Abstracts 7th World Congress Rome 2009
Volume 26, Spec. Issue
ISSN 1868-596X
1-376 (2009)

ALTEX

ALTERNATIVES TO ANIMAL EXPERIMENTATION

Thomas Hartung and Herman B. W. M. Koëter: **Welcome**

Theme 1
Innovative technologies, concepts and approaches

Integrated approaches
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High throughput technologies
Omics and systems biology
Non-invasive technologies
Non-vertebrate models
In silico models
Databases: scientific approaches
In vitro technologies
Current and evolving concepts for the validation of safety assessment methods

Theme 2
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Chemicals and pesticides
Cosmetics
Pharmaceuticals
Food improving agents
Genetically modified organisms

Nanomaterial toxicity testing
Vaccines and biologicals
Education and training
Animal use policies

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Systemic toxicity and target organs
Genotoxicity and carcinogenicity
Reproduction, development and fertility
Disease models
Environmental science
Animal welfare science
Immunology
Neuroscience

Poster sections to
Theme 1
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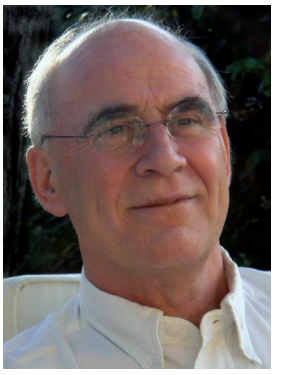
Theme 3

Invitation to WC8
August 21-25, 2011
Montréal, Canada

2009 SEVENTH WORLD CONGRESS
ALTERNATIVES CONGRESS TRUST



Herman B. W. M. Koëter

Thomas Hartung

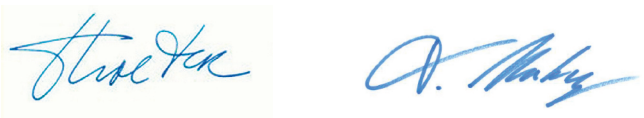
Dear WC7 participants,

since we took on the challenge, in an euphoric moment during WC5 in Berlin, to organize the 7th World Conference on Animal Use and its Alternatives, in short WC7, a lot has changed both in the world of alternative methods and in our private lives. Both of us changed jobs and moved our families away from beautiful Italy: Thomas took the position of Director of CAAT and professor and chair of Evidence-based Toxicology at the Johns Hopkins University in Baltimore, USA and Herman went to Brussels to head a new non-profit scientific organization (Orange House Partnership) with the aim of to provide scientific assistance in the area of chemical and food safety to developing countries.

More importantly, however, we have seen, since the start of REACH and the animal testing ban for cosmetic ingredients in Europe, the milestone publication of the National Research Council for toxicity testing in the 21st century in the US and the creation of JaCVAM in Japan to mention only a few highlights. We have had the successful WC6 in Tokyo and we have experienced that the field of the 3Rs has expanded, with more and more methods available, validated and accepted. We have witnessed that globalization is at work, that our wish to see more formal international collaboration has come true. We are aware that what was until recently termed "alternative" has become mainstream science and an integral part of the life sciences. These developments have created business opportunities and can solve regulatory problems, most notably in priority settings. All together the field is moving to the forefront of scientific developments, leading the path towards improved quality assurance and predictive usefulness.

Thus, it seemed appropriate to give WC7 the motto "CALLING ON SCIENCE", both in the sense of challenging established sciences with new approaches and in calling for a sound scientific base for innovative technologies and concepts. A substantially increased number of sponsors, a record submission of abstracts and the support of many individuals and friends have made it possible to welcome you in this beautiful part of Rome. We strongly believe that we can offer you an outstanding opportunity to meet with colleagues and friends and to share, discuss and return inspired to your offices and laboratories at home. On the occasion of the 50th birthday of Russell & Burch's renowned 3R principles you will experience a flourishing field ready for the challenges of the life sciences in the 21st century.

We hope you will enjoy WC7 as much as we intend to.



Dear WC7 participants, dear readers of ALTEX

ALTEX joins the organisers, Herman Koëter and Thomas Hartung, in welcoming you to WC7. This is the second time that ALTEX is compiling the abstract book for a World Congress - after Berlin 2005, now Rome 2009.

This allows us to draw a small comparison to follow which changes have occurred in the research foci and how the numbers of abstracts contributed by individual countries have developed. Of course the quantity of abstracts does not necessarily reflect the quality of the contributions and the reference to a theme in the abstract does not mean that a solution to a problem has been found.

Regarding the research foci: the interest in alternatives for the cosmetics testing has increased further. The number of contributions in this area has in fact almost doubled since Berlin. Although only 12 posters were submitted for the section "cosmetics", the section "skin and eye toxicity", which is closely related to this area, comprises 73 posters. This shows what a strong influence politics can exert on science, for it was the 7th Amendment to the Cosmetics Directive that set off the strong innovative drive in this area. This number of poster contributions is superseded only by the 89 posters in the section "*in vitro* technologies", a sign of the extreme innovative force that is still driving these technologies. The number of contributions to REACH has also doubled. The contributions to "safety testing" and "risk assessment" remain steady, while the contributions to "refinement" and "animal welfare" have diminished considerably. Contributions to ecotoxicology have also receded significantly as have, very surprisingly, those to QSAR methods, despite the plans of the EU to employ these prominently in the safety testing of chemicals.

Regarding the individual countries: three real "full size 3R nations" actively participate or are co-responsible for more than half (53.7%) of all contributions (in Berlin 2005 this figure was 60.0%). These three are the United States (21.7%), Germany (17.0%), and the United Kingdom (15.0%). In Berlin their shares were 21.5% (US), 22.5% (Germany) and 16.0% (UK). Six "middle class 3R" nations follow, i.e. France (10.3%), Japan (8.9%), the Netherlands (7.0%), the European Union (6.2%), Italy (5.4%) and Switzerland (5.1%).

However the unofficial motto of the Olympic Games is also applicable here, "The most important thing is not to win but to take part!" So we greatly appreciate the contributions, which may not be so numerous but are scientifically highly valuable, from Argentina, Australia, Austria, Belarus, Belgium, Bolivia, Brazil, Canada, China, Columbia, Cuba, Czech Republic, Denmark, Estonia, Finland, Greece, India, Ireland, Israel, Latvia, Lithuania, Malaysia, Mexico, New Zealand, Nigeria, Norway, Peru, Poland, Portugal, Rumania, Russia, Serbia, South Korea, Spain, Sweden, Syria, Turkey, Ukraine, Uruguay, Uzbekistan, Venezuela. The world power Russia, like Poland, is only represented by a single poster contribution in Rome (in 1996 there were 10 and 6 contributions respectively). This may not necessarily mean that an interest in developing 3R methods is lacking in these countries. Financial limitations but also the language barrier may be reasons for staying away from the Congress. The summaries of all ALTEX articles will be translated e.g. into Russian, because it is a simple fact that a large part of unquestionably highly qualified Russian speaking scientists does not publish in English. Here the United States and Russia can repeat what they have demonstrated so well in space research: exchanging scientists, driving collaborative projects forward, improving communication and supporting the humane handling of animals. It should be an important goal of the World Congress to actively integrate a world nation such as Russia in this regard. Comparing the national contribution numbers to an earlier Congress (Utrecht 1996), we could already find the three "3R full size nations" then, i.e. UK (15.0%), US (11.7%) and Germany (11.0%), though all were eclipsed by the host of that Congress, the Netherlands, with 16.0% of contributions. 32 countries contributed in 1996, this number had already increased to 51 in Berlin and remained steady with 50 in Rome (the EU is counted as a separate "country" here; the contributions of ECVAM in Ispra are not attributed to Italy).

In some of the poster abstracts submitted for WC7, especially in some from countries in which no independent efforts have been documented in the 3R area before, a 3R relevance is not immediately obvious. Animal experiments are described without reference to means of reducing these in any way. The Program Review Panel did not oversee these abstracts but came to the decision that participation in WC7 should be enabled for these groups so that they may be introduced to the full scope of the 3R efforts.

The Proceedings of the WC7 shall also be published. This will comprise a written version of all lectures, which requires a certain amount of discipline from the speakers. Only few speakers will arrive in Rome with a finished manuscript. But we must consider that such Proceedings find widespread distribution to universities and use in education. No other medium provides such a good overview of the state of the art in a form that is accessible to students as a collation of manuscripts encompassing the entire area of 3R research. It is thus highly desirable that especially the invited speakers, whose opinions on the developments in their fields are highly valued, contribute their manuscripts to the Proceedings.

The generous sponsorship of the WC7 organisation, CAAT and the DZF has made the provision of the Abstract Book to all ALTEX subscribers possible. Thus persons who are not able to come to Rome can also inform themselves comprehensively of the developments in the 3R field.

The ALTEX Team wished the organisers and all participants of WC7 a successful Congress and a memorable time in Rome. We look forward to seeing you all again at WC8 in Montréal, Canada, on August 21-25, 2011.

With best wishes

Franz P. Gruber

Programme

Sunday August 30

Pre-Satellite Meetings and Congress Registration

<table>
<tr><th>Hrs</th><th>Auditorium</th><th>Room Cavalieri 1</th><th>Room Cavalieri 2</th><th>Room Ellisse</th><th>Terrazza Monte Mario</th><th>Room San Pietro</th></tr>
<tr><td>14.00-15.00</td><td></td><td></td><td></td><td rowspan="3">SYMPOSIUM co-organized by CELLTOX and ESTIV “From tissue engineering to alternatives: research, discovery and development”</td><td rowspan="3">JOINT MEETING of Altweb Project Team and 3Rs Centers organized by the Johns Hopkins Center for Alternatives to Animal Testing (CAAT)</td><td></td></tr>
<tr><td>15.00-16.00</td><td></td><td></td><td></td><td></td></tr>
<tr><td>16.00-17.00</td><td colspan="3" rowspan="3">Registration and Posters set up</td><td></td></tr>
<tr><td>17.00-18.00</td><td rowspan="2">ANIMAL PROTECTION SATELLITE MEETING “Bringing together International Animal Protection Organizations” organized by Humane Society of the United States (HSUS) and Eurogroup for Animals</td></tr>
<tr><td>18.00-19.00</td></tr>
<tr><td>19.00-20.00</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>20.00-22.00</td><td colspan="6">Welcome Cocktail and Get-Together Party</td></tr>
</table>

Monday August 31
Theme 1: Innovative Technologies, Concepts and Approches

Hrs	Auditorium	Room Cavalieri 1	Room Cavalieri 2	Room Ellisse	Terrazza Monte Mario	Room San Pietro
08.00-09.00				*Early Morning Session (MS3)* Animal welfare Associations	*Early Morning Session (MS1)* 3Rs centers 1: Asian regulatory affairs in animal welfare and alternatives	*Early Morning Session (MS2)* Industry activities 1
09.00-09.15	Opening ceremony					
09.15-10.00	*Plenary lecture 1 (PL1)* The US Tox21 community and the future of toxicology testing					
10.10-12.40	*Breakout session (BS9)* In vitro technologies	*Breakout session (BS1)* Integrated approaches	*Breakout session (BS3)* High throughput technologies	*Breakout session (BS7)* In silico models	*Breakout session (BS2)* Chemical and Physical Methods	10.10-11.10 *Extra breakout session (EB1)* Status report on Predict-IV 11.30-12.30 *Extra breakout session (EB3)* Status report on CAESAR
12.40-13.40 **13.40-14.40**	Lunch Visit poster exhibition					*Lunch session (SL1)* Databases: progress report *Lunch session (SL2)* Good Cell Culture Practices
14.50-17.20	*Breakout session (BS4)* Omics and systems biology	*Breakout session (BS5)* Non-invasive technologies	*Breakout session (BS6)* Non-vertebrate models	*Breakout session (BS8)* Databases: scientific approaches	*Breakout session (BS10)* Current and evolving concepts for the validation of safety assessment methods	14.50-15.50 *Extra breakout session (EB2)* Status report on Evidence Based Toxicology Project 16.00-17.00 *Extra breakout session (EB12)* Update on ICH and VICH progress
17.30-18.15	*Plenary lecture 2 (PL2)*					
20.00-23.00	Garden Party					

Tuesday September 1
Theme 2: Areas of Animal use

Hrs	Auditorium	Room Cavalieri 1	Room Cavalieri 2	Room Ellisse	Terrazza Monte Mario	Room San Pietro
08.00-09.00				*Early Morning Session (MS6)* Associations	*Early Morning Session (MS5)* 3Rs centers 2	*Early Morning Session (MS4)* Educational activities
09.10-09.55	*Plenary lecture 3 (PL3)* Brueghel's two monkeys: passing the final exam in the history of mankind					
10.00-12.30	*Breakout session (BS12)* Chemicals & pesticides	*Breakout session (BS11)* Basic research	*Breakout session (BS14)* Pharmaceuticals	*Breakout session (BS16)* Genetically modified organisms	*Breakout session (BS19)* Education and tranining	10.00-11.00 *Extra breakout session (EB4)* Status report on ICATM 11.10-12.10 *Extra breakout session (EB5)* Status report on EPAA
12.30-13.30	Lunch Visit poster exhibition					*Lunch session (SL3)* Communication strategies
13.30-14.30						*Lunch session (SL 4)* Recent progress and future directions in the validation and regulatory acceptance of alternative test methods that reduce, refine and replace animal use
14.45-17.15	*Breakout session (BS13)* Cosmetics	*Breakout session (BS18)* Vaccines and biologicals	*Breakout session (BS15)* Food improving agents	*Breakout session (BS17)* Nanomaterial toxicity testing	*Breakout session (BS20)* Animal use policies	14.45-15.45 *Extra breakout session (EB6)* Status report on ReProTect 16.00-17.00 *Extra breakout session (EB7)* Status report on OSIRIS
17.25-18.10	*Plenary lecture 4 (PL4)* Metabonomics-driven top-down systems biology: techniques and applications					

Wednesday September 2
Theme 3: Progress in Life Sciences Domains

Hrs	Auditorium	Room Cavalieri 1	Room Cavalieri 2	Room Ellisse	Terrazza Monte Mario	Room San Pietro
08.00-09.00				*Early morning session (MS8)* Industry activities II	*Early morning session (MS9)* 3R centers 3	*Early morning session (MS 7)* Lessons learned from the validation and potential regulatory applicability of in vitro alternative pyrogen tests
09.10-09.55	*Plenary lecture 5 (PL5)* Predictive testing strategies: R&D achievements and perspectives					
10.00-12.30	*Breakout session (BS21)* Skin and eye toxicity I	*Breakout session (BS23)* Systemic toxicity and target organs	*Breakout session (BS25)* Reproduction, development and fertility	*Breakout session (BS26)* Disease models	*Breakout session (BS29)* Immunology	10.00-11.00 *Extra breakout session (EB8)* Status report on AcuteTox 11.10-12.10 *Extra breakout session (EB9)* Status report on ToxCast
12.30-13.30	Lunch Visit poster exhibition					*Lunch session (SL5)* Animal use policies: the future of animal welfare legislation
13.30-14.30						*Lunch session (SL6)* Vaccines: acceptance of 3R methods
14.45-17.15	*Breakout session (BS22)* Skin and eye toxicity II	*Breakout session (BS24)* Genotoxicity and carcinogenicity	*Breakout session (BS27)* Environmental science	*Breakout session (BS28)* Animal welfare science	*Breakout session (BS30)* Neuroscience	14.45-15.45 *Extra breakout session (EB10)* Status report on Sens-it-iv 16.00-17.00 *Extra breakout session (EB11)* Introduction and status report on ESNATS EU Project
17.25-18.10	*Plenary lecture 6 (PL6)* Is forefront science considered and applied in regulatory risk assessment?					
20.00-23.00	Congress Dinner (Villa Miani)					

Thursday September 3

Special Event: 50 Years after Russel & Burch

Hrs	Auditorium
08.00-09.45	*Plenary lectures 7 (PL7)* The principles of humane experimental technique: timeless insights and unheeded warnings The principles of humane experimental technique – is it relevant today?
09.45-10.00	Awards ceremony
10.45-11.15	Special recognition event
11.15-12.00	*Plenary lecture 8 (PL8)* Calling on Science
12.00-12.15	WC8 presentation
12.15-12.45	Closing speeches

Early Morning Sessions

MS1: 3R centers 1: Asian regulatory affairs in animal welfare and alternatives

The continuation of Asian activities of alternative research from WC6 to WC7

T. M. Kurosawa

Osaka University Medical School, Suita-Shi, Osaka, Japan

Asian activities in alternatives to animal experimentation were fired up when the World Congress 6 (WC6) was planned to be held in Tokyo. In particular, the western colleagues in this field asked the Japanese Society for Alternatives to Animal Experiments (JSAAE), which was a host of WC6, to extend our activities to other Asian countries. Accordingly, JSAAE asked Korean and Chinese colleagues in this field to organize satellite meetings in their countries. Two satellite meetings were organized in Beijing and Seoul, and they were successfully attended by many participants from all over the world before WC6. WC6 itself was well attended with more than one thousand international participants in 2007, and these gatherings stimulated much research interest in alternatives to animal experimentation among biomedical scientists in Asian countries.

The success of WC6 was extended to academic information exchange among these countries. We of the JSAAE continue the mutual exchange of scientists among Asian countries and, in fact, several delegations were sent to attend the other societies' general meetings. The wave of Asian activities continues into WC7 and the JSAAE is organising the morning session "Asian regulatory affairs in animal welfare and alternatives".

The activities of alternative research should be extended to all countries in which animal experimentation is conducted, including many other Asian countries. Information on Asian alternatives activities will be shared among the international participants of WC7.

Accreditation of animal experiments by Japan Health Science Foundation

Y. Ohno

National Institute of Health Sciences, Kamiyoga, Setagaya, Tokyo, Japan

Japanese animal protection law was revised in 2005 to include 3Rs principles of animal experiments. According to this, general principles of animal experiments were prepared and notified in 2006 separately by Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labor and Welfare (MHLW), and Ministry of Agriculture, Forestry and Fisheries. Main contents of these notifications were almost the same. All includes responsibility of director of the institutes, role of institutional committee for animal experiments, selection of scientifically valid methods and respect of 3Rs principles, appropriate facility and equipments, secure the safety of animal experiments, and culture and keeping of experimental animals.

On the other hands, needs of inspection of animal experiments by third party was not described in the MHLW's notification. However, JPMA companies, who are administered by MHLW, considered it necessary and asked Japan Health Science Foundation (HS) to play the role. HS started to construct the system in 2007 and organized "Center for Accreditation of Laboratory Animal Care and Use" in 2008. Purpose of the inspection is to evaluate the institute conducting animal experiments according to the general principles notified by MHLW. It examines by both document review and site visits. Our institute (NIHS) was the first accredited institute.

Two laws on laboratory animal welfare and use in Korea

J. Park

Seoul National University, Seoul, South Korea

The Animal Protection Law (APL) of Korea was amended in December 2007. It includes two sections on laboratory animals. Section 13 described the principles of animal experiments, including 3R principles. Beside 3R, dogs that have worked for humans may not be used as laboratory animals. Section 14 describes an Institutional Animal Care & Use Committee. Over one third of the committee should be composed of persons who do not work at the corresponding animal experiment facility and do not have any relationship of gain and loss with the facilities. A veterinarian and a person recommended by an NGO on animal welfare should be included in the committee. The law was activated in February 2008. Now, about 600 animal facilities have set up IACUC and started inspection of proposals and animal facilities. The Animal Protection Law (APL) is led by the Korean Department of Agriculture.

The other law on laboratory animal use was adapted in 2008 by the Korean Food and Drug Administration (KFDA). The law on laboratory animal use indicates that animal facilities which provide testing of drugs, cosmetics, food and medical instruments should be enrolled in KFDA. KFDA should supervise and guide these animal facilities. The two laws seem to have some conflicts. According to the laws, many animal facilities must inform both the Department of Agriculture and KFDA of their activities.

Laboratory animal welfare and ethical review of animal experiments in Chinese regulations

R. Rong

Beijing Administration Office for Laboratory Animals, Beijing, P.R.China

In 1988, Chinese State Council has issued "The Regulation for Administration of laboratory animals", and some provincial regulations of laboratory animals are issued in Beijing, Hubei, Yunnan and Heilongjiang. All these regulations pay more and more attention to the animal welfare and ethics on animal use as the issued time successively. The Ministry of Science and Technology has issued "The Guide of Well Treatment for laboratory animal". The guide consists of the requirements for hygiene, safety, watering, feeding, handling, breeding space, anesthesia, euthanasia, transportation and personnel training on animal use. The national standards for laboratory animals are issued and implemented since 1994. These national standards cover all aspects about animal breeding and use. In 2001, "Developing guiding principle during Tenth five years" issued by Ministry of Science and Technology indicate that to establish an animal welfare guarantee system which is agreement with international rules.

