic background and genetic monitoring is crucial.

V-4-714

Breeding: a tool to improve genetically engineered mouse welfare

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The use of proper breeding schemes, appropriate genetic background and sound monitoring of mouse colonies contribute positively to reduction and refinement. The presentation gives a résumé of some of the best practice in mouse breeding and strategies to increase animal welfare.

Session V-4: Poster presentations

V-4-525

Reducing and refining animals used in transgenesis with the use of frozen embryos

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A key step in targeted transgenesis line creation is the production of injectable blastocysts for ES cell injections. To make fresh blastocysts available, most model creation labs must set up an in-house colony of breeder males and purchase breeding age females. This requires significant resources, including dedicated space and equipment, training, and time to provide basic animal care duties to the in-house colony. Also, with this approach, there can be variations in the quantity and quality of blastocysts. Even for experienced teams these variations are challenging to control. Additionally, a full colony is usually maintained for the purpose of producing fresh embryos.

Here we are reporting the development of a commercial product to provide frozen morulas, supported with technical recommendations and appropriate culture media (BlastoKit®, Charles River, Lyon, France) that allows reduction of the number of animals during model creation. After embryo thawing and overnight culture, the BlastoKit® allows researchers to produce injectable blastocysts, using less space and animals, with the benefits of standardisation.

Moreover, this technique allows centralization of embryo production and decreases animal needs for the same production level. In particular, only one colony of males is used more efficiently compared to local low-employed colonies. The final number of embryos produced per breeding male is higher. Through superovulation, this system reduces the risk of having to euthanize females with no embryo production and to thaw valuable ES cells clones without blastocysts ready to perform injections. By using frozen embryos, injection technicians can save time and resources and animals needed in embryo production. Also, by knowing in advance the number of injectable embryos, the transgenic facility team can prepare pseudopregnant females as recipients only when needed, and with a refined number, which again reduces unused female production.

Currently, germ line transmission using BlastoKit®-derived embryos is validated by a range of users in private and academic laboratories for both C57BL/6NCrl and BALB/cAnNCrl embryos. Along with highlighting the reduction of animals used, analysis of blastocyst development rate (at least 70% and 50% for C57BL/6NCrl and BALB/cAnNCrl embryos, respectively), microinjection efficiency, and birth rates (up to 40% for C57BL/6NCrl embryos) obtained during beta-testing will be presented and analysed at this conference.

V-4-587

Reduction of transgenic animal use by simultaneous assessment of *lacZ* and *Pig-a* mutations, micronuclei and DNA adducts in Muta™Mouse

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This study used the transgenic Muta™ Mouse to show that multiple genotoxicity endpoints can be measured in the same animals, thus reducing the use of genetically engineered animals. Muta™ Mouse were exposed to benzo[a]pyrene (BaP) daily for 28 days via oral gavage. Endpoints measured include DNA adducts, indicative of internal dose, micronuclei (MN), indicative of chromosome damage, and gene mutations. Mutations were measured at the lacZ transgene in several tissues, and at the *Pig-a* gene, evaluated as GPI-anchor deficient reticulocytes (RETs) and red blood cells (RBCs).

Dose-dependent increases in DNA adducts were observed in all tissues examined (liver > glandular stomach > small intestine > bone marrow). Dose-related increases in *lacZ* and *Pig-a* mutant frequency (MF) were also observed; doubling dose was the same for these two endpoints; however the *lacZ* MF in bone marrow was approximately 25x higher than that observed for *Pig-a* in RETs. This difference may be related to differences in target size and/or differences in the cell populations examined. Dose-related increases in % MN were also observed, and the doubling dose for % MN was approximately 2.5x higher than the mutation endpoints.

The results of this study demonstrate that measurements of mutation, chromosome damage and internal dose can easily be integrated into a 28-day mouse study. Matching *in vitro* analyses are currently comparing the kinetics observed here to those of cultured cells derived from Muta™ Mouse. The results will contribute to the use of cultured transgenic cells for quantitative hazard assessment, and to the validation of the *Pig-a* assay.

Session V-5: Developments in stem cell research as the basis for sustainable availability of differentiated human cells and tissues

Session V-5: Oral presentations

V-5-422

Focus on stem cells as sources of human target cells for *in vitro* research and testing

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Our knowledge with respect to stem cell technology has been growing steadily. This is not surprising as human stem cells could play a major role in regenerative medicine, offering hope for human cells for disease research and therapy. Sources of stem cells consist not only of embryonic pluripotent cells, but also of multipotent adult stem cells derived from a variety of organs. For clinical purposes, most interest goes to induced pluripotent stem cells, which can be converted into desired cell types by directed differentiation and reprogramming. This huge clinical interest is also a boost for other fields including drug development, *in vitro* modeling and pharmaco-toxicology. Indeed, human stem cell-derived target cells stimulate the introduction of functional *in vitro* models relevant for the human situation. They could be applied in research and in regulatory testing. Of particular interest is the generation of human hepatocytes. They are responsible for major liver functions, including phase I and phase II drug metabolism. The latter often is at the origin of drug activation, making the liver a toxicity target. Unlimited access to human hepatocytes could generate *in vitro* models to study liver function, drug metabolism and the different mechanisms underlying drug induced liver injury, being a major cause of drug attrition. They are important in drug development in general, for screening purposes and in particular in the risk assessment of new biological entities for which human-derived models are crucial.

V-5-702

Dermal stem cells: An accessible multipotent precursor with potential application for drug screening and therapeutics

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Stem cell-based therapies hold great promise for repair and functional restoration following tissue injury and disease. Skin-derived precursors (or "SKPs") are a novel, multipotent somatic stem cell that resides within the mammalian dermis. SKPs persist within the skin throughout adulthood and yet intriguingly, exhibit many similarities to embryonic neural crest stem cells (NCSCs). For example, SKPs give rise to both neural and mesodermal cell types, exhibit similar gene expression profiles and the former appear biased to peripheral nervous system fates. Here I will summarize our current understanding of the biological origin of SKPs and specifically the potential therapeutic utility of SKPs as a highly accessible, autologous and renewable source of neural crest-like precursors.