MS2: Industry activities 1

Building a 3R corporate culture at Johnson & Johnson

S. Sloan

Johnson & Johnson, New Brunswick, USA

With over 250 operating companies in 57 countries, Johnson & Johnson demonstrates its commitment to the 3Rs through a variety of internal and external endeavors. In 1992, a research group in the EU was established with the aim to develop and implement alternatives for animal testing in the area of pharmaceutical research. Several alternative models for eye irritation have been implemented and research is on-going for other domains of toxicological research such as embryotoxicity, vascular irritation, phospholipidosis testing, etc. To acknowledge the importance of research in the area of the 3Rs, the Johnson & Johnson Corporate Office of Science and Technology established an internal Annual 3Rs Award. Each of the awardees is recognized at a major company event to further emphasize the important work they have accomplished and to reinforce the commitment of the company's senior leadership to the 3Rs. In 2006, Johnson & Johnson established a post doctoral fellowship specifically targeting projects dedicated to advancing the 3Rs. The 2006 3Rs post-doc fellow presented the results from her research at the 6th World Congress on Alternatives & Animal Use in the Life Sciences. Externally Johnson & Johnson employees participate with several international organizations such as ECVAM, ICCVAM, COLIPA, EPAA, the OECD and numerous consortiums including the European Industry Initiative and IVTIP. Johson & Johnson maintains a leadership position with several non-profit organizations whose mission is targeted towards the advancement of the 3Rs. Specifically, Johnson & Johnson is a member of the Scientific Advisory Panels of both IIVS and CAAT.

Procter & Gamble's approach to the development of alternatives

M. Lafranconi

The Procter & Gamble Company, Miami Valley Innovation Center, Cincinnati, OH, USA

Procter & Gamble is committed to the eventual elimination of animal testing for evaluating the safety of consumer products. Our approach has three key elements: 1) Development of methods to replace animal-dependent tests and continue to refine, or reduce animal use until a replacement is developed, 2) Development or adaption of tools that maximize use of existing data, and 3) Participation in programs that foster acceptance. Our research efforts focus on gaining a mechanistic understanding of the toxicity and developing methods that target key events for early detection and optimal predictivity such as the Peptide Reactivity Assay. We have developed internal databases, systems, and developed or adapted computational methods, and risk assessment approaches, such as TTC, which enable us to more effectively utilize existing information. Finally, our strategic partnerships with academics, government, NGOs and others in industry accelerate development of methods and acceptance by the scientific community, the public, and regulators.

The *colipa* programme on alternatives

O. de Silva

Colipa Strategic Committee on Alternatives, Brussels, Belgium

The 7th Amendment prohibits the use of animal tests in the EU to meet the requirements of the Cosmetics Directive by introducing testing and marketing bans.

The cosmetics industry has a long standing and continuous commitment to the replacement of animal testing. It already eliminated animal testing for finished products back in the 1980s

and has contributed together with several partners and ECVAM to the development and validation of the replacement alternative methods that are available to date: phototoxicity, skin corrosion and skin irritation, percutaneous absorption.

The development of new alternative methods is a tremendous scientific challenge. Since 1992 ***colipa*** and its members are committed to making all possible efforts in order to achieve the best results in developing alternatives. Currently within ***colipa***, 4 project teams are working on four priority areas: eye irritation, genotoxicity/mutagenicity, skin sensitisation and systemic toxicity. They have the mission to coordinate all the efforts of the European cosmetics industry to ensure development and help acceptance of replacement alternative methods in these areas. ***colipa*** has also established a special Project Team on safety assessment approaches. This work is supported by all members of ***colipa*** and adds to the ongoing substantial efforts of individual companies.

Overall, safety assessment of our products draws on multiple factors: data banks, physico-chemical values, analytical chemistry, exposure, frequency, animal data, *in vitro* data, *in silico* data (mathematical modeling, computer predictions), structure activity, read-across, benchmarking, clinical studies, post-marketing surveillance… and it will be possible to continue business and innovation. However, in some rare cases we may lack the necessary information.

In view of 2013, ***colipa***'s goal is to deliver a first generation integrated testing strategy capable of providing skin allergy information, and research aimed at delivering second generation methods more predictive and more applicable by 2013. In addition, a joint collaboration with the European Commission on the funding of systemic toxicity research has been agreed.

Whilst industry will be able to deal with the 2009 deadline and still remain innovative, the 2013 deadline poses huge challenges.

MS3: Animal welfare associations

The Foundation Animalfree Research: 33 years of replacing animal experiments

S. Schindler

Animalfree Research, Zurich, Switzerland

It all started in 1976 with 5,000 Swiss Francs (3,300 Euro) in Zurich. The founders of the new organization, then called *Fonds für versuchstierfreie Forschung* (FFVFF) were deeply moved by reports on hair-raising cruelties towards experimental animals and decided to do something about it. Aware of the fact that animal experiments could not be completely abolished overnight and inspired by the concept of FRAME, they dedicated their foundation to the financing of alternative methods – pioneer work in the 1970s. From the first moment, the dialogue with the public as well as with scientists and politicians was considered pivotal. The FFVFF published ALTEX for the first time in 1984 and can look back on many successful projects, such as the meta-analysis by Prof. Gerhard Zbinden which, for the first time, demonstrated the low scientific value of the LD_{50} test and found worldwide recognition. In 2007, the name of the FFVFF was changed to Animalfree Research.

The American Society for Prevention of Cruelty to Animals (ASPCA) and 3R initiatives

S. A. Khan[1], M. K. McLean[1], S. Zawistowski[2] and S. Hansen[1]

[1]ASPCA, Uraba, IL, USA; [2]ASPCA, National Programs, New York City, NY, USA

Founded in New York City in 1866, the ASPCA is the oldest humane organization in North America dedicated to animal welfare and protection. Currently, the ASPCA is recognized as a national animal welfare organization with both regional and national programs aimed at fighting animal cruelty, controlling pet overpopulation issues, preservation of population and species, and promoting the health and welfare of animals. The organization is wholly committed to effecting change through nonviolent approaches. It believes in achieving its vision of humane communities across the United States through education, advocacy, and other forms of intervention that support the beneficial relationship between people and animals. The society's policies are based on empirical evidence and are supported by scientific research that establishes animals capacity to feel pain and suffering. The ASPCA strongly supports the development and validation of alternative methods to the use of animals in biomedical research and testing. The organization believes that the 3Rs are fundamental and should be applied to the use of animals in all aspects of biomedical research.

For the last twenty years, the ASPCA has been supporting 3R initiatives by using their expertise in the use of animal alternatives in research and through educational means. These initiatives have included sponsorship, participation and presentations at the alternative world congresses and other forums; participation in the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) in the US; reviewing and approving organizational protocols promoting 3R concepts; publishing scientific information in peer-reviewed journals by using veterinary toxicology databases to characterize sensitivities, syndromes, and interspecies differences from different chemicals; providing toxicological data to pharmaceutical and government regulatory agencies without using traditional animal testing studies to help investigate product safety. The recently started alternative literature search services at the ASPCA will strengthen our 3R initiatives further. This service is aimed at helping industry meet animal welfare act guidelines by performing database searches for existing research and alternatives.

Eurogroup for Animals

K. Reid

Eurogroup for Animals, Brussels, Belgium

Eurogroup for Animals has been deeply committed to improving the way animals are treated and kept throughout the European Union. Since our creation in 1980 this includes protecting the millions of animals used in research every year. As a federation of NGOs, we speak for millions of Europeans who are concerned about animals. Our membership of 42 organisations stretching across the European Union makes us ideally placed to represent the views of animal welfare. The network we have created consisting of about 15 experts, facilitates the sharing of effective campaigning tools and ideas among our members. We work with legislators, experts and industry towards the introduction, implementation and enforcement of EU laws that will improve animal welfare and reduce animal suffering and whereby animal tests are replaced with alternative methods.

Key to Eurogroup's success has been its excellent communication and in-depth knowledge and experience of EU political processes. Through this we strive to influence legislation at an early stage to bring about key improvements at a very early stage. To do this we sit on more than ten advisory committees at five different Directorates General of the European Commission. We also work closely with the European Food Safety Authority (EFSA) and the European Centre for the Validation of Alternative Methods (ECVAM) through their advisory boards.

In our mission to ultimately replace all animal experiments with viable alternatives Eurogroup collaborates with the European Partnership on Alternative Approaches (EPAA) – a unique collaboration between the European Commission (including ECVAM) and major companies from seven industry. ECVAM has to this day validated 32 alternative testing methods and is currently looking at validating many more, which could prevent the suffering of thousands of animals each year. Eurogroup has 2 representatives in the ECVAM ESAC committee. We continue to express our grave concern over the Commission's decision to restructure the Centre as well as question the level of ECVAM's operational funding. We believe that increasing both funding to and the role of this important organisation is key to speeding up the development and use of alternatives. This is an excellent time, as EU policy makers are in the midst of revising the legislation on the protection of animals used for scientific purposes, the very legislation that brought about the creation of ECVAM.

We work with Members of the European Parliament (MEPs) to support the push for our welfare demands and we closely monitor the discussions in the various parliamentary committees and supply MEPs with facts and figures to ensure that the Parliament's reports include substantial improvements for the protection of animals and implementation of alternative meth-

ods. Furthermore, for nearly 25 years we have run the secretariat of the European Parliament's Intergroup on the Welfare and Conservation of Animals. This popular and well-established forum held its 250th meeting in January 2009. To further the cause of animals in Europe we also work hard to ensure that successive EU presidencies are informed of key welfare issues during their six month chairmanship of the Council of the EU.

Our mission to keep animal welfare firmly in the spotlight does not stop at Europe's borders. Through our membership of a number of coalitions, we work closely with international organisations such as the International Organisation for Animal Health (OIE) and the Organisation for Economic Co-Operation and Development (OECD) can also call on Eurogroup to offer expert advice through the International Council on Animal Protection (ICAPO) alliance.

The HSUS and the Three Rs

M. L. Stephens

The Humane Society of the United States, Gaithersburg, Maryland, USA

The Humane Society of the United States (HSUS) embraced the Three Rs approach of replacement, reduction and refinement shortly after its elaboration by Russell and Burch in 1959. In recent years, the HSUS's efforts in promoting alternative methods have been enhanced by the work of its sister organizations, the Humane Society International (HSI) and the Humane Society Legislative Fund (HSLF). Current projects in the area of toxicity/safety testing include coordinating a coalition of corporations and NGOs to promote 21st century testing methods; advocating for Three Rs-friendly provisions in the programs of national and international regulatory and standard-setting bodies; lobbying for funding of Three Rs-related programs; co-managing the AltTox website to provide information and an interactive platform to promote non-animal methods of testing; and pressuring Allergan to replace its use of the LD_{50} test in assessing the potency of Botox products. We are currently in negotiation with the European Union to coordinate a project ("AXLR8"), under the 7th Framework Programme, designed to facilitate the development of 21st century testing methods in Europe. In the area of biomedical research, we are encouraging universities to adopt refinement policies prohibiting the conduct of severe animal experiments, and we are lobbying for a ban on the use of dogs and cats rounded up by "Class B" animal dealers as well as a phase out of chimpanzees used in invasive research. In the area of animal use in education, we promote alternatives to dissection by supporting relevant legislation and school policies and by loaning out alternative materials to students and teachers. Cross-cutting efforts include suing the U.S. Department of Agriculture to secure more meaningful national statistics on animal use to better inform planning efforts on the Three Rs, and bestowing the Russell and Burch Awards to deserving Three Rs scientists.

Vereniging proefdiervrij (Dutch society for replacement of animal testing)

M. Zuidgeest

Proefdiervrij, The Dutch Society for the Replacement of Animal Testing, The Netherlands

In the Netherlands, each year some 600,000 animals are still used for various animal tests. Proefdiervrij is a non-profit organisation that works towards replacing all kinds of animal testing (in the Netherlands). Its focus is on the promotion of alternative research methods. Proefdiervrij seeks to peacefully raise awareness of the plight of laboratory animals by means of promotional activities. The organisation urges politicians, the scientific research community, and industries to stimulate and innovate by using alternative methods and replace animal testing altogether.

It can change, it must change

Naturally, research into life threatening diseases is necessary and Proefdiervrij supports that thought. But it should not be at the cost of hundreds of thousands of test animals. This is not only about medical research but also about tests for cleaning products and foods. There are alternatives for animal testing, which often produce even better results. For instance, testing for skin rashes can be done on artificially cultivated skin and toxicology tests can be performed in an imitation gastrointestinal tract.

Non animal methods

Despite the availability of non-animal methods, a lot of scientist still fall back on using live animals. A force of habit. Moreover, the same tests are repeated all over the world, time and time again. Proefdiervrij holds the view that more money and energy should be spent on developing new non-animal techniques, so that the suffering of animals can truly be brought to an end.

You can find more information on our website www.proefdiervrij.nl (in Dutch) or visit the (English) site of our colleagues www.eurogroupforanimals.org.

It's high time for animal free techniques

MS4: Educational activities

Establishment of dedicated chairs for alternative methods as strategy to promote the 3R

M. Leist [1], B. Blaauboer [2], K. Brune [3], P. Cosson [4], F. P. Gruber [5] and T. Hartung [6]

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The promotion of more 3R approaches classically builds on 4 major pillars. Industry develops and applies new methods, authorities and animal welfare groups are involved to some extent in method development and validation but also play a major role in the implementation of the methods and in creating legal and societal driving forces. Last but not least, academia contributes greatly to the design of new assay systems.

As academia is most transparent to the general public and associated with a large number of students, it has a special role in overcoming conceptual barriers to the use of 3R approaches. Such barriers, in contrast to technological and legal barriers, are frequently linked to a certain inertia or even resistance towards breaking traditions, to a trust issue with regards to new approaches, to misinformation on the benefits and advantages of classical animal experimentation as opposed to newer 3R approaches, and to psychological factors, such as the fear of a loss of academic freedom. One approach to strengthen the academic progress towards the development of 3R approaches is the introduction of university chairs dedicated to this topic. They add credibility to the discipline as a serious topic of modern science, influence student education early on, and show realistic career paths and options for gifted students who want to commit to this research field and may otherwise follow other interests. One difficult issue for the establishment of 3R chairs is that method development as a goal in itself is neither well accepted nor is it well-funded by scientific organizations. Also, the peer acceptance is relatively low, and this would be counterproductive to the major goal of enhanced scientific credibility and attraction of students.

The solution to this problem is relatively simple and has been demonstrated successfully by the Doerenkamp-Zbinden (DZ) Foundation, one of the major sponsors of 3R chairs in the world: The methods development approach needs to be coupled with a conceptual scientific question. For instance, the DZ chair in Erlangen couples the development of refinement methods on the basis of non-invasive imaging techniques with conceptual research on the pharmacology of analgesics, the DZ chair in Konstanz links *in vitro* method development with conceptual research on neurodegenerative conditions and the DZ-Naef chair in Geneva combines the development of test systems with non-sentient invertebrates, with basic cell biological and immunological questions. Such combinations allow the chairs to plug in to key technologies of highly dynamic basic research fields and to use them for the development of 3R methods. In general, medical, veterinary or engineering faculties are more open to method development as major goal of a chair. Therefore chairs with such an affiliation may fully dedicate to 3R method development and the implementation of and lobbying for new concepts of thinking in the field.

Accordingly, the DZ chair at Johns Hopkins University is a major driver for evidence-based toxicology and the DZ chair at Utrecht University is promoting the consideration of biokinetic aspects in the development of 3R methods and their application in risk assessment.

All these DZ chairs are anchored firmly in the academic curriculum and, based on a high reputation of the chairholders, have the best chances of attracting students to the field of 3R research and also to contribute to the education of journalists and the broad public for wider acceptance of the 3R principle.

CAAT's humane science policy, outreach & academic programs

B. N. Merrill

Johns Hopkins Center for Alternatives to Animal Testing (CAAT), Baltimore, USA

The Humane Science Policy and Outreach Program serves to educate US policy-makers and legislators on the need for alternatives to animals in testing, and to advocate for humane sciences in government research and regulations. This program focuses on individuals and institutions that make or implement policies influencing the use of humane sciences and alternatives. The goal is to create a legislative and policy culture that values the lives of animals and promotes the use of the 3Rs.

To bring about this goal, the policy and outreach program sponsors and participates in seminars, prepares articles, and works with members of the policy and legal communities to cultivate a greater understanding of the principles and applications of humane science. An important objective is to identify champions in the policy field to assist in implementing the National Academy of Sciences (NAS) report, Toxicity Testing and Assessment in the Twenty-first Century: A Vision and a Strategy.

CAAT's Academic Programs educate students and professionals in the research field about alternatives, helping them gain a better understanding of the 3Rs and humane science. A central component is CAAT's Humane Science and Toxicology Certificate Program, which has a six-course curriculum and is accessible to the business, legal and regulatory communities. It is anticipated to be available entirely on-line in 2010

The Three Rs in veterinary medical education

F. Ohl

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The goal of veterinary medicine is to promote animal health and welfare. Therefore, the training of veterinary students aims at providing them with knowledge of physiological and pathological processes across the species, an understanding of different animal husbandry systems, clinical competencies such as the diagnosis and treatment of disease, the ability to reason in a scientific manner, a fundamental appreciation of business management, and the development of a professional attitude. The use of (healthy) animals in veterinary education is mainly directed towards learning (anatomy and physiology), observation of animal behaviour and the development of practical skills such as animal handling and surgical technique.

About 75 million vertebrates are used worldwide per year for experimental purposes. On average, about 2% for education and training. Legislation on the use of animals for experimentation includes the use of animals for educational purposes: it is only permitted if the objective cannot be achieved by the use of non-animal methods, competence of the staff involved has to be proven, and unnecessary pain and distress has to be avoided – following the Principle of Humane Experimental Technique, that is, the Three Rs of Russell and Burch (1959).

For veterinary education, a variety of replacement methods are available, such as:

- Audio-visual
- Models and simulators
- Multimedia computer simulation
- Ethically-sourced animal cadavers and tissues
- Clinical work with animal patients
- *In vitro* labs
- Non-invasive field studies

Especially the integration of veterinary students in clinical practice at an earlier stage of their study in addition to training them in virtual laboratories designed to improve their skills seems to represent a highly successful combination.

Whilst the humane use of farm and companion animals and a critical eye on the use of animals for education are hot topics in modern veterinary education, it does not however usually include training in the humane use of laboratory animals. Given the fact that veterinarians regularly use and have to assess the validity of the results of animal experiments (i.e. treatment methods, new pharmacological compounds), veterinary students should at least have an obligatory basic training in ethical aspects of the use of experimental animals and in the application of the three Rs.

MS5: 3R centers 2

The NC3Rs – supporting science and animal welfare

V. Robinson

NC3Rs, London, UK

The National Centre for the 3Rs (NC3Rs) was established by the Government in 2004 to provide a UK focus for driving advances which replace, reduce or refine the use of animals in research and testing. Working with scientists in universities and industry, research funding bodies and regulatory authorities, the Centre's goal is to use the 3Rs as a framework for supporting science and innovation and improving animal welfare. The NC3Rs is developing new approaches to addressing key areas of unmet medical need; exploiting emerging technologies such as tissue engineering and providing an environment for data sharing and discussion on the utility of model systems. The Centre is the UK's biggest funder of 3Rs research and has a number of schemes for supporting high quality research including a recently launched scheme for PhD studentships. This presentation will briefly describe the Centre's work and the resources it provides scientists. Further information can be found at www.nc3rs.org.uk

A proposal of establishing a Brazilian center for validation of alternative methods (BraCVAM)

O. Presgrave

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Many products on the Brazilian market are requested to be controlled by using animal testing. Some groups at official laboratories, universities and industries are studying alternative methods, but there is no improved mechanism for funding collaborative studies and there is no institution responsible for managing and coordinating these studies. Many validated alternatives need to be improved in Brazil, taking into account some country specificities. This includes that some methods must be validated for the specific kinds of products. The Oswaldo Cruz Foundation (FIOCRUZ) assembles all the conditions necessary to become the headquarters of the Brazilian Center for Validation of Alternative Methods (BraCVAM), since it is an internationally recognized scientific institution uniting a large number of scientific fields, including basic and applied research, drug and vaccine production, quality control, teaching, hospitals, etc. The multidisciplinary scientific infrastructure of FIOCRUZ could be used to establish a network in different fields of knowledge. INCQS has been participated in several Brazilian and International Congresses and has a group of about 20 professionals and students working on alternatives for replacing animals in skin, eye and mucous irritation, sensitization and pyrogen tests, as well as vaccine and anti-venom sera potency determination. During this period, a great amount of posters presentations, lectures, organization of meetings and roundtables, papers and post-graduation studies have been developed. The creation of BraCVAM would facilitate the development and validation of tests, not only in Brazil but also in South America and the Caribbean, working together with institutions developing alternative methods around the world.