V-5-429

A stem cell based test battery to detect developmental neurotoxicants

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In order to identify compound effects on different steps and mechanisms involved in neurodevelopment, we designed a test battery consisting of different model systems. In addition we performed a literature search to assemble a list of relevant test compounds of known toxicity, developmental neurotoxicity or proven absence of toxicity. The following assay systems were used: (1) Murine embryonic stem cells (mESC) were converted into terminally differentiated neurons. (2) Conditionally-immortalized human neural precursor cells (LUHMES) were differentiated to a homogeneous population with a complex neurite network and typical biochemical and morphological features of dopaminergic neurons. (3) Human embryonic stem cells differentiated to central nervous system neural precursors or to peripheral nervous system neural crest stem cells.

We used different readouts for compound effects, all measured in a concentration range not causing direct cytotoxicity. Transcript levels were used to characterize disturbed differentiation patterns on the basis of up to 80 endpoints. In addition, functional endpoints were chosen to reflect neurite outgrowth capacity, cell migration and the turnover of neurotransmitters. For instance methylmercury affected neuronal maturation and neurotransmitter uptake at much lower concentrations than required for neurite toxicity. It also affected the migration of stem cells at concentrations lower than those required to inhibit proliferation. This approach allowed profiling of different compounds. A new class investigated was that of epigenetic modifiers. The human cell systems were extensively characterized for chromatin modifying factors, and for instance histone deacetylase inhibitors affected the differentiation pattern towards neuronal precursors.

V-5-410

Reconsidering pluripotency tests: Do we still need teratomas?

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The production of teratomas in immunodeficient mice is regarded as the "gold standard" assay for pluripotency. This assay has been used to demonstrate the pluripotency of embryonic stem cells, induced pluripotent stem cells (iPSC) and other pluripotent cells. However, the teratoma assay raises two main issues concerning animal welfare: (i) the inoculation of animals with potentially malignant tumors, and (ii) the breeding of genetically deficient experimental animals. Both of these issues are associated with suffering of the animals. To explore pluripotency testing in the context of the 3Rs, the German Foundation for the Promotion of Alternatives to Animal Testing (*Stiftung zur Förderung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen, set*) organized an expert workshop in December 2010. This presentation will demonstrate the results of this workshop. Several alternatives to the mouse teratoma assay were discussed, including the directed differentiation of ES and iPS cells into organotypic cells, expression of pluripotency-associated markers such as TRA-1-60, DNMT3B, REX1 that correlate well with the teratoma forming potential of ES and iPS cells, epigenomic footprints, such as DNA methylation, and histone modifications. Each of these assays is capable of addressing one or several aspects of pluripotency. It is imperative that more research be performed in order to standardize such alternative tests. Simple, robust, reliable, standardised tests need to be developed to facilitate the testing of pluripotency of new and existing cell lines.

V-5-184

Characterization of developmental changes and electrophysiological functions in contracting cardiomyocytes derived from human iPS cells

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Cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs) promise to become a useful *in vitro* tool for assessing cardiotoxicity, including QT prolongation, and to contribute to a reduction in animal use in drug discovery. Thus, clarifying their functional properties and the developmental changes in their transcriptional expression pattern is very important for confirming their suitability for safety assessment of drug candidates. In this study, we characterized the properties of contracting hiPS-CMs and assessed their functionality using gene expression analysis and whole-cell patch-clamp recordings. We used the human induced pluripotent stem cell line 201B7 (Takahashi et al., 2007) and the embryoid body method for differentiation. Beating colonies appeared around day 8 of differentiation. In the contracting areas at the later stage (day 38), mRNA expressions of cardiac-related ion channels and markers were detected. After contraction started, expression levels of some ion channel mRNAs were upregulated gradually according to the time in culture. These changes were in agreement with those of cardiomyocytes from human embryonic stem cells and suggest that beating hiPSC-CMs mature progressively. Pharmacological responses of major cardiac ion currents and action potential duration to each known ion channel blocker in hiPS-CMs at the later stage were similar to those in human cardiomyocytes. These results reveal the functional suitability of hiPS-CMs for QT risk assessment and indicate that the developmental profiles of their ion channel mRNA expressions reflect appropriate differentiation and maturation of hiPS-CMs for safety assessment of drug candidates.

Reference

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V-5-703

Skin-derived precursor cells – a promising source for hepatic progeny

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Withdrawal of promising drug candidates is often triggered by the detection of hepatotoxicity in (pre)clinical studies. The availability of reliable *in vitro* screening models capable of detecting hepatotoxicity and in particular chronic liver toxicity in an early stage of the drug development process is thus of utmost importance for the pharmaceutical industry. Today, however, most existing liver-based models suffer from phenotypic instability and are rodent-derived, making them not fully representative of the human situation. The development of a model based on easily obtainable human adult stem cells could overcome this problem. A promising stem cell source is the human skin-derived precursor cell (hSKP), a multipotent neural-crest related precursor capable of generating neuronal, glial and mesodermal progeny.

In the present study, we show that these cells are able to undergo endodermal differentiation. More specifically, upon sequential and gradual exposure to hepatogenic factors, hSKP differentiate into immature hepatocyte-like cells expressing foetal and mature hepatic markers in a time-dependent manner, reflecting the hepatogenesis *in vivo*. Upon intrasplenic transplantation into the $uPA^{+/+}$-SCID mouse model, hSKP-derived hepatic cells are able to migrate to, engraft and survive in the diseased mouse liver for more than 10 weeks. Furthermore, hSKP-derived hepatic cells are able to immunomodulate the immune response both in the presence and absence of inflammatory conditions, indicating their high potential as a promising source for *in vivo* transplantation as both a hepatocyte-like or supportive cell type. To conclude we can say that hSKP are a promising multipotent stem cell source to generate hepatic progeny for both *in vitro* and *in vivo* applications.