A national center for animal alternatives in India: the Mahatma Gandhi-Doerenkamp centre for alternatives to animal use in life science education

M. A. Akbarsha[1], F. P. Gruber[2] and S. Pereira[3]

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"*I abhor vivisection with my whole soul. All the scientific discoveries stained with innocent blood I count as of no consequence*" (Mahatma Gandhi, 1869-1948)

The Mahatma Gandhi – Doerenkamp Centre for Alternatives to Animal Use in Life Science Education will be established as a National Center for alternatives in India, at Bharathidasan University, Tiruchirappalli, Tamil Nadu. The Bharathidasan University is a renowned university under the University Grants Commission of the Government of India. The mandate of the center is to synergize the Gandhian Philosophy of "Ahimsa" or "Non-Violence" in the teaching/research of Life Sciences. For the first time in India, this Center will introduce a new socio-scientific concept of promoting the philosophy of "non-violence" in the teaching and research in Life Sciences. The center will be established with the generous financial support received from The Doerenkamp-Zbinden Foundation, Switzerland, and the establishment of the "Gandhi-Gruber-Doerenkamp Chair" for Alternatives in Biomedical Education. The Center is being established in knowledge that promoting humane science is an imperative scientific, legal, psycho-social, ecological and economic need of the hour. The Center will strive to create a strong positive presence of alternatives in India to the use of animals thereby promoting quality and excellence in Life Science education/research/testing by way of continuous training programs, an alternatives knowledge bank, library and a certificate/diploma/post-graduate diploma program in animal alternatives.

The Center will also bring together stakeholders in the 3Rs – academia, scientific community, industry, government and animal welfare personnel from national/international levels to raise the awareness/facilitate the exchange of information/ideas on alternatives to translate the vision of the 3Rs into policy and curricular changes in India as relevant to education and research. The Center will also help by way of funding research and development of environmentally friendly pedagogical tools and *in vitro* alternative methods for Life Science teaching and research. The twin approach will be to encourage the use of e-tools and help establish virtual learning centers for teaching, and to establish a state-of-the-art cell culture laboratory for training in non-animal methods of research and product testing. The Center will be essentially a service provider in respect of non-animal methods in learning, research and testing.

The Center is a fruitful culmination of a decade's work of People *for* Animals, Chennai, and I-CARE, Chennai, in promoting the concept of the 3R in India.

MS6: Associations

ecopa: realisations and future perspectives

V. Rogiers

ecopa, Brussels, Belgium

ecopa, the European Consensus Platform on 3R-Alternatives, is an International Not-For-Profit organisation, based in Belgium and complying with Belgian law. It is the quadripartite organization at the EU level promoting the 3Rs all over Europe. ***ecopa*** brings together National Consensus Platforms (NCPs) on alternative methods. Consensus means that the major parties concerned are represented, including animal welfare, industry, academia, and governmental institutes. ***ecopa*** actually counts 16 NCPs: 14 full members (Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Italy, Norway,

(the Netherlands), Spain, Sweden, Switzerland) and 2 associated members (Poland, Ireland). It is active through working groups. The fields of interest are concerned with Research, Policy, Education and Ethics.
ecopa is thus uniquely placed and has huge expertise to offer to the debate around scientific and politically-linked topics in the field of the 3Rs.
ecopa is in particular active in dissemination of 3R-information via its website: www.ecopa.eu and its newsletter: ecopa messenger. It organizes yearly workshops of general interest in the field of the 3Rs, expert workshops and 2 yearly eSI (ecopa Science Initiative) workshops bringing young scientists into contact with well established, more senior experts. ***ecopa*** is partner for dissemination of results in an important number of EU research projects on 3R-alternatives: Predictomics, ReProTect, Sens-it-iv, Carcinogenomics and Esnats. Dissemination is done via the website, newsletters, workshops and congresses. ***ecopa*** is also coordinator of the EU coordination action.
START-UP is concerned with the "Identification of bottlenecks/ strengths of 3Rs in different stages of drug development". As such ***ecopa*** stimulates the promotion, development and acceptance of 3R-alternatives. This is highly necessary since all parties concerned need to build up accurate knowledge in the field, understanding of the ethical, scientific, regulatory and economical concerns related to the switch from *in vivo* to *in vitro* testing, and above all realistic expectations.

ESTIV, a bridge between scientists working on alternative methods in Europe

G. E. R. Schoeters

European Society for Toxicology in Vitro, Belgium

The European Society for Toxicology *in Vitro*, ESTIV, connects people and information in the field of *in vitro* toxicology within Europe.

The field of toxicology is undergoing a big change as pressure mounts to reduce animal use while new technologies such as genomics, proteomics, and other cell-based molecular techniques have entered the field and offer new opportunities to develop and improve alternative tests.

The European Society for Toxicology *in Vitro* brings together, every two years, scientists that are committed to alternative tests. The ESTIV workshops (formerly INVITOX workshops) have recently expanded to congresses that are attended by more than 300 scientists from all over Europe. They cover a broad range of topics related to *in vitro* tests for different health endpoints, application of new technologies, needs from stakeholders. ESTIV encourages young scientists by awarding them for the best poster and best oral presentation. ESTIV seeks partnerships with alike organizations and supports and stimulates national scientific organisations that are active in *in vitro* toxicology by organizing joint initiatives. A major task of ESTIV is to advocate the value of the scientific progress made in refinement, reduction, replacement of animals for toxicology testing and as such act as the scientists' voice in the dialogue between different stakeholders.

Specific information on ESTIV is available at our web site www. estiv.org

CELLTOX – the Italian association for *in vitro* toxicology

I. De Angelis

Istituto Superiore di Sanità, Environment and Primary Prevention Department - CELLTOX Chair, Rome, Italy

The Italian Association for *In Vitro* Toxicology (CELLTOX) was founded in 1991; the following aims are the mainstays of our association and are expressed in its statute:
- to promote the use of *in vitro* experimental models and alternative methodologies, in pharmacological and toxicological field;
- to investigate mechanisms of toxicity at cellular and molecular level, with special interest for cell culture models;
- to facilitate the exchanges of information and collaborations among research groups of different public and private institutions;
- to create a network of information about ethical and practical aspects of the reduction of animal use, spreading the 3R principles and philosophy in the scientific community.

The association has also been particularly active in favouring the dissemination of alternative methods in toxicology by organizing scientific events and courses. The education of young researchers is one of our main goals and it is also pursued by giving grants and fellowships to support their participation to scientific events on alternatives.

CELLTOX board, as well as its members, comes from several public and private institutions, allowing a fruitful sharing of knowledge and expertise between them.

CELLTOX has often worked in close collaboration with similar European Societies and the European Society for Toxicology *in Vitro* (ESTIV).

In these last years, the association web-site, www.celltox.it, is become an important point of reference for all Italian people that work in the area of *in vitro* toxicology.

MS7: Lessons learned from the validation and potential regulatory applicability of *in vitro* alternative pyrogen tests

Development, purpose and importance of *in vitro* pyrogenicity tests

M. Daneshian

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Pyrogenic, i.e. fever inducing, contaminations in pharmaceuticals cause adverse effects, therefore pyrogen exclusion is required for all parenteral drugs. The rabbit pyrogen test has been used for this purpose since 1942, but animal protection issues as well as the scientific problem that the human and rodent response is not always congruent motivated the development of *in vitro* alternative methods. The *Limulus* amebocyte lysate (LAL) specifically detects the prototypical pyrogen endotoxin; however, among other limitations, it does not detect other pyrogens.

The *in vitro* pyrogen test systems of the second generation detect mediators, i.e. interleukin-1β or interleukin-6, induced upon exposition of human immune cells, i.e. human whole blood, mononuclear cells or a monocytoid cell line, to pyrogens, thus reflecting the *in vivo* human response. These systems detect the entire spectrum of pyrogens relevant for humans with the respective sensitivity. Quantification may be performed by comparison with a standardized stimulus, e.g. *E. coli* endotoxin. Human blood required for some of these methods can be made available to laboratories without access to fresh blood by cryopreservation. The *in vitro* pyrogen test (IPT) based on human whole blood has been adapted to an air-collecting system for air quality assurance, to the detection of pyrogens on solid biomaterials, and can be combined with albumin-coated beads for accumulation and subsequent detection of pyrogens in the femtomolar range or in immunomodulatory or toxic drug preparations.

In vitro pyrogenicity tests – lessons learned from the European validation study and ESAC peer review

M. Halder

European Commission Joint Research Centre, Institute for Health and Consumer Protection, Ispra, VA, Italy

Five *in vitro* pyrogenicity tests based on human blood cells (monocytes) have been validated within the framework of the EU-funded project "Human(e) Pyrogen Tests" and in a catch-up validation study coordinated by ECVAM. In March 2006, the ECVAM Scientific Advisory Committee approved their scientific validity. Recently, they had been adopted by the European Pharmacopoeia Commission and will be covered in the new General method 2.6.30 – Monocyte Activation Test (European Pharmacopoeia 6th edition). Following the peer review and recommendations of ICCVAM, the US Food and Drug Agency approved the methods in 2009.

The presentation will report on the various phases of and problems encountered during the validation process, summarise the outcome of the ESAC peer review process and the process of regulatory acceptance.

The monocyte activation test (MAT) for pyrogens in year zero of its regulatory acceptance

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

Paul-Ehrlich-Institute, Langen, Germany

The idea to exploit the human fever reaction for pyrogen detection was first described 25 years ago by Charles Dinarello. It took 15 years to develop and standardize such tests to enter validation and now another ten years until their regulatory acceptance by European Phramacopoeia and US FDA this year.

We have learned over these decades about the nature of pyrogens, the challenges of different products and the merits and limitations of different *in vitro* approaches. We have arrived at a situation where the traditional rabbit test is clearly outperformed by the novel assays for essentially any product assessed so far. The earlier partial replacement by the Limulus (or bacterial endotoxin) test has still some advantages with regard to price and duration, but its limitations with regard to species differences, limitations to Gram-negative endotoxins and multiple interferences by test substances are clearly overcome.

The agreement on a pharmacopoeial monograph is a milestone for the broad use of the novel tests. However, similar as in case for the Limulus assay further refinements, adaptations to test challenges and new uses will evolve. The presentation will take stock of the recent developments and discuss future prospects.

MS8: Industry activities 2

Assuring safety without animal testing: Unilever's ongoing research programme to deliver novel ways to assure consumer safety

C. Westmoreland, P. Carmichael, M. Dent, J. Fentem, C. MacKay, G. Maxwell, C. Pease and F. Reynolds

Safety & Environmental Assurance Centre, Unilever, Colworth Science Park, Sharnbrook, Bedford, UK

Assuring consumer safety without the generation of new animal data is currently a considerable challenge. However, through the application of new technologies and the further development of risk-based approaches for safety assessment, we remain confident it is ultimately achievable. For many complex, multi-organ consumer safety endpoints, the development, evaluation and application of new, non-animal approaches is hampered by a lack of biological understanding of the underlying mechanistic processes involved. The enormity of this scientific challenge should not be under-estimated. To tackle this challenge a substantial research programme was initiated by Unilever in 2004 to critically evaluate the feasibility of a new conceptual approach based upon the following key components: 1. Developing new risk assessment approaches. 2. Developing new biological (*in vitro*) and computer-based (*in silico*) predictive models. 3. Evaluating the applicability of new technologies for generating data that can be interpreted for risk-based safety assessment (e.g. "omics", informatics, mathematical modelling). Our research efforts are focussed in the priority areas of skin allergy, cancer and general toxicity (including inhaled toxicity). In all of these areas, a long-term investment is essential to increase the scientific understanding of the underlying biology and molecular mechanisms that we believe will ultimately form a sound basis for novel risk assessment approaches. Our research programme in these priority areas consists of in-house research as well as Unilever-sponsored academic research, involvement with EU-funded projects (e.g. Sens-it-iv, Carcinogenomics), participation in cross-industry collaborative research (e.g. Colipa, EPAA) and ongoing involvement with other scientific initiatives on non-animal approaches to risk assessment (e.g. UK NC3Rs, US "Human Toxicology Project" consortium).

Providing solutions for industry, regulators and the animal protection community

R. D. Curren

Institute for In Vitro Sciences, Inc., Gaithersburg, MD, USA

Current scientific, political and ethical concerns about the use of animals in safety testing are causing increased attention to *in vitro* (non-animal) methods. This interest is not limited to industry toxicologists, but also includes other stakeholders, e.g. regulators and the animal protection community. All three of these constituencies need to have reliable information on the availability and performance characteristics of *in vitro* methods, and, in addition, industry must be able to have a source which can conduct these tests on their products. The Institute for *In Vitro* Sciences (IIVS) – a non-profit organization – was created to fulfil these needs. The approach that IIVS uses to meet the above-mentioned needs is to operate a high quality laboratory facility that conducts *in vitro* safety and efficacy testing for industrial and government clients worldwide. Knowledge of the performance of various test methods and protocols gained from having this unique hands-on familiarity is then shared with others through education and outreach programs. IIVS strives to obtain experience with all types of *in vitro* models, especially those that may commercially compete with one another, so that unbiased information about the performance of the various systems is available. This is especially useful for international researchers who may only be able to conveniently purchase from a supplier in close geographic proximity. The overall goal is to accelerate the use and acceptance of *in vitro* methods through the sharing of practical information. Thus all stakeholders in the *in vitro* field benefit from our experience.

The science strategy of the European centre for ecotoxicology and toxicology of chemicals, ECETOC

D. E. Owen

Shell Chemicals Ltd, London, UK

ECETOC was established in 1978 as a scientific, non-profit, organisation. It has 51 member companies with a broad range of scientific expertise and experience in their respective external fora, including regulatory circuits. Most of these companies offer their senior scientific staff to work together, often with input from non-industry experts, on selected scientific topics. The strategic themes covered by ECETOC will be presented with examples of work programmes that have been running during the past year. One of the strategic themes is Intelligent Testing Strategies (ITS). ECETOC's work on topics such as fish testing and the 1-generation reproduction study will be reviewed, together with the oversight of several projects monitored by ECETOC which fall under the ITS theme.

The economics of animal testing

A. Bottini

Johns Hopkins Medicine International, Baltimore, USA

How do animal testing-based regulatory and economic measures interplay? Which role do European legislations and regulatory entities such as those of the European Commission play? How does globalization impact on animal testing? Is there harmonization of regulation or do differences prevail? If an economic approach is attempted does this mean that industries, the consumer and animals will be better off?

This thesis also investigates the role and networking of several different industries e.g. cosmetic and chemical industries, and looks deeper into how these are connected through animal testing in the validation of alternatives for consumer product safety. The economic implications of animal testing are colossal with sales value of regulated products in Europe alone touching 1.7 trillion € per year (5.6 t€ world-wide). Within this context alone the reader should be able to grasp the sheer enormity and massive effects on manpower, investments and animal numbers related to animal testing. Indeed the classical toxicology of chemical substances on animals costs 620 m€ in the EU (2-2.5 b€ world-wide), directly employs 15,000 people (world-wide 73,000) and involves about 60,000 experimenters (300,000 world-wide) and in terms of animals 23.3% of the 12.1 million animals used in the EU 2005 were for regulatory tests and 31% for industrial R&D!

For brevity reasons but also because of the limited availability of reliable data resources, the analysis discussed here focuses on Europe, but where possible and necessary, a global perspective is also taken, since the effects of globalization cannot be ignored.

MS9: 3R centers 3

CAAT: a 3Rs center for the 21st century

C. Howard

The Johns Hopkins Center for Alternatives to Animal Testing (CAAT), Baltimore, Maryland, USA

The Johns Hopkins Center for Alternatives to Animal Testing (CAAT), founded in 1981, is one of the oldest of the 3Rs Centers. For 28 years, CAAT's innovative programs have served to promote the creation, development, validation, and use of alternatives to animals in research, product safety testing, and education.

CAAT also is, in many ways, one of the newest of the 3Rs Centers, with a new director – former Head of ECVAM Thomas Hartung – and an ever-expanding array of new programs and projects.

This session will offer an overview of CAAT's diverse activities and resources, from our long-standing research grants program, workshops and symposia series, and awards (two CAAT awards will be presented at WC7) to such developments as a Transatlantic Think Tank of Toxicology (t4); a union of Altweb with the journal ALTEX (now all in English), a new website devoted to 3Rs Centers, a variety of policy and outreach programs, implementation of the report, Toxicity Testing and Assessment in the Twenty-first Century: A Vision and a Strategy, and more.

ANZCCART's publication strategy: maintain, update and expand

G. Dandie

CEO, ANZCCART, University of Adelaide, Australia

The Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART) is a not-for-profit organization charged with the responsibility of maintaining an informed and balanced public debate about the scientific use of animals as well as offering well researched, up to date advice to anyone wanting information. This is a role we have now been fulfilling for 22 years.

ANZCCART publishes high quality resource material for use by researchers, teachers and particularly Animal Ethics Committee (AEC) members across Australia and New Zealand. One example of this has been our series of Fact Sheets, which have been published progressively over the past 16 years. Of course times, ideals and attitudes change and during those 16 years, we have also seen two major revisions of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (The Code), with yet another revision currently underway. All of these factors have highlighted the need to institute a programme of regularly assessing the relevance of the material we publish and ensuring that it at least meets if not exceeds current international "best practice" standards.

We have traditionally employed a range of methods to disseminate information and have largely concentrated our efforts in the areas of research and tertiary teaching. While these sectors remain essential target areas, it has become increasingly clear that we also need to expand our area of expertise and influence to include the use of animals in both primary and secondary education. The use of animals in schools is also covered by The Code but has not traditionally received the same level of support as the tertiary sector in all regions.

The challenge for ANZCCART as well as many of our related organizations around the World is to achieve more, without real hope of additional personnel or genuine increases in our funding base. We propose that the answer lies in the strength of collaboration and formation of strategic partnerships and this is a path ANZCCART has actively begun to explore.

The new Three Rs program at the Canadian Council on Animal Care

G. Griffin and C. Gauthier

Canadian Council on Animal Care, Ottawa ON, Canada

For a number of years, the Canadian Council on Animal Care (CCAC) has been viewed as the Canadian Centre for the Three Rs by other like-minded organizations. In 2008 CCAC formally launched its Three Rs Program, providing the opportunity to outline, substantiate and better focus the role of the CCAC in the promotion and implementation of the Three Rs in Canada. The Three Rs Program is fully integrated within the CCAC, including appropriate links with the other three programs: Assessments; Guidelines; and Education, Training and Communication.

The Three Rs Program has already begun to establish a theoretical basis for its work and to prioritize its activities. The focuses of the program are: promoting the Three Rs through communication of the CCAC's ethics of animal experimentation, the maintenance of an up-to-date Three Rs microsite to make new Three Rs information and tools available to investigators, and the consolidation of CCAC's role as Canada's national centre for the Three Rs; and supporting the implementation of the Three Rs in all areas relating to the use of animals in science covered by the CCAC Program.

Plenary Sessions

Lecture PL1:

The U.S. "Tox21 community" and the future of toxicology testing

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In early 2008, the National Institute of Environmental Health Sciences/National Toxicology Program, the NIH Chemical Genomics Center, and the Environmental Protection Agency's National Center for Computational Toxicology entered into a Memorandum of Understanding to collaborate on the research, development, validation and translation of new and innovative test methods that characterize key steps in toxicity pathways.

A central component is the exploration of high throughput screening assays and tests using phylogenetically lower animal species (e.g. fish, worms), as well as high throughput whole genome analytical methods, to evaluate mechanisms of toxicity. The goals of the "Tox21 Community" are to investigate the use of these new tools to (1) prioritize substances for further in-depth toxicological evaluation, (2) identify mechanisms of action for further investigation, and (3) develop predictive models for *in vivo* biological response. Success is expected to result in test methods for toxicity testing that are more mechanistically based and economically efficient; as a consequence, a reduction or replacement of animals in regulatory testing is anticipated to occur in parallel with an increased ability to evaluate the large numbers of chemicals that currently lack adequate toxicological evaluation. The initial focus of this collaboration has been on identifying toxicity-related pathways (and assays for those pathways), establishing a Tox21 library of ~10,000 compounds, and developing the databases and bioinformatic tools needed to mine the resulting data. This presentation will summarize the coordinated approaches being taken to achieve our goals, the lessons learned, and expectations for the future.