Session V-5: Poster presentations

V-5-181

Comparison of three kinds of ES cells using two and three-dimensional culture systems

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In 1997, Spielmann et al. developed the embryonic stem cell test (EST), which is an *in vitro* embryotoxicity test protocol that can be used to estimate the risk of embryotoxicity of chemical substances quickly compared to the conventional methods that involve animal experiments. The EST has been evaluated in a validation study funded by ECVAM, in which two other *in vitro* embryotoxicity tests (micromass test, whole embryo culture test) were validated against a set of test chemicals characterized by high levels of *in vivo* embryotoxicity in laboratory animals and humans.

In the EST protocol, mouse ES-D3 cells have been listed as the only ones available. If other kinds of ES cells become available, the experimental application will be of greater usefulness. We conducted a comparison between non-feeder ES-D3 cells and EL M3 cells or ES-R1-EGFP B2/EGFP cells requiring feeder cells, to explore the experimental possibility of using ES cells requiring feeder cells.

As the present results with ES-D3 and EL M3 cells were similar to those obtained under the two-dimensional condition, these two kinds of cell are thought to be equally available under the present three-dimensional conditions. On the other hand, because ES-R1-EGFP B2/EGFP cells did not show any pulsation at all in the three-dimensional culture, other experimental conditions for the three-dimensional culture method need to be established with those cells. In addition, it was suggested that similar results could be obtained with EL M3 cells requiring feeder cells for cultivation compared to those with ES-D3 cells.

V-5-318

Egg yolk extract – a novel alternative to Fetal Bovine Serum in goat stem cell culture media

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Fetal bovine serum (FBS) is a regular component of animal cell culture media. FBS is harvested from the fetuses of pregnant cows during slaughter. Being alive at the time of blood collection, these fetuses are exposed to severe pain and stress. The amount of FBS produced for the world market is approximately 500,000 litres per year. For this, more than 1,000,000 bovine fetuses are harvested and this number is steadily increasing. Scientific and ethical concerns exist regarding the use of FBS in *in vitro* cell culture. Stem cell research is a fast developing area in life science. Across the world several studies are in progress to find a suitable medium which can maintain the immortality of stem cells. Therefore, the present study was conducted to exploit the potential of Egg Yolk Extract (EYE) as a possible alternative to FBS in goat fetal stem cell culture media. EYE was prepared from fresh unfertilized chicken eggs after proper processing. Goat fetal stem cells were isolated and cultured *in vitro* adopting standard procedure. EYE was used at a concentration of 4% in a standard stem cell culture medium vs. control medium with 10% FBS. In the EYE supplemented media, fetal stem cells had normal growth and multiplication and produced maximum number of clones in subsequent cultures. These clones were later characterized with stem cell specific markers, i.e. Oct4, Nanog and Sox2. The study proved that EYE can be used as an efficient alternative to FBS for culturing goat stem cells.

V-5-477

A human neuronal cell line for the substitution of transgenic neurodegeneration models

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Research in the field of neurodegeneration such as Parkinson's Disease (PD) or Alzheimer's Disease (AD) requires the selective manipulation of genes of interest. Transgenic mouse models of PD-related genes (alpha-synuclein, tyrosine hydroxylase, DJ-1), or AD-related genes (APP, BACE1, gamma-secretase), were successfully generated in recent years. Apart from the labor- and cost-intensive procedures, both in the generation and application of these *in vivo* models, the transgenic mouse approach has led to a significant increase in the number of animal experiments.

We herein introduce the conditionally immortalized human neuronal cell line LUHMES as a new *in vitro* alternative to currently established primary cell, cell line, or *in vivo* models. We have established different protocols that allow differentiation into a dopaminergic phenotype for PD studies, as well as for a dopamine-independent neuronal phenotype for AD research. LUHMES in combination with PD-toxins (MPP+ or methamphetamine), resembles key features observed in the PD brain, including oxidative, proteasomal, and metabolomic stress. Alternatively, the cells can directly be applied as model for AD, as they endogenously express amyloid precursor protein (APP) and the proteolytic machinery for the formation of amyloid beta (A-beta) peptide.

We have furthermore established a lentiviral system for the overexpression of alpha-synuclein, tyrosine hydroxylase, as well as APP and BACE1 in LUHMES, knock-down of all these targets is achieved by siRNA. The LUHMES model hence represents a robust and highly reproducible *in vitro* model on the basis of human neuronal cells that can also be applied in high throughput screening programs.

V-5-514

Generation of post-mitotic neurons from the human LUHMES cell line

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We characterized phenotype and function of a fetal human mesencephalic cell line (LUHMES) as a neuronal model system. Neurodevelopmental profiling of the proliferating stage (d0) of these conditionally-immortalized cells revealed neuronal features, expressed simultaneously with some early neuroblast and stem cell markers. An optimized 2-step differentiation procedure, triggered by shut-down of the myc transgene, resulted in uniformly post-mitotic neurons within 5 days (d5). This was associated with downregulation of some precursor markers and further upregulation of neuronal genes. Neurite network formation involved the outgrowth of 1-2, often >500 μm long projections. They showed dynamic growth cone behavior, as evidenced by time-lapse imaging of stably GFP-overexpressing cells, and grew neurites. This extension was specifically inhibited by a set of tool compounds in a concentration-dependent manner. Voltage-dependent sodium channels and spontaneous electrical activity of LUHMES continuously increased from d0 to d11, while levels of synaptic markers reached their maximum on d5. The developmental expression patterns of most genes and of the dopamine uptake- and release-machinery appeared to be intrinsically predetermined, as the differentiation proceeded similarly when external factors such as dibutyryl-cAMP (cAMP) and GDNF were omitted. Only tyrosine hydroxylase required the continuous presence of cAMP. In conclusion, LUHMES are a robust neuronal model with adaptable phenotype and high value for neurodevelopmental studies, disease modeling and neuropharmacology.