Lecture PL2:

Animal health and welfare in the food chain: food for thought

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Basically we need food only for two reasons: (i) to provide building blocks necessary for the development, growth and repair of our body and allowing it to replace worn-out cells and tissues; and (ii) to provide our body with the energy necessary for it to perform adequately. With respect to the latter we are all well aware that while people starve to death in a greater part of the world, in our 'western societies our energy intake has reached levels which can be considered as severely hazardous. With respect to the former, today's major protein source is animal meat, a very inefficient source which, with a growing glo-

bal shortage of food, may not be sustainable. Intensive farming was thought to be the solution but the consequent poor animal health and welfare conditions seem to meet with increasing societal criticism these days. Fish farming, while still in its early days, apparently has not learned from the mistakes made by the bio-industry, and thus high fish density in fish farms has again already resulted in antimicrobial resistance in fish exposed to 'preventive' high levels of antimicrobials.

Next to the use of animals as a source of food, animals are used in considerable numbers to assess the safety of food and feed. Extensive legislation in the EU and elsewhere require at least some form of animal testing of food ingredients prior to its marketing. In addition, the call for more animal testing of food is sometimes used for political reasons, rather than being based on scientific concerns (i.e. for Genetically Modified Organisms – GMO's). On the other hand, the huge numbers of food addditives, enzymes, flavours and food contact materials to be assessed for safety, have forced regulatory scientists to develop more pragmatic and yet scientifically sound alternative approaches requiring substantially less experimental animals. Such approaches include: the use of QSARS (quantitative or qualitative structure-activity relationships), TTC (threshold of toxicological concern), and QPS (qualified presumption of safety).

The lecture will address the health and welfare of both food producing and experimental animals. In addition to science based arguments and suggestions the lecture will touch on some more philosophical aspects with an eye to the future of 2040-2050.

Lecture PL3:

Brueghel's two monkeys: passing the final exam in the history of mankind

I. Newkirk

People for the Ethical Treatment of Animals, USA

As society's ethical values expand over time, we understand that we must have consideration for more than just ourselves, our race, our gender, and our species. This speech will confront our biases and provide food for thought in moving beyond our current understanding of human-animal relations. It will help enable us to say what needs to be said about the use of animals, their suffering, and the appropriateness of the behavior of those around us.

History provides a lens through which our current norms can be viewed. It allows us to discern how our behavior might be perceived by future generations and this perspective can help us understand how to improve our behavior. While it is easy to be appalled by what has been done in the past, it is more challenging to uncover the actions taking place today that will be regarded with horror in the future and most important by far to be a part of the necessary change. By modifying one's perspective to include a more empathetic view of other animals, our obligations and potential become clear.

Examples will be provided of both resistance to change and the growing understanding that scientific knowledge does not have to be based on animal experimentation. Recent developments in science that allow – indeed demand – non-animal approaches to chemical testing are being embraced and developed even by institutions that have long been great promoters of animal testing. The new advances are building momentum to replace the use of animals and reinforce the animal rights community's long-standing contention that, where there is a will, there is a way to obtain information without the use of animals.

Lecture PL4:

Metabonomics-driven top-down systems biology: techniques and applications

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There has been a greater understanding of "druggable" targets through their characterisation involving measurement of gene expression (transcriptomics) and protein expression changes (proteomics). Metabonomics is a crucial and integrating component of a systems biology view of an organism because it allows inclusion of environmental factors such as diet, age ethnicity, life-style and gut microfloral populations can have a large influence.

Metabonomics is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" and involves the generation of metabolic databases, based on tissue or biofluid samples, for control animals and humans, diseased patients, animals used in drug safety testing, etc., allowing the simultaneous acquisition of multiple biochemical parameters on biological samples. It is also possible to use cell culture supernatants, tissue extracts and similar preparations and is hence directly implicated for animal replacement. Because animals an act as their own controls in many studies, it is particularly useful when considering animal reduction and refinement. Metabonomics now impacts many areas including animal models of disease, preclinical evaluation of drug safety, assessment of safety in clinical trials, improved understanding of idiosyncratic toxicity, improved differential diagnosis and prognosis of clinical diseases, better understanding of environmental population effects through epidemiological studies, patient stratification and the effects of interactions between drugs, and between drugs and diet.

The two most information-rich analytical techniques are mass spectrometry and nuclear magnetic resonance spectroscopy and the metabolic response of an organism is then extracted from the complex data sets by application of appropriate multivariate statistical analyses. Metabonomics also allows time-dependent patterns of change in response (metabolic trajectories) to stimuli to be measured.

This talk will cover the concept of systems biology and put into context some recently developed metabonomics data analysis methodologies and show how the information is integrated. The effects of symbiotic gut microflora on metabolic profiles will be covered. Some pharmaceutical applications of the approach will be illustrated, including the COMET project for evaluating drug adverse effects, and the prediction of an individual's response to therapy before drug administration. These examples will serve to show the usefulness of the approach in animal sparing and indicate how metabonomics is an integral part of systems biology.

Lecture PL5:

Predictive testing strategies: R&D achievements and perspectives

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Since the last world congress, a sustained and massive investment in the R&D for Alternative Safety can be acknowledged from academics, industries, trade associations and regulatory bodies in Europe and worldwide. The resulting scientific progresses and promises remain nevertheless driven in an industry perspective by realism and the only objective of perpetuating and insuring the Consumer Safety in the regulatory context of the 7th amendment to the Cosmetic Directive.

In a first part, mostly based on large number of data and case studies, an overview of the Research achievements for the development and the implementation of useful and usable batteries of alternative assays will be illustrated. The ongoing refinement and improvement of the currently existing or validated methods in cutaneous irritancy and genotoxicity will be discussed in the double perspective of the applicability domains expansion and/or the continuous improvement of specificity-sensitivity perform-

ances. The progresses in the development of rapid, efficient and cost effective skin bioavailability assays will be also presented as an another example of the optimization/refinement of the already implemented OECD TG428 *in vitro* skin bioavailability guideline. This effort was seen as a necessity since the availability of exposure estimates is indeed key for the implementation of new alternatives strategies for Safety assessment. The ocular irritancy and the acute toxicity endpoints will then be analyzed as study cases of the progresses and difficulties in the development and implementation of integrated testing strategy in these fields.

In a second part the lessons in terms of research policy will be drawn from the concrete study cases discussed previously. One major output of the R&D progresses and efforts reside in the work (done and ongoing) for development and integration of *In Silico* tools in the processes of safety evaluation. The R&D of *in silico* tools and strategies can thus certainly be seen as a paradigm of what is needed for an effective R&D in alternative toxicology: a close R&D collaboration effort between academic, regulatory bodies and industry, sharing the objectives, some data, and understanding of the needs of each parties.

Naturally mirroring the emergence of *in silico* technologies will also be discussed the place and role of new promising (bio) technologies in that quest for a new way of assessing the human safety of chemicals. Many trans-national and trans-organizational initiatives such as the US-EPA ToxCast program, research projects funded by the European Partnership for Alternative Approaches to Animal Testing (EPAA) and/or EU, rely on these new technologies marrying biology, micro electronic, microfluidic and data-mining. Illustrations of the promises and anticipated difficulties raised by this essential and necessary trend for the post 2009 challenges and their remaining scientific gaps will be presented and discussed. The skin-sensitization and systemic toxicity endpoints are domains in which such technologies could facilitate the emergence of new possibilities.

Finally the question of how the R&D efforts developed these last years, the amount of data generated on chemicals, the weight of evidence and the best industry practices could be used to support and accelerate the scientific validation and regulatory acceptance of the resulting Alternative Integrated Testing Strategies (ITS) will be discussed. The ocular irritancy, where hundreds of ingredients from a wide range of chemical classes have served for the optimization and development of such an ITS, will be here again used as a study case. What could be the place of the weight of evidence in the future, how to validate a battery of in silico plus *in vitro* assays, how to use these progresses in the frame of the European REACh regulation will be questioned.

To conclude a call will be made for a coordinated and sustained worldwide R&D effort for the raise of this new era of the alternative toxicology.

Lecture PL6:

Is forefront science considered and applied in regulatory risk assessment?

V. Silano

EFSA Scientific Committee, Parma, Italy

Modern societies are confronted with major uncertainties and complexities when making decisions, particularly in the public health sector. Achieving specific health targets requires the ability to predict possible interactions of many factors determining the end result of each decision. To this end, the approach known as risk assessment has been developed to identify the likelihood of unwanted end results.

Science is an absolutely necessary tool to understand the impact of health determinants and their complex interactions and to increase the likelihood of success of specific policies or proposals under consideration for possible adoption by decision makers. Science is also necessary to follow up the results of any decisions adopted, to assess the causes of success or failure and to understand what improvements may be needed on a case by case basis. Therefore, science and derived evidence-based knowledge are the key factors to ensure the success of public health policies and interventions.

Considering the nature of science and its development as a prerogative of human development, attributing an adequate role to science and ensuring regular use of science (and particularly of forefront science) in decision making could be seen an indicator of the social and technological development reached in a given country. Such an objective can be achieved only if an adequate regulatory framework is established to recognize such a need and clear procedures are adopted to ensure science independence. The answer to the question "is forefront science considered and applied in regulatory risk assessment?" depends, therefore, largely on the specific sector and country it applies.

While the current situation cannot be seen as satisfactory in all cases, an outstanding successful approach is represented by the EU Food Law, adopted in 2002 and in the following years mainly to overcome the consequences of the "mad cow disease". Such a regulation and its current implementation will be used to highlight key issues in addressing the above question.

Lectures PL7:

The principles of humane experimental technique: timeless insights and unheeded warnings

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In The Principles of Humane Experimental Technique, Russell & Burch said that "the central problem is that of determining what is and what is not humane, and how humanity can be promoted without prejudice to scientific and medical aims". They then explained how the Three Rs can be used to diminish or remove direct inhumanity ("the infliction of distress as an unavoidable consequence of the procedure employed") and contingent inhumanity ("the infliction of distress as an incidental and inadvertent by-product of the use of a procedure"). They concluded that "Replacement is always a satisfactory answer, but Reduction and Refinement should, whenever possible, be used in combination".

Many of the commonsense insights in The Principles are no less relevant today than they were in 1959. However, their warnings about the limited value of models and, in particular, the danger of succumbing to the high-fidelity fallacy (whereby it is assumed that the best models for humans are always placental mammals, because they are more like humans than other animals), appear to have largely gone unheeded. Of particular importance is their discussion on toxicity testing, which they saw as one use of laboratory animals "which is an urgent humanitarian problem, for it regularly involves considerable and sometimes acute distress". How, then, can it be that mammalian models are still routinely used in attempts to detect chemical carcinogens and reproductive toxins, despite the fact that the relevance to humans of the data they provide has not been, and perhaps could never be, satisfactorily established?

However, as Alan Goldberg points out, there are signs that some significant changes in attitude are taking place, which could be more in line with the thrust of The Principles, that good science and human technique inextricably go hand in hand. As Russell & Burch put it, "If we are to use a criterion for choosing experiments, that of humanity is the best we could possibly invent. The greatest scientific experiments have always been the most humane and attractive, conveying that sense of beauty and elegance which is the essence of science at its most successful." If we are to live up to that ideal in the 21st century, all concerned should take to heart the insights and heed the warnings.

The principles of humane experimental technique – is it relevant today?

A. M. Goldberg

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In the 1959 publication on "The Principles of Humane Experimental Technique", Bill Russell and Rex Burch stated, at the end of the chapter on Replacement, that "As new fields of biology open in the future, it may become a matter of routine to apply the lessons of the past and turn as soon as possible to the techniques of replacement." They recognized that *in vitro* techniques, in their infancy at that time, would become the science of the future.

Today, in the US, the National Academy of Sciences publication of "Toxicity Testing in the 21st Century – a Vision and a Strategy", proves their point. The recognition in this publication that the future of toxicity testing lies in the use of human cells in culture and methods that Bill Russell and Rex Burch could not have possibly conceived of in 1959 but identified generically as the future.

To truly establish the approach will now require very specific training in translational toxicology (the use of clinical observations to develop *in vitro* methods to understand pathways and systems biology) , the development of transnational programs, and ways to evaluate the accuracy, validity and importance of new and/or traditional studies (these evaluations are known as evidence based toxicology (EBT).

Science is the "Art of the Question". The concepts identified above are the tools to answer these questions. The principles that Bill Russell and Rex Burch developed during the 1954-59 writing of The Book are possibly more important today than they have been in the last 50 years. Their concept that the newest science and the most humane science is the very best science is being proven as each of us contribute to the worlds body of knowledge.

Lecture PL8:

Calling on science: making alternatives the new toxicity-testing gold standard

M. Andersen

The Hamner Institutes for Health Sciences - Program in Chemical Safety Sciences, Research Triangle Park, USA.

All of life's great journeys start with a goal in mind. The 2007 NAS report, "Toxicity Testing in the 21st Century - A Vision and A Strategy", has proposed a clear goal. This report envisions a not too-distant future where routine toxicity testing will be done in human cells *in vitro*, by evaluating perturbations of cellular responses in a suite of toxicity pathway assays. Dose response modeling would comprise computational systems biology models of the circuitry underlying each toxicity pathway; *in vitro* to *in vivo* extrapolations would use pharmacokinetic models, ideally physiologically based pharmacokinetic models, to predict human blood and tissue concentrations under specific exposure conditions. These toxicity assays and dose response tools would become the new gold standard for chemical risk assessment rather than high dose studies in animals. This talk focuses on the scientific challenges required to make this vision a reality, including characteristics of assay design, prospects for mapping and modeling toxicity pathways, concepts of assay validation, and biokinetic modeling. All of these tools are either available or in advanced development. Science must lead this transformation. However, the scientific community, regulatory agencies, and funding organizations will also have to muster resolve to quickly make this vision a reality.

Theme 1: Innovative technologies, concepts and approaches

Breakout Sessions

Session BS1: Integrated approaches

Exposure based waiving under REACH

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This presentation aims to describe criteria for exposure based waiving (EBW) as foreseen in the REACH regulation. This presentation is based on research done within the EU Sixth Framework project OSIRIS.

Within the REACH framework, but also within OECD, there is understanding that for reasons of animal welfare, costs and logistics, it is important to limit the number of tests to be conducted. Integrated Testing Strategies (ITS) will make it possible to increase the use of non-testing information for regulatory decision making of chemicals, and to effectively reduce animal testing without increasing the overall uncertainty. Exposure is one of the decision elements in ITS. Testing can be waived on the basis of exposure considerations (Exposure Based Waiving, EBW).

The principle behind EBW is that there are situations when human or environmental exposures are so low that there is a very low probability that the acquisition of additional effect information may lead to an improvement in the ability to manage risk. If absence of exposure cannot be argumented in a qualitative sense, a quantitative exposure assessment and risk characterization based on hazard and exposure may be needed, considering the exposure scenario developed in the REACH Chemical Safety Report. Quantitative justification for EBW needs an assessment that exposure is below a "no further action level" such as PNECs (Predicted No-Effect Concentrations), DNELs (Derived No-Effect Levels) or TTCs (Thresholds of Toxicological Concern). The "no further action level" should be applicable even when little toxicological information is available.

Chemical category evaluation as non-test component of ITS

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For reasons of resources and animal welfare, it is important to limit the number of tests to be conducted where this is scientifically justifiable. One approach is to consider closely related chemicals as a group, or chemical category, rather than as individual chemicals. In the category approach, not every chemical needs to be tested for every required endpoint. Rather, the data for chemicals and endpoints that have been tested are used to estimate the corresponding properties for the untested chemicals and endpoints. The categories could be defined by the structural, parametric, mechanistic and metabolic boundaries specifying its domain as well as the category members. They could be characterized by their structural and mechanistic consistency, which defines the category robustness. The missing data in a chemical category could be filled in by one of the following approaches: read-across, trend analysis or (Q)SAR models. Read-across involves identification of the closest analogues according to appropriate measure for similarity and the assumption that the analogues and the target chemical behave similarly. Trend analysis involves ordering of analogues according to molecular parameters associated with bioavailability, such as molecular weight, water solubility, partitioning coefficients, etc. (Q)SAR models belong to the estimation methods used to predict the behaviour of a chemical in a biological or environmental system based on the qualitative or quantitative relationship between an endpoint (activity) and one or more molecular descriptors. The categorization and data gap filling approaches will be illustrated by well selected case studies within the regulatory context and REACH legislation.

ID ABS: 64

Tiered approaches to the use of alternative approaches to animal testing for the safety assessment of cosmetics: skin and eye irritation, genotoxicity

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The use of strategies based on alternative approaches for hazard and safety assessment of cosmetic ingredients/products was investigated in a series of COLIPA scientific meetings in 2008. The focus was on skin and eye irritation (1,2) and genotoxicity (3).

For skin irritation, EpiSkin™, EpiDerm™ and SkinEthic™ models are validated as stand-alone replacements for the rabbit Draize test. Results from these *in vitro* tests are usually evaluated in combination with all available data. For eye irritation, no single *in vitro* assay has been validated as a full replacement for the rabbit Draize test. So far, organotypic assays only are accepted for specific and limited regulatory purposes. Further *in vitro* models (or combinations thereof) have been used by the cosmetics industry for decision-making in weight of evidence approaches.

There are many accepted *in vitro* mutagenicity/genotoxicity assays, but no single test detects all types of carcinogens. Therefore, a battery of tests is recommended but is prone to yield high numbers of non-relevant positive results (4). In order to replace follow-up *in vivo* animal tests, the specificities of *in vitro* assays need to be improved.

Here we present decision-tree approaches using available methods for skin and eye irritation and genotoxicity as part of weight of evidence assessments. Furthermore, recommendations are given on how remaining data gaps can be addressed.

References:
(1) Macfarlane et al. (accepted by *Regul. Toxicol. Pharmacol.*)
(2) McNamee et al. (accepted by *Regul. Toxicol. Pharmacol.*)
(3) Pfuhler et al. (submitted to *Regul. Toxicol. Pharmacol.*)
(4) Kirkland et al. (2005). *Mutation Research 584*, 1-256.

Use of TTC under REACH – are TTCs applicable to industrial chemicals?

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Thresholds of toxicological concern (TTC) are in discussion for exposure based waiving (EBW) to reduce animal testing under REACH. The TTC concept describes thresholds below which no appreciable risk to human health is assumed. TTC values have been derived for chemicals likely to be "encountered in commerce" (Munro et al., 1996) for three structural classes of chemicals; class 1 being non toxic, class 2 moderately toxic and class 3 toxic. They are currently applied to food contaminants, flavouring substances and residues in pharmaceuticals. So far the TTC values are specific to oral exposure.

We evaluated, whether the TTC-concept is applicable to industrial chemicals by using the database RepDose (Bitsch et al., 2006). It focuses on repeated dose toxicity studies with rodents for existing chemicals. Only about 18% of the substances are common with the Munro Database. The overall L(N)OEL distribution in Munro and RepDose as well as the distribution into the 3 Cramer Classes is very similar with slightly lower values in RepDose. For inhalation in RepDose lower values are obtained than for the oral route. In both databases, only a small number of chemicals belong to Cramer Class 2, although the L(N)OEL distribution indicates that the majority of chemicals are of moderate toxicity. Further there is a major overlap in the NOELs/LOELs in the 3 classes, i.e. toxic and non toxic chemicals are not well distinguished.

Overall, the data show that TTCs are also applicable for industrial chemicals, yet further refinements are desirable.

Weight-of-evidence based Integrated Testing Strategy (ITS) approach in human toxicology

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This presentation aims to describe the weight-of-evidence approach for combining testing and non-testing information in an Integrated Testing Strategy (ITS) under development within the EU Sixth Framework project OSIRIS.

Under REACH the health impact of over 30,000 substances has to be assessed in a relatively short time period. This implies that large amounts of information on their potential toxic effects on humans have to be generated. In principle this can be achieved by conducting a large number of relevant toxicity studies according to accepted guidelines. However, for reasons of animal welfare, costs, time and logistics, it is recognized as well that vertebrate testing should only be carried out as a last resort.

In order to facilitate a reduction in vertebrate testing, the REACH Regulation outlines a number of waiving options for guideline tests: when it can be demonstrated that *in vitro* data suffice, and/or the required test data can be replaced by non-testing information obtained *via* grouping (chemical categories) and analogue approaches (read-across), computational methods such as (Q)SARs or exposure estimates proving exposure is absent or too low to be toxic.

The REACH guidance doesn't, however, clearly outline any criteria the available information from the above referred testing and non-testing sources has to meet in order to be considered adequate for classification & labeling or risk assessment. Therefore, within the EU Sixth Framework project OSIRIS, we are designing a formal weight-of-evidence approach, based on Bayesian networks, providing these criteria.

Towards a theory of tiered testing as approach within ITS

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In regulatory applications, toxicological tests are combined into test systems. A test system contains rules for when and in what order the different tests should be performed. With limited resources it is necessary to use tiered test systems in which relatively simple tests are performed for a large number of chemicals, and the outcomes of these simple tests are used to prioritize substances for further, more resource-intensive testing. However, no general theory seems to be available for the combination of single tests into efficient tiered testing systems. In this contribution, the possibility to develop such a theory is discussed as well as what factual and value-related information is needed for the analyses. A method for the optimization of test systems for industrial chemicals, based on the calculation of efficiency ratios for tests and test systems will also be briefly presented. The efficiency ratio of a toxicity test depends on the monetary cost of performing the test and the probability that the test will identify a chemical of concern according to the rules for classification and warning labelling. Analyses of different standard tests according to the efficiency ratio indicates that, within the classification and labelling system, it is currently more efficient to perform short-term testing of a larger number of substances rather than to perform subacute toxicity studies on substances already tested for acute toxicity.

Session BS2: Chemical and physical methods

Optical biochips

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Earlier detection of diseases via biomarkers leads to better patient treatments and higher survival rates, but it also requires more sensitive and more specific tools. Higher sensitivity and specificity are gained through an optimization of all steps from sample preparation to signal transduction.

With the ever improving photon generation, propagation, confinement, interaction, collection and detection, bio-molecule recognition has relied more and more often on optics as the biosensing technique of choice to achieve unsurpassed sensitivity. In parallel, thanks to its access to molecule specific signatures through infrared spectral information, optical biosensing is now in a position to offer selectivity and specificity on top of sensitivity, placing it in the ideal position to solve the upcoming bio-molecule recognition challenges.

Due to its amenability to integration and compatibility with standard silicon, glass and plastic manufacturing, miniaturized optical systems for the detection of bio-active molecules, also called optical biochips, are gaining increasing market share in optical biosensing. Depending on their primary function, optical biochips can be segmented into microarrays, microfluidic chips to complete microsystems known as lab-on-a-chip (micro-TAS). The sensitivity, selectivity, functionality, speed, simplicity, degree of miniaturization and cost-effectiveness of such optical biochips can be enhanced if custom photosensors with application-specific smart pixels are employed.

The realization of well-known optical biosensing techniques using cost-effective application-specific chips will allow the monolithic integration of complete optical biosensor systems on one single, self-contained biochip of unprecedented complexity and functionality. This holds the promise of solving the upcoming challenges of increasingly rapidly moving bio-molecular targets.

Liquid chromatography-mass spectrometry detection of marine biotoxins in seafood: a technique to reduce mouse bioassays

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Relatively few species of naturally occurring microalgae of the world's oceans have the ability to produce potent marine biotoxins which can accumulate in filter feeding shellfish. Contaminated shellfish consumed by humans can cause gastrointestinal and neurological illnesses which, in certain cases, can be lethal. Thus, marine biotoxins pose a serious hazard to public health and can cause severe economic losses to aquaculture globally. To protect consumers, most countries have adopted shellfish monitoring programmes. Two types of mouse bioassay (MBA) are used as reference methods for routine detection of paralytic shellfish poisoning (PSP) toxins and lipophilic marine biotoxins. An advantage of the MBA is that overall toxicity of a sample is expressed, but toxin profiles cannot be monitored, the effects of combinations of toxins are unknown and assays can be prone to false positive and/or false negative results. Furthermore, ethical arguments against the continued use of live animal assays that cause substantial animal suffering has led the scientific community to develop alternative instrumental methods. Over the last decade, an increasing number of analytical methods involving the use of liquid chromatography-mass spectrometry (LC-MS, LC-MS/MS) have been successfully developed for the qualitative and quantitative determination of marine biotoxins. LC-MS/MS allows efficient toxin separation, high selectivity and sensitivity with lower limits of detection than the MBA, and accurate and precise quantification. Although a lack of certified reference biotoxin standards has hampered implementation of LC-MS/MS into monitoring programmes, once validated, such methodologies shall provide an alternative technique to the MBA.

Single molecule force spectroscopy of specific blood protein interactions

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Proteins found in blood have evolved the unique ability to function under the dynamic forces of the circulatory system. For example, fibrin, the polymeric network that forms the structural scaffold of a blood clot, must maintain its intermolecular bonds (known as knob-hole bonds) to stabilize the clot in a range of environments. Examining these interactions in traditional static biochemical assays contributes to an incomplete understanding of the complex function of these proteins. We developed a method to examine the behavior of specific bonds between such proteins under force with single molecule force spectroscopy (SMFS). We modify both an atomic force microscope tip and substrate with whole proteins or protein fragments containing the active site of interest. With these tools and the use of an atomic force microscope we acquired force-curves to examine unbinding behavior. By effectively eliminating non-specific interactions with a unique buffer system we were able to characterize the complexities of the specific interaction. Further, we studied the behavior of the proteins prior to rupture with custom analysis techniques. We found that the forced unbinding of the knob-hole fibrin interaction causes protein unfolding prior to rupture. To our knowledge, this was the first example of protein unfolding due to force applied to a specific bond. We expanded our method to study the behavior of FnBP binding to fibrinogen. With this highly specific method, we are able to elucidate the intricate nature of protein interactions that have evolved to function in blood.

ID ABS: 314

Saving animals by *in vivo* imaging with PET and MRI: proposed innovations in developmental neurotoxicty testing

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To protect children against adverse effects of exposure to chemicals in environment, drugs and food chain the law demands mandatory animal testing according to specific guidelines. Guidelines for Developmental Neurotoxicity (DNT) require large numbers of rats to test behavior and neuropathology at different ages. We hypothesize that implementation of non-invasive *in vivo* functional PET (Positron Emission Tomography) and structural MRI (Magnetic Resonance Imaging) may, in time, replace part of the behavior and neuropathology survey, thereby reducing the number of rats required for testing.

The potential of functional [18F]FDG (Fluorin Deoxy Glucose) micro-PET imaging was investigated relative to motor activity testing proposed in current DNT guidelines and structural MRI relative to neuropathology. Rats were exposed to a model developmental neurotoxicant methylazoxymethanol (MAM: 0 or 5 mg/kg BW/day, gestation days 13-15). In the offspring [18F]FDG uptake was measured by micro-PET (n=2x4=8) on PN18,21,35,61; motor activity (n=2x10=20) on PN13,17,21,61; MR imaging (n=2x6=12) and neuropathology (n=2x2x10=40) on PN21,61.

Regional cerebral [18F]FDG uptake was quantified and appeared significantly lower in the MAM-treated group, in particular on PN 18, whereas no significant differences in motor activity were observed between the two groups. Linear measurements in MRI scans confirmed size differences measured also during gross macroscopy. However, as demonstrated also during microscopic neuropathology, volume estimations (Cavalieri principle) are needed to detect the selective size reduction in predilection areas for MAM (e.g. frontal cortex and hippocampus). Together, the results show that [18F]FDG PET and MRI are very promising animal saving alternatives to assess DNT.

Session BS3: High throughput technologies

The role of quantitative high throughput screens in toxicology testing

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Assessment of toxicity has traditionally been *in vivo*, but such studies are low-throughput, costly, and inconsistently predictive of human toxicity. *In vitro* approaches using testing on human and rodent cells would potentially be more efficient, be more easily connected to molecular mechanisms, and allow construction of predictive algorithms to refine and reduce the use of animals in toxicology. The Tox21 collaboration was formed by the National Toxicology Program (NTP), the Environmental Protection Agency (EPA), and the NIH Chemical Genomics Center (NCGC), to advance this vision. At the NCGC, rapid parallel profiling of thousands of chemicals in cell-based and cell-free assays relevant to toxicity is being performed. A collection of 10,000 compounds is being assembled and tested across a broad range of *in vitro* assays of cellular toxicity phenotypes and biochemical/genetic pathways implicated in toxicity. All assays are performed at 15 concentrations, using the NCGC's titration-based quantitative High Throughput Screening (qHTS) paradigm (PNAS 103:11473, 2006), generating a concentration-response relationship for each compound in each assay. These data are being correlated with historical animal data from NTP and EPA, and computational algorithms are being developed which are initially aimed at prioritizing chemicals for further testing.

IHCP/ECVAM activities on high throughput and high content screening for hazard profiling of chemicals and nanomaterials

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The new REACH Directive on chemicals, the recent Amendment to the Cosmetics Directive, the emerging EU policy on Environment and Health, and the potential risk from engineered nanoparticles have all created a significant regulatory demand for validated *in vitro* test methods. The IHCP-ECVAM has recently established a high throughput screening (HTS) facility to support the assay validation process, to generate data useful for the development and assessment of computational toxicology tools, and to provide critical input for its programme on integrated testing strategies. The facility is also used to evaluate new assay technologies, with particular emphasis on micro, nano and optically based devices. This talk will give an overview of the facility and the associated data management system and will describe how the automation platform has been used for the purpose of assay validation. Recently a quantitative-HTS approach has been implemented and an explanation will be given on how throughput can be optimised without compromising quality. Examples of high (information) content methods currently under evaluation will be presented including a neurotoxicity test system based on micro electrode arrays (MEA) for electrophysiological measurements on primary neuronal cultures, and automated quantitative imaging platforms based both on fluorescent staining and label-free approaches. Some technical issues arising when testing nanomaterials will also be discussed, such as the challenges of reliable dispersion of the materials in cell media, the potential interference of nanomaterials with typical assay reagents, and the difficulties in quantifying the concentrations of nanomaterials in a matrix.

R&Ds of *in vitro* and *in silico* alternatives to animal testing under the strategic R&Ds of chemical risk analysis technologies by the NEDO, Japan

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Around 5,000 chemicals with a high production volume (HPV chemicals) are now used by industries internationally although we do not have enough safety data for most of them. The Ministry of Economy, Trade and Industry (METI) of Japan shares the responsibility for gathering biodegradation, bioaccumulation and health/ecological effect data for HPV chemicals.

To accelerate the HPV data gathering and, more generally, to promote chemical risk analysis among industries, the New Energy and Industrial Technology Development Organisation (NEDO), the R&D funding organisation supervised by the METI, started strategic R&Ds of chemical risk analysis technologies in 2000. The chemical risk analysis technologies include high throughput (HTP) assay systems, which target carcinogenicity, teratogenicity and immunotoxicity tests, as alternatives to animal testing, because it seems to be technologically difficult and time-consuming to develop alternatives to them. A cell transformation assay (CTA) using Bhas 42 cells has been established to detect both initiators and promoters among non-genotoxic carcinogens. The HTP assay systems on teratogenicity and immunotoxicity have also been developed using a multicolour luciferase reporter gene assay method. These are simple and mechanism-based alternative assay systems.

In addition, R&Ds of a QSAR *in silico* system and gene expression profile datasets, based on 28-day repeat dose oral toxicity studies, have been progressed as NEDO projects. The outlines of R&Ds on chemical risk analysis technologies and project formations of R&Ds of *in vitro* and *in silico* alternatives will be presented as a brief introduction to presentations to be made by Japanese scientists who carry out the projects.

OECD activities related to the international development and evaluation of high throughput technologies for hazard assessment

M. Oi

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The OECD Molecular Screening Project is led by the United States and supervised by the joint OECD/IPCS Advisory Group on Toxicogenomics. It evaluates a number of selected chemicals in a series of molecular screening *in vitro* assays (high throughput screening (HTS)) with the aim of establishing a strategy for rationally and economically prioritizing chemicals for further evaluation based on molecular properties and categories linked to potential toxicity.

The Extended OECD/IPCS Advisory Group met twice to discuss the details of the project in 2007 and 2008. At its second meeting held in Utrecht, the Netherlands (June 2008), it was agreed to set up several subgroups focusing on specific pathways, mechanisms and effects, nomination of target chemicals and database development.

The presentation of the OECD Molecular Screening Project will focus on (i) objectives and work items of the project, (ii) recent progress of each subgroup's work, as well as (iii) future steps and expectations.

Biokinetic factors in *in vitro* systems: analytical tools to analyse the freely available concentration

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The application of *in vitro* techniques in toxicology has mainly been restricted to studies on the mechanisms of toxic action and was therefore limited to hazard identification. When *in vitro* toxicity data are to be used quantitatively in risk assessment, an *in vitro-in vivo* extrapolation (IVIVE) must be performed. The first prerequisite for being able to do this is the relevance of the quantitative data regarding concentrations causing an effect in *in vitro* systems. ›

Since it is usually the freely available concentration that is the driving force for toxic reactions on the (sub-)cellular level, processes such as binding to proteins, to the culture plastic and evaporation will influence the free concentration. It is therefore necessary to estimate or measure this concentration, especially when it can be expected that the free concentration will differ from the nominal concentration on the basis of the physico-chemical properties (e.g. lipophilicity). One technique to do this is to sample the culture medium with solid-phase micro-extraction (SPME) devices and to analyse the compound. These devices consist of small rods covered with material absorbing the compound in equilibrium with its free concentration. This technique allows the identification of processes influencing the free concentration. This in turn enables the modelling of the *in vitro* system. The application of these techniques showed that for some compounds the free concentration could differ up to two orders of magnitude from the nominal concentration, showing the importance of understanding, measuring and modelling the "biokinetics *in vitro*".

Session BS4: Omics and systems biology

Gene expression profiling as a tool to develop alternatives to animal toxicity testing

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Genome wide modulation of gene expression as a result of exposure to chemicals can be monitored in cells and target tissues by DNA microarray technologies. This strategy is currently undertaken in various EU sponsored projects for different toxic effects, including carcinogenicity. Two main strategies for the application of toxicogenomics tools in hazard and risk assessment of carcinogens can be distinguished. First, is the identification of molecular mechanisms. Second, is the prediction of toxic properties of chemicals using gene expression signatures as biomarkers. Current standard assays for genotoxicity and carcinogenicity testing have several drawbacks, the main ones being 1) the high false-positives rate of *in vitro* genotoxicity tests, 2) the lack of an *in vitro* test for non-genotoxic carcinogens, 3) the high false-positives rate of the cancer bioassay, and 4) the requirement of many animals for a long period and with high inconveniency for the bioassays. Most of the current studies focus on liver as target tissue, applying both cell lines or primary hepatocytes cultured *in vitro* and tissue from rats. In summary, these studies prove the principle that gene expression profiling is capable of discriminating carcinogens with major differences in their modes of action, such as genotoxic from non-genotoxic carcinogens, and carcinogens from non-carcinogens.

Metabolic profiling as a tool in biomarker research and systems biology

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Metabolic biomarkers have much potential in biomedical and toxicological research. They can be measured non-invasively via imaging or body fluid profiling, which is better for the welfare of both patients and animals and facilitates longitudinal studies and translation of results between models and man. Metabolites are also defined chemical entities without genetic variation or post-translational modifications, which also helps to translate analytical methodologies directly between the bench and the bedside. A substantial

body of research has shown that metabolic profiles can report sensitively and specifically on a number of pathological states, both in terms of clinical disease processes and laboratory studies of genetic manipulation or chemical exposure. Certain conditions, such as Type II diabetes or cancer have defined metabolic phenotypes that are already exploited in diagnosis and therapy. Importantly, metabolic biomarkers have been shown to be predictive of the way that individual people or animals metabolise and respond to drugs. In this lecture I will review some of this evidence, and go on to present data from our own laboratory to show that metabolic profiling (metabonomics/metabolomics) is a crucial element of systems biology, enhancing the information "pathway" recovery from other "-omics" datasets.

Evaluation of chemically induced epigenetic alterations through whole genome methylation analysis

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Historically, to assess safety, toxicologists have focused on measurement of DNA damage, DNA adduct formation, and mutations induced by any given factor as the most appropriate indicator of carcinogenic potential. However, increasing recognition that exposure to carcinogenic agents can alter the expression of genetic information not only by genetic but also by epigenetic mechanisms, coupled with a better understanding of the role of epigenetic mechanisms in carcinogenesis, has challenged our current approach to carcinogenicity testing and indicated the need for a new generation of exposure biomarkers. The results obtained in a number of studies have demonstrated the role of epigenetic alterations as early indicators of carcinogenic exposure. We have conducted experiments to examine the role and contribution of epigenetic dysregulation to rodent liver carcinogenesis induced by genotoxic and non-genotoxic carcinogens. Long-term exposure of rats to genotoxic carcinogens, i.e. tamoxifen and 2-acetylaminofluorene, and non-genotoxic carcinogens, i.e. peroxisome proliferator WY-14,643 and lipogenic methyl-deficient diet, resulted in profound epigenetic alterations, characterized by aberrant DNA methylation, histone modifications, altered gene expression and altered miRNA expression. More importantly, our data demonstrate that the cellular epigenetic status, particularly DNA methylation, may predetermine sensitivity to carcinogen exposure and susceptibility to tumorigenesis. This has a great significance for the identification of vulnerable subpopulations and, considering the potential reversibility of epigenetic alterations, opens novel approaches to cancer prevention.

Systems modeling as an iterative approach towards understanding mechanisms of toxicity and carcinogenesis

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Recent developments in experimental high-throughput technologies and computational analysis have given rise to a paradigm shift in the analysis of biological systems. Systems biology aims at analysing biological processes and their perturbations at the network level rather than the single protein level. With the translation of these biological processes into computer readable networks and with the kinetic analysis of these networks, mathematical modelling tries to predict properties of these systems on different levels of granularity. The components of the models (genes, metabolites and their interactions) are typically linked to phenotypical read-outs, giving rise to testable hypotheses. The level of approximation that can be achieved with these models is dependent on the size of the model and the knowledge of the model parameters.

In this presentation we describe the development of mathematical models and the identification of their essential parts (components, interaction networks, kinetics) with respect to the analysis of toxicity and carcinogenesis. We present tools and resources, such as the ConsensusPathDB, a database that integrates comprehensive information on human molecular interactions, and the modelling and simulation software PyBioS, that are suitable for the construction of these models from scratch. Additionally, we describe a logical framework for predicting the effects of disturbances of the biological processes under study caused by, for example, toxic effects of chemical compounds.

Using systems biology to develop the gene-environment disease interactome for humans

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Understanding how the environment, broadly defined to include the natural, chemical, social and cultural environments, combines with our individual genetic make-up to initiate and/or promote human disease is key to the future of personalized medicine and improved public health. Using data on genetic polymorphisms in humans and the structurally-enhanced pathway enrichment algorithm (SEPEA), we are able to identify human signaling and metabolic pathways which, when perturbed, increase the risk of diseases in humans. Using this information, we are then able to link a large number of environmental compounds (vitamins, pharmaceuticals, food ingredients, man-made chemicals) to the same pathways and set priorities for further study. Specific examples will be given of pathways and diseases that are similar to each other due to the pathways that seem to trigger the disease. Parallels across diseases are then used to suggest mechanisms to be explored in order to find additional triggers and better understand the disease. Additional examples are given showing the potential for false hypothesis generation from different methods, illustrating how difficult these types of predictions could be. Suggestions for future research will be discussed.

Session BS5: Non-invasive technologies

Bioimaging of laboratory animals: reduce animals numbers, increase animal to human translation

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The numbers of animals required to effectively study dynamic *in vivo* processes is one of the many inherent limitations to using static tissue-based techniques. Experimental designs of longitudinal studies also pose particular limitations, because a certain degree of understanding about the kinetics of a disease process is critical to the appropriate timing of tissue evaluation. A large number of animals is needed for every experiment to enable the processing of tissue at given time points of interest. Imaging modalities available for use with laboratory animals provide a means to explore the molecular mechanism of diseases, minimize many of the limitations of static tissue-based techniques, and, most importantly, decrease the numbers of animals required. In fact, depending on the application, it is possible to reduce the number of animals required per study by as much as 80% to 90%. Imaging techniques that have been customized for laboratory animals provide scientists with the unprecedented ability to link detailed molecular understanding with the complexity of whole organism physiological responses and anatomical detail. This talk will highlight the successful use of specific imaging methods in lab animals by many individuals and laboratories to make the point that this technology can serve to reduce and refine the use of animals in drug discovery research and study of disease pathogenesis. Perspectives and considerations reviewed in a recent ILAR Journal Issue on this topic will also be discussed.

Neuroimaging techniques and applications to pain perception

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Over the last 15 years there has been a rapid development in neuroimaging technologies that have been applied to the study of human brain function. In particular, functional MRI (fMRI) and Magnetoencephalography (MEG) provide unprecedented spatial and temporal resolution of brain function in health and disease in human volunteers. Despite these technological and methodological advances, there continues to be a strong defence of the use of non-human primates for *in vivo* studies of brain function. The rationale for this lies in the defence of the exquisite targeting of site-specific neurons, yielding unparalleled spatial and temporal information. It is undoubtedly true that this approach has yielded invaluable data that has been translated to the human model and has produced valuable medical benefits. Whilst this continues to be the case in some areas of research, it can be argued that for some experimental procedures, non-invasive measures in man provide not only comparable, but in some cases superior data. In support of this argument, data from the application of magnetoencephalography and functional Magnetic Resonance Imaging, particularly in the study of pain perception in human volunteers, is compared to invasive non-human primate studies.