Session V-6: Animal reduction through the better use of mechanistically-based translational animal disease models

Session V-6: Oral presentations

V-6-654

Computational models for predicting human toxicities

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Predicting the potential for human toxicity from a molecule structure is feasible by learning from large numbers of known compounds or understanding structure activity relationships for proteins involved in toxicity. Several examples will be presented including recent models for drug-induced liver injury (DILI), which is one of the most important reasons for drug development failure at both pre-approval and post-approval stages. In addition, the results of combined *in vitro-in silico* studies have suggested new, structurally diverse inhibitors for human transporters that may possibly cause clinically significant toxicities such as rhabdomyolysis. The versatility and potential of using such models in drug discovery may be illustrated by increasing the efficiency of screening and rapid identification of potential interactions for FDA approved drugs or new molecular entities. This may lead to insights into potential off-target effects of molecules as well potential repurposing uses.

V-6-406

A model for inter-institutional 3Rs co-operation – sharing *in vivo* research resources

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With the broad goal of reducing and replacing research animal use where possible, MedImmune entered into an inter-institutional agreement to make ferret trachea tissue available to a neighboring academic institution so as to enable them to carry out vital *in vitro* research work without having to obtain additional live animals. Over the course of 3 years, MedImmune provided the Cystic Fibrosis Research Library with over 200 ferret tracheas which enabled them to work, without further use of live animals, in a major area of unmet medical need. Key to this successful effort were communication within the laboratory animal science community and more rare but essential: the institutional recognition of the 3Rs in ethical research beyond one's own walls and the willingness to engage in a collaborative process to reduce and refine animal use where possible.

Cystic Fibrosis (CF) is a severe genetic disease that affects several organ systems in people but most destructively the pulmonary system. In CF, tracheal mucous production is abnormal, leading to inadequate removal of airway microbes. Repeated pulmonary infections then result from usually innocuous bacteria getting into the lungs. Severe scarring causes increasing impairment of respiratory capacity and often early death. Victims often succumb as children or young adults. In order to understand and then treat afflicted patients, normal and abnormal tracheal mucous production needs to be studied which cannot readily be done in people. Ferrets and pigs most closely mimic the human respiratory tract. The CF laboratory developed a system to study mucous production in the excised trachea of the ferret.

The IACUC at both institutions supported developing the tissue sharing agreement and the legal departments also engaged in the process to successfully pave the way for the tissue transfer. To date, one study has been published with others in preparation.

V-6-295

The Three Rs and the one P: Predictability

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Recent technological advances and broad initiatives to improve the predictability of animal models can significantly reduce the number of animals used in biomedical research. Factors which have historically influenced animal model development and use, such as technological advances, discoveries within a vast diversity of species, medical initiatives, and evolving public opinion, will be discussed. This will set the context for discussing how recent shifts in medical approaches toward personalized health care and technological advances will likely influence preclinical biomedical research in the future.

An example will be presented from a strategy used to compare gene profiles from healthy and diseased human populations with those of *in vivo* models in order to increase the confidence in, and predictability of, preclinical studies. Additionally, advances in molecular imaging technologies have enabled us to obtain more contextual, mechanistic information than previously possible. As a case in point, continued advancement in this area may result in the increased applications of orthotopic mouse tumor models which are believed to be more relevant to cancer in patients than the traditional subcutaneous tumor models. In short, as these approaches are applied to more closely align molecular/genetic profiles of healthy and diseased people and animal models, preclinical research efficiency will likely increase and ultimately have a positive impact on the 3Rs.

V-6-711

Development of a flow chamber test to replace animal research on arterial thrombosis and bleeding *in vivo*

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Arterial thrombosis is a main cause of death in the western world. At present, antithrombotic treatment is only partially effective, due to incomplete knowledge of the molecular determinants of this disease. Arterial thrombus formation occurs by increased activation of blood platelets and the coagulation system. On the other hand, insufficient activity of these processes leads to bleeding. Genetic approaches, where platelet or coagulant proteins are knocked down in mice, are of indispensible value in the finding of new molecular targets. However, current tests of thrombus formation and bleeding are carried out with anesthetized, living animals and are therefore cumbersome. We have developed and miniaturized flow chamber technology as an alternative for this *in vivo* testing. Herein, we perfuse blood under arterial shear rate over a spotted array of purified vessel wall proteins, and measure the buildup of a thrombus at each spot with microscopy. Application of this *in vitro* test with mouse blood shows that it is sensitive to the expression of >50 proteins (platelet signaling proteins, transcription factors and coagulation factors). Furthermore, pharmacological inhibitors and antithrombotic drugs, active *in vivo*, also suppress thrombus formation of human blood *in vitro*. This technique is now being used in many other laboratories. For the area of atherosclerosis, thrombosis and haemostasis, it provides a novel way for reduction, refinement and replacement of animal use in experimental research.

Session V-6: Poster presentations

V-6-074

Comparative study of propensity for amyloidogenesis in male and female mice

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Reactive amyloidosis is a condition that complicates a long list of chronic inflammation, chronic infectious, malignant, and hereditary disorders. In the present study, the propensity for amyloidogenesis in male and female mice on spatio-temporal pattern was evaluated. For this purpose a total of 40 male and female Swiss mice after being weighed were randomly divided into 2 treatment groups including 2 groups [10 male (Group A1) and 10 female (Group B1) each], and 2 control groups [10 male (Group A2) and 10 female (Group B2) each]. Chemical compounds included vitamin-free casein as an amyloid inducer. For induction of amyloidosis the following protocol was met: Group A1 and B1: subcutaneous injection of 0.5 ml of 12% vitamin-free casein per day, 5 days per week. Group A2 and B2: subcutaneous injection of 0.5 ml saline per day, 5 days per week. At the end of the 3rd, 5th and 7th week of the experiment three mice were randomly selected from each group and were subjected to necropsy. Liver, lung, kidney and heart samples of each animal were obtained and embedded in paraffin blocks and stained by alkaline Congo red techniques. A green birefringence under the polarized microscope was considered to be a positive criterion for the presence of amyloid, indicating development of amyloidosis. Amyloidosis scale was assigned from 0-3. The data obtained from microscopic quantitative evaluation did show significant differences between groups A1 and B1. A preferential expression of reactive amyloidosis is concluded in male, indicating sex differences in amyloidosis. The maximum and minimum amyloid density/deposition was observed in the lung and the heart of male mice, respectively.

V-6-118

Hydra as an alternative model system to study the role of dysfunctional receptor tyrosine kinases in toxicity and disease

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Receptor Tyrosine Kinases (RTKs) are key components of cell-cell signalling required to regulate cell proliferation, differentiation and apoptosis, making them potential proto-oncogenes. Mutations in RTKs have been linked to many human congenital syndromes and diseases. Functional specificity of RTKs is attained by activation of the tyrosine kinase domain following cognate ligand binding. Hydra, being a simple organism with tissue level organization and sophisticated molecular pathways, provides a powerful alternative model system to study the roles of RTKs in induction of toxicity and initiation and progression of diseases. We have carried out genome-wide screening in hydra for RTKs. The domain-based screening using Hidden Markov Models (HMMs) for RTKs in Genomescan predicted gene models of *Hydra magnipapillata* genome resulted in identification of 15 RTKs. Only 5 of these RTKs have been previously reported and a few of these have been partially characterized. These RTKs have been classified into 8 families based on domain architecture and homology. We have identified most of the RTK family members, including DDR, Eph, FGFR, IR, MuSK, PTK7, Ror and RyK in hydra. Identification of these RTKs and presence of cell types comparable to humans (musculo-epithelial cells, nerve cells and stem cells) potentially make hydra a powerful alternate model organism for the study of various aspects of RTK-related toxicity and disease promotion.

V-6-131

The justification of animal numbers – The role of sample size, precision and power analysis in assigning and justifying animal numbers

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The US Animal Welfare Act (1966, 1985) empowered every institution using animal models for research and development to have their study protocols and related support policies reviewed by its Institutional Animal Care and Use Committee (IACUC). Recently, the USDA has begun to look more closely at the justification and assignment of animal numbers, and more generally, at identifying reduction alternatives to the related study practices.

To meet immediate and potential compliance concerns, as well as those related to basic study design and animal sample size, many research investigators have turned to biostatisticians for statistical support and justification of the animal numbers used in their studies. One response has been to develop web based sample size and power analysis software tools to assist in writing the Statistics Section of the IACUC Protocol. These tools use software previously employed by the biostatistician for routine power analysis for studies using a t-test, analysis of variance, or multiple treatment comparison for their data analysis, in addition to generating a report.

An immediate consequence has been to define an empirical statistical standard for the support of animal numbers. In particular, we can now improve control over the risk of using too few animals, where variation can hide potential activity of the research drug. Similarly, we can control the risk of using too many animals, thus avoiding the unnecessary pain and distress of extra animals. Either case supports real or potential animal reduction.

V-6-159

Improved design of animal experiments

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Animal experiments are an important phase in the development of new therapeutic agents against diseases. Tests to investigate efficacy of new therapeutic agents typically have a fixed design with two control groups (diseased without treatment and healthy without treatment), and three treatment groups (low, middle and high dose of the experimental treatment).

It is common practice to randomize the animals over equally sized groups. However, since not all comparisons between groups are of equal importance to answer the research question, the choice for equal group sizes is often not the most efficient one. The optimal experimental design provides maximum information with minimum sample size. In statistical literature, optimal experimental design has been described before. However, a set of useful guidelines and tools to aid investigators in efficacy research to optimally design their own experiments is lacking. Literature addressing this issue is sparse, technical in nature and unknown.

In an example, we highlight how optimizing experimental design will lead to an increase in experimental power with the use of fewer animals. The aim of this project is to develop a set of guidelines and tools to aid investigators in efficacy research to optimally design their own experiments, potentially leading to a 10-15% decrease in the number of animals needed.

V-6-208

Reduction through statistical tools and design – impact & implementation experiences

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The Pre-Clinical Statistics Group at AstraZeneca has achieved considerable success in the area of animal reduction through close collaboration with scientists in Discovery based in UK, Sweden and USA.

A number of statistical tools and techniques are now advanced in their application (including power analysis, factorial experimental design – http://dels-old.nas.edu/ilar_n/ilarjournal/43_4/v4304Shaw.shtml, sequential design and quality monitoring) and have been providing significant impact in overall reduction of animals, and in better decision making based on available animal data. As one example, factorial experimental design provides a set of tools for rapid learning about which factors are important for an animal model. It is possible to use significantly fewer animals in comparison to more conventional approaches. A variety of methods have been used, including web-based tool delivery, scientist training and empowerment and statistical consultancy. A key element for successful implementation of these important techniques is to understand what aspects the scientist can take ownership for and what aspects require consultation with a statistician. The group has developed working processes and practices which have proved to be highly effective and have also developed strong inter-personal skills to build relationships with customers to bridge the gap between statistics and biology. The focus of the presentation will be to introduce some of these statistical concepts in a user-friendly way and to demonstrate how the techniques can be applied effectively, including quantifiable impact on animal reduction, with real examples from AstraZeneca R&D.

V-6-235

In vivo predictive efficacy

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Animal experiments are used to make decisions about which new compounds should be progressed to clinical development and ultimately used for patients. But how do we know whether a positive signal registered in an animal model will also yield a positive response in a diseased human? What are the criteria associated with a particular animal model that provide confidence in its utility as a tool for making progression decisions? More importantly, which models are we currently using for this purpose which are misleading and could be eliminated, reducing the use of animals for irrelevant work and reducing the risk (and cost) of clinical development based on a false premise? The answers to these questions are fundamental to the use of animals in research but often are not clearly understood. This work aims to understand the predictive confidence associated with models used in cardiovascular disease by comparing both clinical and pre-clinical data and establishing a set of criteria that relate to animal model performance. These criteria can then be used to consider the likely predictive utility of models for which there is currently no clinical precedent and, if required, either modify them to yield a high level of confidence or replace them with more appropriate alternatives. In this poster we present the criteria, in the form of a questionnaire, which we have used to begin to explore this area and present some preliminary data from a selection of models used in the cardiovascular disease area.