Non-invasive fMRI of the pain system in rodents under mild anaesthesia

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There is a demand for novel analgesics to provide relief for different types of pain. During development of new analgesics, traditional pain tests are performed measuring behavioural reactions of conscious animals, which consequently suffer from these painful stimuli. A solution could come from measuring responses to painful stimuli in the anesthetized animal non-invasively and by functional magnetic resonance imaging (fMRI). This method provides highly resolved, objective, functional information on the processing of nociceptive stimuli throughout the whole brain as has already been demonstrated in human pain studies. Consequently, this method could also improve the objective measurement of modulatory effects of analgesics. Moreover, optimized data-analysis strategies can help to reduce the number of experiments needed to obtain a representative group average response e.g. for different analgesics.

We established such an fMRI testing system in anesthetized rats and (transgenic) mice using a mild noxious heat stimulation applied to the hindpaw. Because testing is applied to animals under anaesthesia and stimulation is mild, we minimize the stress. We obtained 1) reliable information of different pain competent structures throughout the whole brain 2) representative group average results at a minimal number of experiments by applying modern image analysis 3) analgesic drug effects. This would open a new avenue for pain research, and it may contribute to the evaluation of novel, more specific analgesics.

Non-invasive imaging of small animals in biomedical research: multimodal imaging with PET and MRI

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Non-invasive anatomical or functional imaging of small laboratory animals has a growing impact in the field of biomedical research. Dedicated high resolution imaging modalities like positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT), optical imaging (OI), single photon emission computed tomography (SPECT) or multimodality systems like PET/CT or PET/MRI have evolved into mature diagnostic scanners. While PET reveals quantitative functional information about the biodistribution or metabolism of radiolabeled biomarkers in the picomolar range, MRI or CT provides morphological information in the sub-millimeter, down to the micrometer area. Latest multimodality systems combine anatomical with functional information in one single imaging session. Small animal imaging has spread to many fields in biomedical research beyond the conventional core areas of diagnostic imaging oncology, cardiology and neurology towards immu-

nology, infectiology and inflammation. Non-invasive molecular and anatomical imaging can contribute significantly to the 3R concept, specifically by reducing the total number of animals needed for one study. The talk will provide a comprehensive review of latest high resolution small animal imaging technology and review applications from different fields of biomedical research. Furthermore, an outlook to the next generation of multimodality imaging systems will be provided.

ID ABS: 109

Urinary metabolites as non-invasive biomarkers for the pleiotrophic effects of peroxisome proliferator activated receptor-alpha agonists in rats

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A diverse group of chemicals, including hypolipidemic and antidiabetic drugs, herbicides, plasticizers and fluorinated compounds, possesses PPAR-α agonist properties. Traditionally, investigation of these agonists requires a large number of animals for the assessment of known and putative endpoints such as hepatomegaly, peroxisome proliferation, fatty acid β-oxidation, oxidative stress and tryptophan-NAD metabolism. Recent advances in metabonomic and assay techniques enable evaluation of urinary metabolites as non-invasive biomarkers of some of these effects. Male rats were administered orally corn oil (control) or di(2-ethylhexyl)phthalate (DEHP), clofibrate (CLO), perfluorooctanoic acid (PFOA) in corn oil at 100 mg/kg (day 1-3, corn oil only, day 4-6, corn oil or test chemicals, day 7-9, non-dosing period). Urine was collected daily and analyzed for (1) ascorbic acid (AA), a biomarker associated with hepatomegaly, (2) quinolinic acid (QA) and N-methylnicotinamide (NMN), metabolites in the tryptophan-NAD pathway, and (3) thiobarbituric acid reactive substances (TBARS) and 8-hydroxy deoxyguanosine (8-OHdG), biomarkers of oxidative stress. PFOA, a potent PPAR-α agonist, and clofibrate caused 11 and 4 fold increases in hepatic palmitoyl Co-A oxidase activity, and 70% and 9% increase in liver weight. The most responsive urinary biomarker was AA with a peak elevation of 19 (day 4) and 6 fold (day 5) following PFOA and CLO treatments. PFOA alone caused a peak 4 fold increase in QA and 3-fold elevation of NMN. PFOA also produced a mild increase in urinary 8-OHdG but no significant increase in TBARS. Conclusion: urinary metabolites have potential as alternative, non-invasive endpoints in the toxicological and time course studies of PPAR-α agonists.

ID ABS: 313

Ultrasonic vocalizations (USV) in rat pups. An animal friendly marker for neurotoxicity during development

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To protect children against exposure to chemicals in the environment the law demands safety testing, including mandatory research in animals (mainly rats). The developmental toxicology studies require daily testing of hundreds of animals to reflect on their development. This behavioral survey during the pre-weaning phase requires intensive handling of the pups to study the development of senses and reflexes. Although not invasive, these activities greatly disturb normal housing and nursing of the litter in the home cage. We argued that at least some of this tedious testing could be replaced by an animal friendly marker with high discriminative power. Hereto, we studied the Ultrasonic Vocalizations (USV) of the rat pups, i.e. calls of 35-65 KHz emitted by rodent pups to their mothers. USVs were counted during 30s each day, from postnatal day 4 to 18. The rats were exposed perinatally to known developmental toxicants (MeHg, DOTC and TBTO). The question was raised whether USVs could serve 1) as a marker to study normal development and 2) as a marker to study developmental (neuro-)toxicity. The results showed that the number of calls emitted by the pups changed over time from day 4 to 18 postnatally, with a peak around day 12. TBTO and MeHg, both neurotoxicants, clearly affected the number of USV calls, whereas DOTC, a known immunotoxicant, did not. These results support the suggestion that USVs form an animal-friendly marker to study neural development and developmental neurotoxicity and warrant a role for USVs in developmental safety testing in time.

Session BS6: Non-vertebrate models

ID ABS: 327

Dictyostelium amoebae: a model host to study infectious bacteria

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Bacterial infections are a major health problem worldwide, aggravated by the rise of antibiotic-resistant strains. The study of pathogenic bacteria is essential to develop new strategies to control infectious diseases. In order to study the complex relationship between infecting bacteria and the host, it is necessary to infect a host, often a rodent, and to observe the infection as it progresses. For both ethical and practical reasons, it can be advantageous to replace rodents with non-mammalian model hosts.

In our laboratory, we use *Dictyostelium amoebae* as an alternative model to study host-pathogen interactions. We have previously shown that this system can be used to study the pathogenic mechanisms of many bacteria. Our recent studies illustrate how this system can also be used to identify and to study host resistance genes. We will notably present the identification and characterization of Kil2, a new gene product involved in the killing of bacteria.

Kil2 encodes a P-type ATPase transporter present in the phagosomal membrane. Our results suggest that Kil2 pumps magnesium ions into the phagosomes, and that this is a process essential to kill certain bacterial species. These results should enable a better understanding of the mechanisms allowing host cells to kill bacteria and to control infections.

ID ABS: 10

Reducing animal-based drug toxicology testing using a single cell organism

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Dictyostelium is a simple, single celled organism – an amoeba – used as a model system for biomedical research. For example, we use *Dictyostelium* to better understand the mechanism of action of several commonly used psychiatric drugs. Our current study investigates using *Dictyostelium* as a novel system for reducing and possibly replacing animal use in testing new drugs that may have vomiting as a side effect (emetic liability). Current models for these experiments employ ferrets, dogs, shrews and rats in procedures that are often distressing to the animal. In this project, moving *Dictyostelium* cells were subjected to a range of compounds with known emetic or "taste" aversive effects, and the speed and shape of cells were recorded and analysed using image analysis software. Results of the study showed that an emetic metal (copper) and different bitter (aversive) compounds have large and rapid effects on cell speed and shape. However anti-cancer drugs such as cisplatin (with known emetic liability) were without apparent effect during the short exposure periods employed and must be tested in more detail. These results indicate that *Dictyostelium* is sensitive to some emetic/aversive compounds. Further studies are required to determine the spectrum of compounds to which this organism is sensitive prior to its potential use in reducing the number of animals used in screening novel drugs for emetic side-effects.

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Medium and high-throughput toxicity screens using *C. elegans*

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The use of the free-living soil nematode *Caenorhabditis elegans* as an alternative model organism in high-throughput chemical screening is being assessed. Several characteristics of *C. elegans* biology indicate that it can serve as a model in studies of human disease and toxicology. First, a high degree of evolutionary conservation between *C. elegans* and higher organisms is observed in many of the signal transduction and stress-response pathways. In addition, homologues for many of the genes induced in response to toxicant exposure in vertebrates have been identified in *C. elegans*. For these reasons, it is likely that responses elicited in *C. elegans* will be applicable to understanding similar processes in humans. Methods have been developed to rapidly measure sublethal toxicity endpoints including growth, reproduction, feeding, and movement. These assays utilize COPAS Biosort flow cytometry technology to simultaneously measure size and florescence of individual nematodes under different chemical exposure conditions or automated microscopic observation. In addition to rapidly collecting nematode data, new mathematical and statistical models have been developed to quantitatively measure chemical toxicity. Using automated high-throughput technologies with statistical modeling, several chemical libraries have been tested, including the NTP 1408 and the ToxCast 320. Currently, high-throughput assays are being developed, using GFP-based transgenic *C. elegans*, to measure the effects of toxicants on neuronal development, DNA damage response, and the transcriptional activation of stress-response genes. Results from these studies demonstrate that *C. elegans* can be used to rapidly screen large numbers of chemicals as part of a toxicity testing program.

ID ABS: 47

Galleria mellonella as a flexible mini-host system to study virulence of *Aspergillus fumigatus* and efficacy of the antifungal drugs used in treatment

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Background: Traditionally, mammals are used to determine pathogenicity and drug efficacy. Mini-hosts provide a flexible, economical replacement. Here, *Galleria mellonella* (Gm) were used to determine drug efficacy and virulence of *Aspergillus fumigatus* (AF), an important pathogen with mortality rates of ~50%. AF pathogenicity and therapeutics can be studied using Gm survival.

Methods: 9 clinical and 6 mutant AF isolates were used for pathogenicity studies based on historic virulence assessment in mice. Groups of 30 Gm were injected with AF using inoculae of 80-800,000 CFU/larva. For efficacy studies AF 1163 was injected into groups of 10-20 Gm at 200,000-850,000 CFU/larva. 3, 24, 48 and 72 hours post infection 4 antifungal drugs were administered at the same doses as in mouse models. All larvae were injected into the last pro-leg, incubated at 37°C for 7 days, and daily survival was recorded.

Results: Rank order of virulence for clinical and mutant strains based on LD_{90} compared excellently with mice, with highest and lowest ranking strains identical in both models. A good dose response was noted with 3/4 drugs (the cyclodextrin vehicle was toxic for one compound). Uninfected, vehicle treated Gm had 100% survival except for cyclodextrin. Uninfected controls in both models showed ~100% survival.

Conclusions: Gm are an excellent invertebrate model to study AF pathogenicity and screen drug efficacy. This data demonstrates a strong correlation between virulence in Gm and mice and replicates efficacy studies in rodents. This model is a robust, economical, extremely flexible system with few ethical constraints.

Drosophila melanogaster: an invertebrate genetic model system for complex-disease screening and testing

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For over a century, the fruit fly, *Drosophila melanogaster*, has provided a powerful model system for revealing the functions of genes and chromosomes. Using techniques for transgenic manipulation of fruit flies, mechanisms underlying development, behavior, neoplasia, and xenobiotic detoxification, among others, have been revealed. Recently, *Drosophila* has emerged as a system for testing and screening drugs for potential therapeutic indications. Evidence is also emerging that fruit flies could be used to identify gene-X-environment interactions that are proposed to underlie complex disease, including developmental neurobehavioral disorders. The value of *Drosophila* in this domain emerges from the combination of remarkable phylogenetic conservation of genes controlling cognitive function, a complex behavioral repertoire, and a relatively simple nervous system. Our data indicate that some behavioral features of autism, notably stereotyped repetitive behaviors, can be modeled in *Drosophila*. Autism is associated with fragile X syndrome (FXS) in a significant fraction of affected children. *Drosophila* fragile X mental retardation 1 (dfmr1) is the fly ortholog of human FMR1, mutations of which cause FXS. Using videomicroscopy of individual flies, we demonstrated a highly significant increase in spontaneous grooming by dfmr1 mutants compared with their genetic controls. Ethogram analysis revealed that the excessive grooming is not random; rather, mutants engage in repetitive patterns of grooming. The mutant grooming behavior is reminiscent of stereotyped repetitive behaviors in children with autism spectrum disorders. We are now in a strong position to test the hypothesis that chemical exposures during development can modify the excessive, repetitive grooming behavior of dfmr1 mutants.

Session BS7: *In silico* models

3R systems for biomedical discovery acceleration

L. Hunter

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The profusion of high-throughput instruments and the explosion of new results in the scientific literature, particularly in molecular biomedicine, are both a blessing and a curse to the bench researcher.

Even knowledgable and experienced scientists can benefit from computational tools that help navigate this vast and rapidly evolving terrain. However, effective design and implementation of computational tools that genuinely facilitate the generation of novel and significant scientific insights remains poorly understood. In this talk, I will describe a set of efforts that combines natural language processing for information extraction, graphical network models for semantic data integration, and some novel user interface approaches into a system that has recently played a pivotal role in making a significant biomedical discovery. I will also speculate about possible applications in predictive toxicology.

Predicting cyp-mediated xenobiotic metabolism in human skin

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Metabolic stability is crucial in allowing drugs to reach therapeutic concentrations or to avoid formation of toxic metabolites. Recently *in silico* methods have been developed with the aim to predict soft spots (the molecular site of metabolic reactions) and chemical pathways in the human liver and proved very successful. However, such methods do not provide information on the soft spots for the compound in human skin, limiting rational design to overcome potential degradation and toxic effects in this body compartment.

This paper describes an advanced *in silico* procedure for soft spots identification coupled with mass spectrometry (MS) data from high-throughout clearance assays, allowing the location of soft spots in human skin much faster and earlier in the drug discovery process.

The procedure, experimentally tested and validated in a batch of cosmetic compounds, shows that data on skin metabolism mediated by cytochrome P450 (CYP) enzymes can now be obtained using high-throughput prediction with a software program called MetaSite, without microsomal assays or metabolic degradation with artificial skin rhCYPs cocktail composition.

It therefore seems that incorporating this procedure in early discovery research could considerably increase the likelihood to improve the metabolic stability of compounds and provide also chemical strategies for stabilization. In turn, this may have a beneficial effect on toxicity prediction and safety care.

In silico prediction of the toxic potential of drugs and chemicals

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A fully automated technology has been developed at the Biographics Laboratory 3R to assess the toxic potential of drugs and chemicals *in silico*. It simulates and quantifies the binding of any small molecule of interest towards a series of 12 proteins known or suspected to trigger adverse effects. The underlying "mixed model approach" – a combination of automated, flexible docking and multi-dimensional QSAR – considers the protein-ligand interaction, induced fit, solvation and entropic effects. The toxic potential is derived from the normalized binding affinities towards those 12 proteins and weighted using their standard deviations as well as the quality of the underlying models (number of training and test compounds and the covered activity range). The toxic potential is based solely on thermodynamic considerations and does not include any ADME (adsorption, distribution, metabolism, elimination) aspects. A low toxic potential does not necessarily prove a compound's safety but indicates a low probability for triggering endocrine-disrupting effects. The technology allows rationalizing a prediction at the molecular level by interactively viewing and analyzing the binding mode of the tested compound at all 12 target proteins in 3D. In the recent past, we have calculated the toxic potential of over 2,000 compounds, including 500+ chemicals; the results are posted on http://www.biograf.ch. In the presentation, selected compounds shall be discussed on a mechanistic basis and in the context of REACH and the 3Rs. Reference to the technology: http://www.biograf.ch/downloads/VirtualToxLab.pdf and references cited therein.

The cyberbiohybrid lung: a prototype cell-based computational model for pulmonary drug development

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Cell-based molecular transport simulations have been developed to help interpret drug absorption, distribution, metabolism, and excretion (ADME) data across species, from the microscopic cellular level to the macroscopic organism level. Here, we elaborate a computational pharmacokinetic model for ADME prediction in the lung. In this model, mathematical equations represent the passive transport of lipophilic molecules across cellular membranes. In turn, transmembrane transport processes are coupled to each other to capture the three dimensional organization of the airways, the lung's histological architecture, and pulmonary physiological parameters such as alveolar surface layer thickness, pH, lipid content and mucociliary clearance. For a weakly basic drug-like molecule, varying the molecule's pKa and lipophilicity is shown to affect

the rate at which the molecule appears in the blood and its distribution in the lung. Based on experimental data, the model's parameters can be optimized to fit measurements of transcellular permeability (*in vitro*) or organ-associated drug mass (*in vivo*). The model's input parameters can be adjusted so as to mimic delivery from inhaled (aerosol) formulation. Using in-house and published data of experimental pharmacokinetic measurements, we are evaluating this "cyberbiohybrid organ", integrating transport simulations with *in vitro* bioassay results as a complementary – and potentially alternative – approach to animal models for pulmonary drug development and toxicity screening.

ID ABS: 240

The patient specific virtual cardiac ventricular wall: quantitative prediction of pro-arrhythmic effects of anti-arrhythmics

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Methods for the experimental investigation of cardiac arrhythmias are limited to inferring propagation within the myocardium, from optical recordings (using voltage sensitive dyes) or multiple electrode surface measurements, or from plunge electrodes at a few sites within the cardiac wall. Clinical methods can use intra-cardiac electrodes (via a catheter) or multiple surface electrodes. None provide information about activity within the ventricular wall, and ventricular arrhythmias are intrinsically three-dimensional. Biophysically and anatomically detailed computational models of cardiac tissues offer a powerful way to study the three dimensional electrical propagation processes and arrhythmias within the virtual heart. We use virtual tissues to study and visualize the effects of patho- and physiological conditions, and pharmacological interventions on transmural propagation in the virtual ventricular walls. We illustrate the automated construction of a virtual anisotropic ventricle from Diffusion Tensor MRI for individual hearts, and use it to explore mechanisms leading to ventricular fibrillation. Case studies are based on personalised structure (hypertrophy), electrophysiology (mutations in hERG, and the long-QT syndromes) and anti-arrhythmic actions on the transmural dispersion of action potential duration – (Dronedarone and NS1643). The virtual ventricular wall provides an effective tool for exploring, evaluating and visualizing processes during the initiation and maintenance of ventricular arrhythmias.

This research was supported by the Dr. Hadwen Trust.

Session BS8: Databases: scientific approaches

Data integration in the life sciences: from data to networks, models and phenotypes

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We are witnessing the emergence of the "data rich" era in biology. The myriad data in biology ranging from sequence strings to complex phenotypic and disease-relevant data pose a huge challenge to modern biology. The standard paradigm in biology that deals with "hypothesis to experimentation (low throughput data) to models" is being gradually replaced by "data to hypothesis to models and experimentation to more data and models". And unlike data in physical sciences, that in biological sciences is almost guaranteed to be highly heterogeneous and incomplete. In order to make significant advances in this data rich era,

it is essential that there be robust data repositories that allow interoperable navigation, query and analysis across diverse data, a plug-and-play tools environment that will facilitate seamless interplay of tools and data and versatile user interfaces that will allow biologists to visualize and present the results of analysis in the most intuitive and user-friendly manner. This talk with address several of the challenges posed by enormous need for scientific data integration in biology with specific exemplars and possible strategies. The issues addressed will include:

- Architecture of Data and Knowledge Repositories
- Databases – Flat, Relational and Object-Oriented; what is most appropriate?
- The imminent need for Ontologies in biology
- The Middle Layer: How to design it?
- Applications and integration of applications into the middle layer
- Reduction and Analysis of Data – the largest challenge!
- How to integrate legacy knowledge with data?
- How can we generate networks and models of living systems?
- Can we compute and predict phenotypes with these models?

The complex and diverse nature of biology mandates that there is no "one solution fits all" model for the above issues. While there is a need to have similar solutions across multiple disciplines within biology, the dichotomy of having to deal with the context, which is everything in some cases, poses severe design challenges. For example, can a system that describes cellular signaling also describe developmental genetics? Can the ontologies that span different areas (e.g. anatomy, gene and cellular biology, functional imaging) be compatible and connective? Can the detailed biological knowledge accrued painstakingly over decades be easily integrated with high throughput data? These are only few of the questions that arise in designing and building modern data and knowledge systems.

Mining on expressed sequences: examples from plant genomics

M. Chiusano

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The success of bioinformatics approaches is directly dependent on the efficiency of data integration, which in turn is determined by the diversity of data sources, the quality of their annotation and the level of details of the information produced. Here we present results from Plant EST (Expressed Sequence Tag) database analysis to support expression patterns detection from organism specific libraries.

EST collections are certainly no substitute for a whole genome scaffold and show high levels of sequence redundancy and low quality sequence attributes. However, they currently represent the core foundation for understanding genome functionality and the most attractive route for broad sampling of the transcriptome from specific libraries. Here we discuss our strategy to enhance data quality and increase data information content. Moreover, we describe our effort to exploit data classification and the analysis of co-expressed genes. Preliminary results and possible strategies to focus on genes of specific interest are discussed.