V-6-266

Evaluation of dried blood spotting – dog study

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Microsampling has been under investigation in AstraZeneca for a number of years focusing on a technique of accurate blood collection in a microtube for analysis after solvent extraction. The interest in dried blood spots (DBS) was seen as an alternative way forward in microsampling and was evaluated in a study on a compound that showed a potential paediatric indication. In this study a blood microtube method was compared to DBS with additional data collected from the conventional plasma analysis. A further study was undertaken to investigate the effect on PK by sampling from the jugular vein compared to a peripheral sample collected from the ear. The compound was validated over the range 20 to 1000 ng/ml in plasma and DBS for a 1 month toxicity study. The data for the tox study was shown to be equivalent. Correlation plots derived from the sample data combining the plasma and DBS results gave R2 values of 0.92 and 0.96 (x=y plot) for the day 1 and 28 data respectively. For the sampling site comparison study two dose levels were investigated and samples collected on DBS and as a wet blood sample in microtubes. The comparative data for the sampling sites showed good correlation with an R2 value for the plot of 0.99 (x=y plot), indicating equivalence for the two data sets across the dose ranges. No lag time was observed with a T_{max} of 6 h for both the peripheral and venous samples. The final part of the evaluation was the comparison of the wet sample and the DBS dry sample.

V-6-275

Improvements in behavioural pharmacology study design saves animal lives and cost, whilst enhancing quality of pre-clinical data

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Applying pharmacokinetic-pharmacodynamic (PKPD) modeling principles pre-clinically is essential in understanding how a drug's systemic exposure relates to the magnitude and time profile of its pharmacodynamic response in an animal model. The ultimate objective is to predict effect and duration of effect in man, and help simulate and guide design of clinical studies. Pre-clinical behavioural studies in analgesia consist of at least three different studies; efficacy (dose response), effect-duration, and tolerance development. In such studies, different sets of animals are normally used to collect PK and PD data. Historically, PKPD modeling would be applied to these existing data sets, but the precision in describing PKPD relationships is reduced since group mean values are used instead of individual animal values. The aim of this study was to improve the design of PKPD studies, more specifically, investigate if PK and PD data can be taken from the same animal.

AZ100 was orally administered daily for 5 days and tested 1, 2, 4, 7, and 24 h later on day 1, 3, and 5. PK measurements were done on day 2, and 4. Spinal Nerve Ligation pain model induced tight ligation of the L5 and L6 spinal nerve in the rat. Heat hyperalgesia was assessed using the plantar test.

PK and PD data collection from the same animals but taken on alternate days did not produce undesired stress in rats. AZ100 produced a dose- and time-dependent reduction in heat hyperalgesia. This effect is related to the plasma concentration and tolerance is not developed following repeated dosing.

This design study has proven to be very efficient; it allows for the simultaneous evaluation of effect-duration, tolerance development, and PKPD relationship, and as a result, saves time/money, and animal lives (60% reduction).

V-6-283

A bibliography on the care and use of zebrafish

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In 2010, the USDA's Animal Welfare Information Center, released a comprehensive, on-line bibliography on the use of zebrafish (Danio rerio) in multiple areas of research. This versatile vertebrate provides an alternative model organism for numerous research studies of human disease and the study of biochemistry, genetics, development, embryology, cancer, toxicology, pharmacology, physiology, hematology, ecology, cardiology and many other research areas. The publication contains more than 3,600 recent abstracts that also cover husbandry and other aspects of welfare for the fish. The transparent body of the zebrafish makes it a prime candidate for "time lapse" photography, which is very useful in the study of organ development. A recent breed of *Danio rerio* enhances the transparency to improve the study of melanomas. The fish was first recognized as a valuable, inexpensive, vertebrate research model in the 1970s when a small number of laboratories were using it in research. It can now be found as the animal of choice in 1000's of research projects worldwide. As many projects transition from warm blooded animals to the zebrafish, this bibliography will provide an excellent resource for the numerous scientific disciplines it covers and the various methods used for those experiments. Many of the records include the National Agricultural Library call numbers, but all can be easily located as the full text document with the other journal information provided.

V-6-297

Single Site Laparoscopy (SSL) abdominal access simulator – development and validation

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Design verification and validation studies for laparoscopic access systems and devices are typically performed in the porcine model. Data collected from this process is variable, subjective, expensive and limited to specialized facilities. This simulator was developed and designed with multiple objectives in mind: 1) reducing and replacing the need for live animals in the laparoscopic access new product development process, 2) creating a highly repeatable and reproducible comparative testing capability, 3) allowing market research and customer visits to occur in locations that do not allow animal testing and research and 4) ensuring a compact and portable solution was created to extend the ability to use the simulator through the sales force training and global product launch phases.

Simulator design and function was collaboratively developed through a partnership with Industrial Design, Research & Development and Preclinical Affairs. Critical steps of key laparoscopic surgical procedures were analyzed and transformed into a mechanical activity board, which was enclosed in a custom case representing an insufflated adult abdomen. The exterior was a selectable-thickness (up to 8 cm) material that was representative of the anterior body wall, subcutaneous fat and fascia. The simulator could be entered and used with any commercially available laparoscopic access devices and visualization systems.

Resource estimates and model calculations show that more than 380 large animals were not used because the SSL simulator was developed and validated. For the same amount of data generated through the use of the SSL simulator, the business impact in terms of cost savings by not using live animals is estimated conservatively in excess of $ 1.1 million dollars for this single development project. Validation of simulator design and function was performed with laparoscopic surgeons who were asked to evaluate the SSL simulator on a scale of 1-5, with 5 being "excellent".

The SSL Abdominal Access Simulator delivered on all objectives – exceptional business results were and are being obtained while striving towards higher standards in the ethical use of animals in biomedical research.