Go3R, http://www.Go3R.org, the first semantic search engines for alternative methods

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Consideration and incorporation of all available scientific information is an important part of the planning of any scientific project. As regards research with sentient animals, EU Directive 86/609/EEC for the protection of laboratory animals requires scientists to consider whether any planned animal experiment can be substituted by other scientifically satisfactory methods not entailing the use of animals or entailing fewer animals or less animal suffering before performing the experiment. Thus, collection of relevant information is indispensable in order to meet this legal obligation. However, no standard procedures or services exist to provide convenient access to the information required to reliably determine whether it is possible to replace, reduce or refine a planned animal experiment in accordance with the 3Rs principle. The search engine Go3R, which is available free of charge under http://Go3R.org, promises to become such a standard service. Go3R is the first search engine on alternative methods world-wide, building on new semantic technologies that use an expert knowl-

edge based ontology to identify relevant documents. Owing to Go3R's concept and design, the search engine can be used without lengthy instructions. It enables those involved in the planning, authorisation and performance of animal experiments to determine the availability of non-animal methodologies in a fast, comprehensive and transparent manner. Thereby, Go3R strives to significantly contribute to the avoidance and replacement of animal experiments.

Data mining and knowledge extraction from scientific data bases

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In this talk we consider some state of art methods to extract knowledge from databases. In particular, we focus on methods based on the logic representation of data and knowledge, report on the main computational issues related with this task, and highlight the role of such methods in the discovery process of many sciences, with particular attention to bio-engineering applications. We describe feature selection problems, that need to be solved when the number of features observed in the samples is very large, and logic separation problems, used to identify compact logic formulas that represent the knowledge contained in the data. Finally, we describe some of the software tools available for their solution and discuss some recent experimental results obtained.

ID ABS: 560

Chemical safety assessment – evaluation of *in silico* predictive system

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Purpose: The aim of this study was to investigate how *in silico* methods based on systems biology can help to identify potential safety issues and help to create hypotheses about the mechanism of action based on the chemical structure of the compound.

Methods: ToxWiz is a database solution that was evaluated for application in chemical safety assessments. This system contains more than 1,000 pathologies and 1,300 toxic endpoints linked to 100,000 molecules, with over 4 million expert curated articles. It was used to explore the effect of chemicals widely used in everyday life with the special focus on cosmetics.

Results: We have demonstrated how the power of networks can help to investigate the problems that might arise during the exposure to multiple chemicals at the same time. We were able to get quick access to information, such as association of some chemicals used in cosmetics with skin sensitization and eye irritation. Relations were easily visualized and investigated further. The ToxWiz database was able to make a collection of viable hypotheses on mechanisms and interactions, and demonstrated significant advantages over conventional trawling through literature.

Conclusions: By saving time and optimizing experimental design, this kind of tool proves to be valuable in the process of prescreening novel and existing chemicals. Although it is not yet possible to fully replace animal models in chemical safety assessment, present development of *in silico* systems is without any doubt a crucial step towards achieving this goal.

Session BS9: *In vitro* technologies

The development of tissue engineering models for the study of human liver diseases

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The development of experimental systems for the long term *in vitro* culture of functional liver tissue would have profound impact in three areas of biomedical science:

- *in vitro* pharmacological screening and toxicology studies
- the design of bioartificial liver devices for the metabolic support of liver failure
- the development of robust models of human liver diseases

Monocultures of primary hepatocytes, however, rapidly de-differentiate following removal from the highly spatially arranged and multicellular *in vivo* environment. We hypothesised that co-culture of hepatocytes with hepatic stellate cells (HSC) may provide environmental signals necessary for the preservation of hepatocyte specific functions *in vitro*. HSC are the major non-parenchymal cell in the liver and have a central role in the liver response to injury. HSC may therefore have utility in disease modelling. Co-culture of human hepatocytes and HSC on non-adherent PDLLA surfaces led to the formation of 3D spheroids. Spheroids showed enhanced longevity and preservation of hepatocyte specific functions as assessed by urea secretion, albumin synthesis and CYP450 functions. These effects were further enhanced by culture of human spheroids in extra-cellular matrix. We are now using tissue engineering techniques to form complex porous scaffolds with nutrient channels and the capacity to incorporate adhesion molecules and other peptides. We hypothesise that this highly ordered environment will further enhance primary hepatocyte function. Finally, we present the development of a novel model for the detailed study of HSC activity and demonstrate the use of this system to identify novel pro-inflammatory signals in human liver.

ID ABS: 39

Advances in the development of an *in vitro* high-throughput test system to evaluate the carcinogenic potential of chemicals

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Up to the present time, the "gold standard" method to prove whether a chemical is carcinogenic or not is to test the chemical in whole animals. However, this procedure makes use of a high number of animals, is extremely time-consuming and cannot be used to screen a high number of compounds at a time. Because of these limitations great efforts have been undertaken in the last few years to develop test systems that could be used to evaluate the carcinogenic potential of chemicals *in vitro*. The aim of our project is to combine the BALB/c-3T3 cell transformation assay with an automated version of the so-called soft agar colony formation assay. In a first step we show that the soft agar colony formation assay can be run in an automated way in a 96-well format by making use of a commercially available liquid handling system and that the assay delivers within one week the answer to the question whether cells having previously been incubated with a test compound show anchorage-independent growth. In a second step, we show that the combination of a shortened version of the BALB/c-3T3 cell transformation assay with the above-mentioned automated soft agar colony formation assay allows concluding within five weeks whether compounds are able to malignantly transform mammalian cells. Taken together, the *in vitro* test system presented could very well help to significantly reduce the number of animals needed for the *in vivo* testing of carcinogenicity in the near future.

ID ABS: 266

In vitro assays of ventricular cardiomyocytes as screening tools in drug development and safety pharmacology

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Pharmacological intervention for the treatment of disease syndromes can increase the vulnerability of some patients to life-threatening heart rhythm disturbances. An important parameter in assessing heart function is the QT-interval of the electrocardiogram. According to the latest guidelines (SB7), safety pharmacology for human pharmaceuticals is supposed to include *in vitro* assays assessing the potential of QT-interval alterations and cardiac ion channel function. Thus, screening for drug effects on cardiac function has become crucial in pharmaceutical drug development.

To assess drug effects on ion channel function, usual electrophysiological *in vitro* assays are either based on artificial cell systems, or on acute heart preparations requiring one heart for testing one drug. The use of cultured cardiomyocytes as an alternative was so far limited to acute drug testing due to rapid cellular dedifferentiation within 1-2 days in culture.

Here we present for the first time an adult ventricular cardiomyocyte culture system that shows minimized dedifferentiation over several days. Electrophysiological characterization using patch-clamp recordings of the most important ion channels displayed a constancy of channel properties throughout the culturing time. In addition, we have developed an automated cardiomyocyte assay based on microelectrode array technology. This approach allows cardiac field action potential recording of embryonic or neonatal cultured cardiomyocytes simultaneously from up to 96 recording sites.

In summary, our systems enable drug screening on cardiac function over a much longer time period in culture or with an enhanced throughput, resulting in a significant reduction of animals for this kind of studies.

3D tissue engineered models of skin and oral mucosa for investigation of normal and abnormal epithelial biology

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3D tissue engineered models sit uniquely between 2D cell culture models and animal experiments. They are demonstrably better than 2D cultures but currently lack vasculature and any immune components found in immune competent animal models. However, they offer something not available in animal models – one can do mix and match experiments in which a particular combination of cells (and extracellular matrix proteins) can be studied in what are essentially tissue physiology experiments. Using this approach we have made a number of 3D epithelial tissues over the last 15 years: skin, oral mucosa, bladder, gut and most recently oesophagus. We have used these to study normal and abnormal epithelial physiology – wound healing, dermatotoxicity, skin contraction, regulation of pigmentation, melanoma invasion and head and neck cancer invasion and the development of psoriasis and as models of human bacterially infected wounds.

For all of these studies there appear to be "rules" which apply equally to make reliable 3D tissue engineered epithelial models. In brief the recipe is to combine epithelial cells with stromal cells in a relevant stromal matrix then culture them at an air-liquid interface in media containing physiological calcium.

The freedom to put cells together in particular combinations and ask questions of physiological relevance of them is leading to some surprising conclusions. Examples related to skin pigmentation, melanoma invasion and the induction of early stage neovascularisation will be discussed. In summary 3D tissue engineered models of epithelia are contributing to our understanding of normal and abnormal epithelial biology.

ID ABS: 483

Using clinically relevant celecoxib concentrations to identify potential biomarkers in human colon polyp cells

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Celecoxib, a Cox-2 inhibiting non-steroidal anti-inflammatory drug, is an example of a cancer prevention agent that has been studied extensively both *in vitro* and in the clinic. It has demonstrated the ability to inhibit Cox-2 at very low clinically-

relevant concentrations. Celecoxib has many other reported mechanisms with potential relationships to cancer prevention that have been identified from numerous studies using various cancer cell lines. Examples of these mechanisms include: induction of apoptosis; disruption of the mitochondrial membrane; activation of caspase-9; cell cycle arrest; and inhibition of angiogenesis: inhibition of metastasis. When the published data are evaluated, it is clear that most of the reported mechanisms require concentrations that are not clinically relevant or have shown increased potential for producing adverse events. Other than the primary mechanism, Cox-2 inhibition, the mechanisms of action for celecoxib at clinically relevant concentrations are not well defined. To address this issue, gene expression data were collected using a human colon polyp cell line, VACO 235, following exposure to celecoxib. The VACO 235 cell line grows as a 3D-culture that resembles the colon polyps found *in vivo*. Both time and concentration dependent effects were evaluated using clinically relevant concentrations with limited adverse response potential *in vivo*. Gene expression changes and alterations in specific pathways were evaluated to identify potential biomarkers with relevance to colon cancer prevention. The changes in gene expression patterns suggest that celecoxib can alter both apoptosis and growth in the VACO 235 cell line at clinically relevant concentrations.

Supported by NCI Contract #N01-CN-35130.

Session BS10: Current and evolving concepts for the validation of safety assessment methods

Validation of innovative technologies and strategies for regulatory safety assessment methods: challenges and opportunities

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Advances in understanding of the pathways and mechanisms by which substances cause adverse health effects along with innovative technologies are providing new opportunities to develop test methods and strategies that may improve safety assessments. These include high throughput screening and other approaches to rapidly measure various molecular, genetic, and cellular perturbations caused by test substances. Integrated testing strategies that combine biomarker assays with existing test methods or with one or more sensitive biomarker assays are being developed. However, before such test methods and strategies can be used for regulatory decision-making, they must undergo validation studies to determine their usefulness and limitations for specific purposes. Validation studies must be designed to adequately determine the extent that reproducible results can be obtained in different laboratories, and to adequately determine the extent that the proposed use of the test method can provide equivalent or improved protection compared to existing methods. Reference substances must be selected for which there is high quality data and that cover the spectrum of chemistry and biological activity for which the new method is applicable. The adequacy and reliability of proposed dose- or concentration-setting procedures must also be evaluated. Comprehensive and optimal validation study designs are expected to expedite the validation and regulatory acceptance of new test methods and approaches that support improved safety assessments and contribute to reduced animal use for regulatory testing.

Note: The views expressed above do not necessarily represent the official positions of any federal agency.

Validation of innovative test methods for safety testing: drawbacks and advantages of Japanese validation studies

H. Kojima

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We have a long history of Japanese validation studies. To date, several studies have been performed in Japan, and we have not only obtained a significant amount of data but also learned from these experiences. As a result, we have learned the drawbacks and advantages of Japanese validation studies.

Advantages:

1) Most Japanese researchers are volunteer-driven and honorable.
2) Many Japanese researchers observe GLP.
3) Most Japanese researchers have strong technical skills.
4) Statistical analyses are performed by high-caliber biostatisticians.
5) The system-managed validation study is confirmed by the JSAAE (Japanese Society for the Alternative to Animal Experiments).

Drawbacks:

1) Validation studies are of a lower standing than research development studies.
2) There is capital shortfall.
3) Most studies are conducted at non-GLP facilities.
4) Many laboratories participate in a validation study.
5) The data management system is insufficient.
6) Most researchers misunderstand the validation study and have not read OECD Guidance Document No. 34.
7) Most validation studies are conducted with no reference to performance standards.

This year, JaCVAM (the Japanese Center for the Validation of Alternative Methods) has participated in the International Cooperation on Alternative Test Methods (ICATM). We must use this opportunity to discuss the advantages of Japanese validation studies with the ICATM members. In addition, we must learn many of the international rules from the ICATM members and should be open-minded about future validation studies.

Validation of innovative test methods for safety testing: ECVAM's experience and lessons learned

J. Kreysa

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Since its establishment in 1993, ECVAM has developed its widely accepted validation method that exists of basically two phases: pre-validation and validation. In order to streamline and structure these phases, the concept of a modular validation has been developed, allowing to stop at any module should the test not meet the module-specific requirements. With this approach ECVAM was able to validate more than 30 innovative test methods of which a significant share are in use and accepted for regulatory purposes.

While scientific validation is already difficult, assuring application by industry and approval by regulators is at least as complex. Very often regulators criticise the applicability of the tests on the basis that the used validation reference chemicals do not adequately reflect the relevant use-spectrum, e.g. in cosmetics, or they simply have problems with accepting that the innovative method delivers as good information as the normal in vivo method. As a consequence of this ECVAM will intensify and better organise its communication and collaboration with the regulatory community. It will also intensify its dialog with its other stakeholders, i.e. those that will have to use innovative methods for producing data and information for regulatory processes and their own risk assessment, and those who have an interest in alternative methods because of their impact on the 3Rs.

This presentation will outline ECVAM's experience with regard to the pitfalls of validation and of the complex processes leading to the application of alternative tests and their acceptance by regulators and risk assessors.

The use of test method performance standards to expedite validation of innovative and improved versions of test methods and testing strategies

M. Wind[1] and W. Stokes[2]

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Regulatory acceptance of a scientifically valid new test method is often followed by similar versions that incorporate innovations and enhancements that provide for improved accuracy or other advantages. The concept of test method performance standards was developed to allow for more efficient validation of new and revised test methods that are structurally and functionally similar to adequately validated and accepted test methods. As new innovative technologies become available that can be used to improve existing test methods or to develop similar test methods, there will be an increased need for performance standards to more rapidly evaluate these methods. Performance standards are based on the validated reference test method and consist of essential test method components, a minimum list of reference substances, and standards for accuracy/reliability. Essential test method components are the structural, functional, and procedural elements that a proposed test method must have in order for it to be evaluated using performance standards. Accuracy and reliability standards are those that must be met following evaluation of the minimum list of reference substances in the new test method. The routine development and availability of scientifically sound performance standards is expected to expedite the efficient validation of innovative and improved test methods and testing strategies that provide for improved hazard assessments and other advantages.

Note: The views expressed above do not necessarily represent the official positions of any federal agency.

Use of results of alternative testing methods for regulatory purposes in the EU

F. Pedersen

European chemicals agency (Echa), Helsinki, Finland

The new EU chemicals legislation (the REACH Regulation (EC) 1907/2006) requires manufacturers and importers of substances in a quantity of more than or equal to 1 tonne/year to register their substances at the European Chemicals Agency. The registration shall, a.o., include information on the intrinsic hazards of the substance, depending on the tonnage manufactured or imported.

The hazard information to be provided is specified with reference to studies conducted in accordance with the EU Test Methods Regulation (EC) 440/2008, which for the human health endpoints mainly includes the traditional test methods using vertebrate animals. However, results of other testing and non-testing methods may also be used as long as the scientific validity of the method has been established and the result is adequate for the purpose of classification & labelling and/or risk assessment. In addition, even positive results of "suitable" *in vitro* studies can be used. In this respect "suitable" means that the method is sufficiently well developed according to test development criteria.

The new Regulation (EC) 1272/2008 on classification, labelling and packaging further defines criteria for classification & labelling, which are mainly based on results of the same traditional testing methods described above.

The presentation will discuss various options and limitations for use of results of alternative testing methods for the above mentioned regulatory purposes.

International harmonisation and regulatory acceptance of OECD test guidelines and validation principles

P. Amcoff

OECD, Environment Directorate, Environment, Health and Safety Division, Paris, France

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries meet to co-ordinate and harmonise policies and work together to respond to international problems. The OECD Test Guidelines (TG) are a collection of the most relevant internationally agreed test methods used by government, industry and others to assess the safety of chemical products[1]. Animal welfare and the 3R-principles are always considered in test method development. To promote alternative test method development and their regulatory acceptance, OECD has developed a Guidance Document (GD34) on "the Validation and International Acceptance of New or Updated Test Methods for Hazard assessment[2]", based on internationally acclaimed principles. These principles are now requirements for all new or updated TGs. Examples include prospective validations of endocrine disruption test methods e.g., draft TG 455 (ERalpha), and retrospective validations of the acute inhalation TGs 403&436. Draft TG 487 (*in vitro* Micronucleus) was initially retrospectively validated followed by prospective validation. Performance Standards are commonly used for new *in vitro* TGs (e.g., TG 435 and new draft skin irritation TG) and will be introduced into existing TGs (e.g., 430&431), and provide means for developing proprietary test methods into TGs. New, tailored validation concepts for specific applications, such as Performance-Based TGs, are under evaluation. Implementation of the OECD principles of validation and regulatory acceptance enhances international harmonisation of validation of alternatives, saves resources and animals and facilitates international collaborations and adoption of OECD TGs.

URL's:

[1] http://www.oecd.org/department/0,3355,en_2649_34377_1_1_1_1_1,00.html

[2] http://appli1.oecd.org/olis/2005doc.nsf/linkto/env-jm-mono(2005)14

Extra Breakout Sessions

Session EB1: Status report on Predict-IV

Predict-IV, a new approach for integrating *in vitro* toxicity testing into the early stages of drug development

C. Burek

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High-throughput safety testing for drugs in the early stage of development is still hampered by the existence of qualified alternative testing methods that are either accepted by regulators to replace laboratory animal use or lack the practical experience to allow an adequate comparison to currently used test systems. This is highly fretful; hence preclinical drug testing still represents a lag phase during drug development. The pharmaceutical industry spends an enormous amount of money and time in this phase to end up, very often, by killing a potential drug molecule.

Predict-IV aims to improve the drug safety assessment at this stage of development by using a non animal-based integrated approach. With the combination of analytical chemistry, cell biology, mechanistic toxicology, *in silico* modeling and new advanced technologies, such as "omics" and high-content imaging, a link between classical *in vitro* toxicology and modern systems biology will be set. The talk discusses the framework of the large collaborative integrated project and summarizes the current progress of Predict-IV not covered by subsequent speakers.

Cell culture approaches chosen to predict adverse effects of therapeutic compounds

W. Pfaller[1], S. O. Müller[2], P. Hewitt[2], A. Price[3], A. Wolf[4] and P. Jennings[1]

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Predict-IV is aimed at adopting and optimising existing *in vitro* models for the assessment of potentially adverse effects of therapeutic candidate compounds. The target organs modelled in this project are the kidney, the liver and the CNS. Liver and kidney are essential organs for any testing strategy due to their role in bio-activation/inactivation and concentration and excretion of xenobiotics. CNS models will be adopted as there is a need for *in vitro* models that are predictive for CNS toxicity. At present there are no test strategies available to investigate long term neurotoxicity of pharmaceutical compounds *in vitro*. This is of importance, as neuro-degenerative diseases such as Alzheimer's and Parkinson's are increasing, with little information on the contribution of therapeutic pharmaceuticals to the induction of these diseases.

Preferably, the *in vitro* systems used for analysis of chronic toxicity will represent existing simple organ level mono- and co-culture models that are amenable to medium- or high-throughput methods. These models will be adopted in order to be easy to handle and to allow exposure of cells over prolonged periods of time. The *in vitro* models utilised should further enable the efficient acquisition of reliable and relevant indicators of injury over the whole exposure period.

The assessment of cellular injury will comprise the use of -omics approaches as well as the application of classic cytomic assays to identify disturbed intracellular signalling pathways. The data collated will be utilised by other project partners to model kinetic aspects of drug effects.

The crucial role of biokinetics in *in vitro* testing

E. Testai

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In vivo, the actual internal dose reaching the target is the more relevant parameter for evaluating human and experimental animal exposure in the quantitative risk assessment. Although kinetics have often been evoked to explain *in vivo*/*in vitro* differences, only very few studies have addressed the issue of *in vitro* biokinetics. The actual intracellular concentration may greatly differ from the nominal applied concentrations due to altered bioavailability (interactions with the medium, the plate, the cell itself) or to physiological cellular processes (mechanism of transport across the membranes, biotransformation, bioaccumulation). In repeated treatments for prolonged time of exposure to mimic exposure during pharmacological therapies, the uncertainty about the actual level of exposure of cells *in vitro* is greatly enhanced.