V-6-399

New use of an old animal model (wound healing in the ears of rabbits) to significantly reduce the total number of animals utilized in medical device product development

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We are developing products to address obstructive sleep apnea and for facial reconstruction. At issue is how to evaluate and screen prototype products while minimizing animal usage. Our first product in this area gained regulatory approval, but clearly demonstrated the need for a better animal model (single implant in the nasal septum of weanling rabbits). No validated *in vitro* assay or computer-model could address the questions we must answer. The intended applications dictate the need for *in vivo* product evaluation with the product located adjacent to cartilage. A single rabbit had four products implanted along the cartilage of each ear. This simple model was more than adequate for product evaluation. More importantly, we discovered we could easily evaluate the product in real-time for the first time. We "watched" dye diffuse from the implanted products, "watched" the products absorb, and could macroscopically evaluate the tissue response to the implanted products. We decreased our animal usage by 8-fold for our sleep apnea and facial reconstruction product development and have used the model elsewhere (e.g. product performance evaluation) to further reduce our animal use. The potential for this model to further reduce our overall animal use is significant. For example, *in vitro* degradation studies for many polymers are not acceptable to regulatory agencies; therefore, we generate such information using multiple study intervals. With this model, we can generate the absorption (and other) information in real-time with 1 to 2 animals. With time, this model could replace some definitive large animal studies and, hopefully, replace some of the established animal models required by regulatory agencies.

V-6-445

Mechanical characterizations of traumatic brain injury tests on mice using computer models

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Currently, *in vivo* traumatic brain injury (TBI) animal experiments are commonly used to provide insight into brain injury mechanisms as well as to test candidate therapeutic interventions. For example, controlled cortical impact (CCI) is largely used to induce brain contusions. However, different laboratories use different CCI parameters, making comparison of experimental findings very difficult. Furthermore, many animals are sacrificed during preliminary trial-and-error stages in an attempt to create the desired brain contusion severity. In this study, a computer mouse brain model was developed using advanced multi-block techniques. The mouse intracranial responses under various CCI scenarios were calculated computationally and compared favorably with published experimental results. Such computer-predicted intracranial mechanics could minimize the use of mice from unnecessary trials, and also serve as a general platform for comparison and analysis of published results from different laboratories. The developed blocking system can be further used to generate high-quality computer brain models for different transgenic mice with varying intracranial geometries. Additionally, computational analysis could be conducted before other types of experiment, such as fluid percussion and weight drop, to provide clear descriptions of regional brain injury intensity. Ultimately, incorporating current computer models (with around 100 micron spatial resolution) with molecular/cellular level modeling will allow TBI experiments to be performed entirely on computers. In the meantime, it is important to increase awareness and acceptance of such technologies so that fewer animals are sacrificed in TBI experiments.

V-6-491

Deployment of the Vitrocell system for *in vitro* toxicity assessment of aerosols and vehicular emissions at an air-liquid interface

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The Vitrocell system is an exposure device that mimics the *in vivo* conditions of the lung by allowing *in vitro* exposure of cells at an air-liquid interface. *In vitro* toxicity assessment of gaseous substances is problematic due to the fact that cultured cells need to be suspended in liquid medium or adhered to a surface that is completely covered by liquid medium. Therefore, the test article (e.g., aerosol) must be bubbled through or suspended in the medium, with neither option providing a realistic exposure scenario. Thus, the use of animal studies has been a preferred practice. The Vitrocell offers a practical alternative, in which cultured cells are grown on porous membranes and exposed, in real-time, under highly controlled experimental conditions. This has the potential for a reduction in animal-based research.

Human adenocarcinoma epithelial cell line A549 was used in all exposures. Cells were exposed to NO_2 (5 ppm and 20 ppm) and dilute diesel exhaust (1:8), as well as synthetic air controls, at a previously established flow rate of 8.3 ml/min for one hour. Cell viability was determined by examining cleavage of the tetrazolium salt WST-1 (metabolic activity) and relative ATP activity (ATPlite luciferase). All results were compared to incubator controls. Cell viability was observed to decrease with increasing concentrations of NO_2. Cells exposed to 20 ppm showed 56.1% ±9.2% less metabolic activity and cells exposed to 5 ppm exhibited a 52.7% ±7.6% reduction in metabolic activity. ATP activity was observed to be 57.0% ±5.1% less in 5 ppm NO_2 exposed cells, and 65.4% ±12.7% less in 20 ppm NO_2 exposed cells. Cells exposed to synthetic clean air were slightly less viable than incubator control (24.7% ±14.3%). Cells exposed to dilute diesel exhaust showed a significant reduction of 42.6% ±3.4% in ATP activity compared to incubator controls.

These results confirm that the Vitrocell exposure device with A549 cells is suitable for toxicity assessment of gaseous substances. Future work will include cytotoxic and genotoxic assessment of further engine exhaust exposures, representing a host of engine design, fuel formulation and after-treatment conditions. The use of primary human airway epithelial cells will be employed to assess inter-individual responses to vehicular emissions.

V-6-554

Advancing technology and the 3Rs: use of cross-over study design for pharmacological assessment in rats

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Innovation leading to development, qualification, and implementation of new technologies produces Refinements in data quality, Reductions in animal use, and better de-risking of pharmaceutical efficacy & safety. We developed a chronic cannulated rat model wherein cross-over study design is used to evaluate 2 or more compounds at weekly intervals in one cohort. Han Wistar rats (n=16) were obtained from the vendor, surgically implanted with jugular and femoral venous cannulas, for dosing and blood collection, respectively. Cannula patency was maintained through technique and use of a heparin-loc solution when not in use for several weeks. A swivel-cannula system permitted drug dosing and blood withdrawal while maintained in home caging. In the first study rats are randomized to test article 1 and control groups; a week later the groups are crossed-over to receive control and test article 2, respectively.

Dopaminergics (D) can produce unwanted endocrine side-effects in rats by affecting central prolactin release. Test articles 1) exogenous dopamine (dopamine agonist, bromocriptine), 2) endogenous dopamine (dopamine transporter, DAT inhibitors, mazindol and GBR12909) and 3) endogenous norepinephrine (NE, NE transport inhibitor, nisoxetine), and 4) peripheral-acting exogenous dopamine (dopamine agonist, carmoxirole) were evaluated (iv) for inhibition of estradiol-stimulated (E_2) prolactin release using double cannulated ovariectomized rats, incorporating the cross-over design. Rats were dosed with E_2 (0.6-20 μg/rat, iv) and test article, and blood collected every 30 min between 1.5 to 5 h, and evaluated for prolactin and luteinizing hormone (LH). Bromocriptine (1 mg/kg) inhibited the E_2-induced prolactin release, as did the DAT inhibitors mazindol (5 mg/kg) and GBR12909 (3 mg/kg). Carmoxirole (15 mg/kg) induced a rapid independent release of prolactin, and inhibited E_2-induced release. E_2-induced prolactin release was not inhibited by the central acting NE uptake inhibitor nisoxetine (10 mg/kg). Order of control of TA administration did not impact the findings. This study qualified the double cannulated ovariectomized rat model and cross-over design for evaluating the impact of central and peripheral acting compounds, suggests that the mechanism may be activated both inside and outside the blood-brain barrier with D but not NE, and demonstrates both Refinement and Reduction as a result of employing technology permitting use of the cross-over study design.

V-6-615

Comparative study of amyloidogenic potential of $AgNO_3$ and Freund's Adjuvant with that of vitamin free casein on spatio-temporal pattern of experimental amyloidosis in mice by polarized microscope

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Reactive amyloidosis is a condition that complicates a long list of chronic inflammation, chronic infectious, malignant, and hereditary disorders. In the present study, the potential effects of two amyloidogenic substances, $AgNO_3$ and Freund's Adjuvant (FA), with that of vitamin free casein on the spatio-temporal pattern of experimental amyloidosis in mice were compared. A total of 40 male Swiss mice, obtained from Pasteur Institute Tehran, were weighed and randomly divided into 4 groups: 2 treatment groups, 1 control (vitamin free casein) and 1 negative control (normal saline). At the end of the 3rd, 5th and 7th weeks of the experiment, 3 mice were randomly selected and euthanized. A spleen sample from each animal was obtained and preserved in 10% neutral buffered formalin. The samples were then processed through different stages of dehydration, clearing and impregnation, and embedded in paraffin blocks. Sections of 5 μm thickness were then cut and stained by alkaline Congo red techniques. As an indicator of developing amyloidosis, a green birefringence under the polarized microscope was considered to be a positive criterion for the presence of amyloid. For optical evaluation of amyloidogenic potential, amyloidoic areas were observed in 10 randomly selected high power fields. A light microscope equipped with polarized light optics was used to determine the birefringence intensity of the amyloid deposition in Congo red stained sections. This system was assigned to represent changes in the quantitative appearance and intensity of various microscope fields. Spleen weights and the data obtained from microscope quantitative analysis showed no significant differences between groups A (vitamin free casein) and B ($AgNO_3$) A and C (FA), and B and C. However, significant differences were observed between groups A and D, B and D, and C and D, respectively. It is concluded that two compounds, $AgNO_3$ and Freund's Adjuvant, have the same potential, as does vitamin free casein, to induce the spatio-temporal pattern of experimental amyloidosis in mice.

Session V-9: Improving reporting of animal-based research

Session V-9: Oral presentations

V-9-311

Improving the reporting of animal research: The ARRIVE guidelines and ILAR guidance

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The publication of animal studies in research articles is central to many disciplines in biomedicine. Lack of sufficient experimental data in the research literature has both scientific and ethical implications, including the inability to confirm and build on research findings and the unnecessary use of animals in studies that fail to reproduce reported results. Yet most scientific journals provide relatively little specific guidance for authors and reviewers. The NC3Rs (UK) and an ILAR (US) committee believe that scientific reporting is an important component in the system of quality assurance and that journal editors have a role to play in promoting the proper and ethical use of animals in research through the publication of adequate information.

The NC3Rs developed the ARRIVE guidelines as a consensus among scientists, statisticians, research funders, and journal editors. They cover the main aspects of a scientific publication and make recommendations on the reporting of the study design, experimental procedures, animal characteristics, housing and husbandry, and statistical analysis. The ILAR consensus report provides the evidence-based rationale and references supporting the need for adequate data reporting for the description of research animals and the research animal environment.

Publication of the ARRIVE guidelines and ILAR report is only the beginning. Wide dissemination and uptake are essential. The ARRIVE guidelines were published in eight bioscience journals in 2010 and are now endorsed by a growing number of high-quality journals. Future strategies will include assessing the impact of guidelines on the quality of animal research reporting and revisions to ensure that guidelines evolve and continue to represent consensus.

V-9-717

Improving the reporting of animal research: guidance from the US National Academy of Sciences' Institute for Laboratory Animal Research (ILAR)

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The ILAR committee will also consider implementation of reporting guidelines for journals:

– The report will encourage journals to tailor guidelines for their specific area.
– Many journals have limited space for the publication of supplemental information; how can such restrictions be accommodated while at the same time making available the information necessary to ensure the reproducibility of the reported studies?
– What are the challenges and possible approaches to providing supplemental information – e.g., online posting, databases?

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ALTEX is devoted to the publication of research on the development and promotion of alternatives to animal experiments according to the 3R concept of Russell and Burch: Replace, Reduce, and Refine. Animal experiments are defined by the editors as all experimental procedures involving the use of animals in testing, research and education or to obtain tissues, organs, and other animal-derived products which may cause pain or distress to animals.

Besides covering the biomedical aspects of animal experimentation, ALTEX is also devoted to the bioethics of the complex relationship between humans and animals. Articles published in ALTEX must express a basic concern for the dignity of living creatures and the recognition of animals as our partners. All articles will be judged according to stringent scientific standards.

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