The aim of WP3 within the project is the development of a strategy for measuring/estimating the real exposure of cells to drugs and/or their metabolites in the *in vitro* test systems as a key element for the extrapolation of *in vitro* results to the *in vivo* situation relative to drug safety. The ultimate goal in strict collaboration with other partners is to derive the NOEC in model systems based on human cells representative of *in vivo* target organs, from which it would be possible to extrapolate the corresponding *in vivo* dose.

How to integrate *in vitro* pk/pd information for toxicity prediction

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Predicting drugs' *in vivo* effects from *in vitro* testing requires modelling processes that are not reproduced by *in vitro* systems. The most obvious difference between the two situations is the absence of the absorption, distribution, metabolism, and excretion (ADME) processes that govern target tissue exposure *in vivo*. For identical input doses, the concentrations to which *in vitro* systems are exposed may not correspond to those found *in vivo*. The partners of Predict-IV work package 5 will develop prediction models for ADME processes on the basis of *in vitro* information. They will assemble all of these models into a global, generic, physiologically-based pharmacokinetic (PBPK) model able to simulate concentration-time profiles in human blood or tissues. *In vivo* drug toxicity will then be predicted by coupling the predicted profiles to the pharmacodynamic (PD) relationships observed *in vitro*. Human inter- or intraindividual variability will be evaluated for each component of the approach.

The progress made on the following specific objectives will be discussed:

- Predicting ADME processes based upon *in vitro* data or *in silico* tools;
- Developing a generic PBPK model for humans and rats;
- Developing dose-response models for *in vitro* toxicity endpoints in liver, kidneys and central nervous system;
- Coupling the PBPK and dose-response models;
- Predicting the impact of human variability on the drugs' toxicity;
- Simulating the PK/PD of drugs on sensitive sub-populations;
- Confronting predictions to published observations using a meta-models approach.

Session EB2: Status report on evidence-based toxicology project

Evidence-based toxicology: breaking the chain of uncertainty?

C. Griesinger

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The paper will discuss the emerging concept of evidence-based approaches in toxicology, discussed during the First International Forum towards an Evidence-Based Toxicology, held in October 2007 in Como, Italy. This conference gathered about 170 participants from Europe, the US, Africa and Asia (http://www.ebtox.org). The proceedings are in press in Human and Experimental Toxicology.

As every human undertaking, science is based on assumptions. As long as conscientiously used as such, they play a constructive role for forming testable hypotheses. However, if not identified or mistaken as "proven" knowledge, assumptions can severely hamper scientific progress. Toxicological practice rests heavily on assumptions, e.g. mechanisms of action, causative relationships, inter-species extrapolations and the reliability/predictivity of standardized tests. Consequently, hazard/risk characterisations suffer from uncertainty. This is exacerbated by the fact that toxicology depends on the integration of scientific information from various sources showing intrinsic uncertainty, thus leading to a multiplication of uncertainties in basic research to applied decision making (e.g. risk management).

Evidence-based approaches may help breaking this "chain of uncertainty". Evidence-based tools (e.g. systematic reviews analysing all available information on the basis of pre-defined criteria) may help assessing: (1) where and to which extent species differences exist with regard to toxicological responses; (2) possible relevant physiological pathways hitchhiked by toxicants ("toxicity pathways"); (3) sources of uncertainty in toxicology; (4) the effectiveness of toxicological decision-making. Moreover, quantitative data integration tools using pre-defined criteria/weighing factors may increase the consistency/transparency of toxicological practice.

ToxRTool: a tool to assess the reliability of toxicological data

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Mainly because of the REACH programme of the EU, where the use of existing information is emphasised, the assessment of the inherent quality of toxicological information is receiving renewed attention. In addition, in the validation process of alternative test methods, quality assessments of reference test data play an important role. Reference data should be of known and good quality to allow for a sound performance assessment of the *in vitro* test under validation.

Currently, the so-called Klimisch criteria are usually used to evaluate the quality of toxicological data. Starting from these basic criteria, ECVAM and the contractors FoBiG/DKFZ – with the support of a group of European experts – have carried out a project aiming for an unambiguous and transparent assessment of the quality of toxicological data by developing a comprehensive and detailed scoring tool.

Initially, existing approaches of reliability assessment were reviewed and variables related to quality were identified focusing on experimental toxicological data. These toxicological data mainly come from peer-reviewed publications and studies undertaken for regulatory purposes. The tool was developed to be equally applicable to both *in vivo* and *in vitro* data from these two data types. The tool was challenged in two experiments, in which volunteering scientists applied the tool to a broad range of toxicological data/studies. The scoring tool will facilitate and harmonise this first pivotal step of data evaluation and will increase its transparency, qualifying this project as one of the first activities towards an evidence-based toxicology (EBT).

The tool is available for free download on the website http://ecvam.jrc.it/.

Session EB3: Status report on CAESAR

CAESAR's approach for alternative *in silico* methods for REACH

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CAESAR is an EC funded project (Project no. 022674-SSPI) specifically dedicated to develop in silico models for REACH. Five endpoints are addressed within CAESAR: bioconcentration factor, mutagenicity, carcinogenicity, skin sensitisation and developmental toxicity. The approach has been to develop models with the following main characteristics:

- based on the best possible data in order to provide a sound basis for regulatory purposes,
- with high performance,
- validated with several methods, including external validation,
- easy to use.

Models are freely available at the site: http://www.caesar-project.eu.

They use simple chemical information, such as SMILES or an sdf file as input. Models are based on chemical descriptors obtained from the structure. The algorithms calculating toxicity have fixed parameters in order to produce the same prediction from all users. This characteristic is important in case of models for regulatory purposes.

Models include a tool to evaluate the similarity of the compound of interest with the data set used to build up the model. The user can visualize the compounds that are more similar, their predicted values, and the errors for these compounds. This gives a good indication of the suitability of the model for a specific compound.

The *in silico* model for bioconcentration factor (BCF) in fish

A. Roncaglioni, E. Boriani, A. Lombardo, C. Milan and E. Benfenati

Istituto di Ricerche Farmacologiche Mario Negri – Laboratory of Environmental Chemistry and Toxicology, Milano, Italy

Bioaccumulation of chemical compounds is relevant information for the REACH legislation, whose potential uses include C&L, prioritization (according to B criterion) and Chemical Safety Assessment. Within the CAESAR project we developed a QSAR model for BCF based on a set of about 500 compounds. Chemical descriptors were calculated with DRAGON, MDL and other software. Several algorithms have been tested to search for the best relationship. The models have been checked and validated according to strict statistical rules, using an external test set.

The performances of CAESAR model were also compared with estimations based on LogP and other predictive software.

The selected model has been fully implemented in a piece of software and is now accessible through the CAESAR project portal for predicting BCF on the basis of the input structure alone. Appreciation of similarity with the compounds in the dataset has been implemented and alerts about the quality of the prediction have been included.

The final outcome is a user-friendly, intuitive and very rapid tool for assessing bioconcentration potential, useful within the REACH framework.

The *in silico* model for mutagenicity

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Mutagenic toxicity is the capacity of a substance to cause genetic mutations. This property is of high public concern, because it has a close relationship with carcinogenicity and eventually reproductive toxicity.

In experiments, mutagenic toxicity can be assessed by the Ames test on *Salmonella*. An interesting point is the reliability of such experimental tests: the estimated inter-laboratory reproducibility rate of *Salmonella* test data is 85%. This observation

shows the intrinsic limitation of the *in vitro* test and opens the road to other assessments, e.g. *in silico* assessments.

So far, a widely used method is to check for the presence of structural alerts. However, the presence of SAs alone is not a definitive method to prove the mutagenicity of the compound towards *Salmonella*; the substituents present are in some cases able to change the classification. So, statistically based methods will be proposed and developed, with the final target being to obtain a cascade of systems with tailored properties, as the reduction of false negative, or the best accuracy. The system has been developed and validated on a set of a few thousand molecules.

In silico models for carcinogenicity, skin sensitization and developmental toxicity

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Biochemics Consulting SAS, Olivet, France

Several binary classification models were developed on three priority regulatory endpoints selected inside CAESAR, i.e. skin sensitization, carcinogenicity and developmental toxicity associating 209, 805 and 292 chemicals, respectively. They were established by using different (Q)SAR methods based on fuzzy logic, neural networks, nearest neighbour, etc. A strict validation procedure was used in order to carefully evaluate the models' performance, and the presence of false negative predictions is carefully evaluated to reduce the risk of predicting potentially harmful compounds to be non-toxic. Good classification results were obtained for each endpoint, by different (Q)SAR approaches and by using only 2D molecular descriptors. The prediction power on the test set is 90%, 70% and 86%, respectively for skin sensitization, carcinogenicity and developmental toxicity. The best (Q)SAR models developed in this project, and their protocols, were placed on the project's Internet site (http://www.caesar-project.eu/software/index.htm).

Formation of mechanistic categories and local models to facilitate the prediction of toxicity

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In silico models to predict toxicity include the application of quantitative structure-activity relationships (QSARs) and the formation of meaningful chemical categories to allow for read across. These types of models span a range in terms of their extent and application in chemical space from being local to global. Within the CAESAR EU project both global and local models have been developed. The aim of this investigation was to develop local models for the prediction of skin sensitisation and teratogenicity. Local models for skin sensitisation were formed on a mechanistic basis, relying on knowledge of electrophilic reaction chemistry to form meaningful groups of compounds. The structural features associated with the chemistry have been defined, allowing for a qualitative assessment of hazard. Within mechanistic categories, quantitative predictions of skin sensitisation potency were made through read across, using a computational molecular orbital derived electrophilicity index. For grouping of chemicals with teratogenicity data, computational indices for chemical similarity were applied, using the freely available Toxmatch software. Groupings of chemicals can be achieved, which allow for the successful read across of teratogenic effects. The results show that global models are applicable to larger areas of chemistry and hence are more general. In contrast, local models may be transparent and provide more accurate results within specific areas of chemistry that can be rationalised on a mechanistic basis.

The funding of the European Union 6th Framework CAESAR Specific Targeted Project (SSPI-022674-CAESAR) is gratefully acknowledged.

Session EB12: Update on ICH and VICH progress

No abstracts arrived

Lunch Sessions

Session SL1: Databases: progress report

Wealth and diversity of the 3Rs online

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Alternatives information is as essential to quality research as etiological, phenotypical, and physiological information; it is but one aspect of a thorough, scientific consideration of a research question and proposal. Where and how one searches for alternatives information, however, is perhaps less intuitive and, as a result, more challenging.

Many specialized online databases and websites exist, created by government agencies, advocacy organizations, and publishers; this diversity of resources allows accessing different views and encourages the sharing of knowledge and expertise among research communities. Due to this dispersed wealth of 3Rs information online, searching for alternatives requires first identifying and accessing the relevant resources. Where to search for this authoritative comprehensive information will be discussed in this session.

The AnimAlt-ZEBET database: a unique resource for comprehensive and value-added information on 3Rs alternatives

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Federal Institute for Risk Assessment – ZEBET, Berlin, Germany

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

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

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

Altweb + ALTEX: a 21st century union

M. Hughes

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In an era of ever-increasing globalization, the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) has brought together Altweb, the premier global alternatives resource, and ALTEX, a journal of alternatives to animal testing and the official journal of CAAT, to create a resource for the worldwide promotion of the 3Rs and evidence-based toxicology. This synergy of print and electronic media provides free, open access to news, scientific articles, editorials, databases, workshop reports, educational materials, and multimedia.

The union of Altweb and ALTEX promises to broaden the fields of humane science and evidence-based toxicology, with potential to influence not only scientific research, but policy, regulation, education, and animal welfare across the globe.

Animal welfare information center (AWIC): providing information for improved animal care and use in research, teaching and testing

K. Adams

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The mission of the Animal Welfare Information Center (AWIC) was established by Congress in the 1985 amendments to the US Animal Welfare Act. Since that time, the focus of AWIC's information products, services, and activities is to help the regulated community with employee training and to promote the humane care and use of animals by providing information on alternatives (improved methods of animal experimentation which could reduce or replace animal use or minimize pain and distress to animals). AWIC's website provides selected resources, full-text newsletters, and topical bibliographies for people working with animals. In addition, new Web 2.0 technologies such as news feeds allow the Center to provide customers with updated animal welfare information on a daily basis. This talk will introduce users to the AWIC website, teach them how to search and navigate for resources, and demonstrate the use of new technologies that allow for the dissemination of information in a timely manner.

AltTox.org: connecting stakeholders on issues concerning non-animal methods of toxicity testing

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AltTox.org is an interactive website devoted exclusively to non-animal methods of toxicity testing. The emerging paradigm shift in toxicity testing away from adverse outcomes in animals and towards a mechanism-based approach largely *in vitro* and *in silico* was recently endorsed by the US Environmental Protection Agency, and appears to have broad support among many stakeholders. This shift will necessitate communication and coordinated efforts by stakeholders in government, industry, academia, and NGOs. AltTox is designed to provide a common platform for these diverse stakeholders to exchange information and perspectives on relevant science and policy issues. The website consists of two interconnected components: a series of message boards, or forums, and an informational resource center. The forums serve as a communication tool for stakeholders to rapidly exchange information and perspectives. The resource center features concisely summarized and comprehensive information on toxicity testing and alternative test methods. There are numerous ways that stakeholders can communicate with other stakeholders *via* AltTox and/or contribute content that will then be available to all users. AltTox stakeholders are encouraged to contribute forum postings, meeting reviews, Informational Resources suggestions (meetings, *in vitro* testing labs and products, funding sources, etc.), and more. Users can nominate experts who will be invited to submit Way Forward opinion articles. A survey is available for providing feedback on the website itself. We welcome comments on stakeholder interest in possible new interactive tools for the website such as a Webinar portal for stakeholder use or social media links (LinkedIn, Twitter, etc.).

Session SL2: Good Cell Culture Practices

The Bologna statement on Good Cell Culture Practice (GCCP) – 10 years later

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In the 3Rs, replacement of animal testing by *in vitro* methods is one of the most ambitious goals. In this respect, cell and tissue culture techniques serve as important tools. However, cultured cells *in vitro* often represent ambiguous models of living organisms. The uncertainty results from the biology of mammalian or human cells in culture and from their variability depending on the *in vitro* protocols applied and the culture systems used, respectively. Therefore, all efforts should be undertaken to best mimic the *in vivo* situation for cultured cells and to standardize the high number of variables that are inherent to cell and tissue culture. The maintenance of high standards is fundamental to all good scientific practice and is essential to securing the reproducibility, credibility, acceptance, and proper application of any results produced. Thus, well-defined and precisely described culture protocols are mandatory to ensure optimal and reproducible culture conditions, and to enable the interlaboratory comparability and exchange of experimental data obtained with *in vitro* culture systems, e.g. in pre-validation and validation studies of *in vitro* alternatives. To this end, in analogy to Good Laboratory Practice (GLP), guidelines for Good Cell Culture Practice (GCCP) were proposed at the 3rd World Congress on Alternatives & Animal Use in the Life Sciences in Bologna, 1999. Since then, GCCP guidelines for cell and tissue culture work were elaborated and were published by two consecutive ECVAM Task Forces (*ATLA 30*: 407-414, 2002 and *ATLA 33*: 261-287, 2005).

Quality control in cell culture: simultaneous authentication and cross-contamination detection

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Recent reports have shown the growing perception in the scientific community that cross-contamination (CC) of mammalian cell lines represents a major risk for generating false scientific data. The key problems are known and are chronic in nature: neglecting guidelines for quality control and disregarding adequate cell culture techniques are the main reasons why cell lines have been misidentified or cross contaminated. Short tandem repeat (STR) microsatellite sequences are highly polymorphic in human populations, and their stability throughout the lifespan of individuals renders STR profiling ideal for forensic use. Since human cell lines are cross-contaminated by intra- and interspecies cells, DSMZ has established a sensitive and PCR-based technique for detection of animal cells of murine, rat and chinese/syrian hamster origin in human cell cultures and *vice versa*. Furthermore, we have piloted the generation an international reference STR profile database for human cell lines. At present, about 2370 such cell lines have been STR typed and are represented as reference sets on the database. In order to render it user friendly, a simple search engine for interrogating STR cell line profiles has now been made available on the homepages of DSMZ, JCRB, and RIKEN. Online-verification of cell line identity should prove to be a useful weapon to wage war on the havoc of CC, which has dogged cancer research for far too long.

Standardisation in the culture of stem cell lines

G. Stacey

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The UK Stem Cell Bank is required to prepare, quality control and distribute human somatic and embryonic stem cell lines both for research and for clinical use on an international basis. This challenging remit also requires that cells supplied by the Bank have been banked and qualified to ensure that ampoules of cells yield reproducible cultures for use. In addition to comprehensive quality control of the cell lines this has meant careful capture of consensus culture protocols from the depositor's laboratory and accurate definition and control of environmental factors that affect cell growth such as carbon dioxide levels, temperature and tissue culture plastic-ware. Implementation of a well-defined and controlled culture environment is intended to assist in the delivery of standardised stem cell cultures. Quality control methods also need to be standardised and improved. Furthermore, considerable work is still required to improve the reliability of stem cell culture and preservation. These issues will be critical to implementation of acceptable stem cell therapies.

GCCP initiatives in Japan

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In Japan, we have two big cell banks, which have maintained high quality.

Firstly, the cell bank of the MHLW (the Ministry of Health, Labor and Welfare, MHLW) is operated cooperatively by the JCRB (the Japanese Collection of Research Bioresources) and the HSRRB (the Health Science Research Resources Bank). While the HSRRB functions as the distribution center of cells, the JCRB cell bank collects cell lines and is doing research and quality control of the collected cell lines. The roles of this bank are storage, multiplication and quality control of normal and disease-related cells derived from human and other mammalian sources to supply researchers. Especially, it takes account of the detection of the cross culture contamination, mycoplasma contamination, and virus contamination, and high-quality human-derived cell lines are being offered worldwide.

On the other hand, The RIKEN BioResource Center (RIKEN BRC) is a not-for-profit institution and totally supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). The Cell Engineering Division (Cell Bank) in the RIKEN BRC provides not only conventional human and animal cell lines but also human stem cells, such as ES cells and iPS cells. The human and mouse iPS cell lines established by Dr. Yamanaka are currently available from RIKEN BRC.

At present, the facilities run the following bank operations in order to supply research resources to researchers in industry, academia and government and, particularly, in the fields of the health sciences including medicine, pharmacology and basic biology.

Poster Section

PO1: Integrated approaches

ID ABS: 7

A framework for using structural, reactivity, metabolic and similarity to evaluate the suitability of analogs for SAR-based assessments

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The use of structure activity relationships (SAR) to fill data gaps using toxicological data from related compounds is among the most actionable short/mid-term strategies for reducing animal use. However, approaches for such "read across" are not well defined. This paper describes a systematic, expert-driven process for identifying and evaluating analogs for use in the SAR toxicological assessments. The approach involves categorizing potential analogs based upon their degree of structural, reactivity, metabolic and physicochemical similarity to the chemical with missing toxicological data (target chemical). It extends beyond structural similarity, and includes differentiation based upon chemical reactivity and addresses the potential that an analog and target could show toxicologically significant metabolic convergence or divergence. The approach relies heavily on the analysis of chemical reactivity and metabolic pathways. In addition, it identifies differences in physicochemical properties, which could affect bioavailability and consequently biological responses observed *in vitro* or *in vivo*. The result is a comprehensive framework to apply chemical and biochemical expert judgment in a systematic manner to identify and evaluate factors that can introduce uncertainty into SAR assessments, while maximizing the appropriate use of all available data.

ID ABS: 80

Bayesian assessment of skin irritation potential, combining the results from individual physico-chemical exclusion rules

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Probabilistic rules determining the absence of skin irritation potential, using calculated physico-chemical properties, are defined by us (presented elsewhere at WC7). Each of these rules gives a probability that a substance is not a skin irritant, based on the distribution of all experimentally determined skin irritants and non-irritants over a single physico-chemical parameter range. For example, substance A could have a 60% probability of being a non-irritant, based on its extreme log Kow value. The same substance A would have 50% probability based on its water solubility, and maybe 40% based on its molecular weight. A methodology will be presented, using Bayesian networks, enabling the combination of probability predictions from these

physico-chemical exclusion rules. An overall probability that a substance is not a skin irritant can then be calculated. Conditional dependence between the physico-chemical parameters is taken into account, giving little added probability when two parameters are strongly correlated (e.g. log Kow and aqueous solubility in our example), but significant increase in probability if parameters are unrelated (e.g. log Kow and molecular weight). The huge benefit of this approach is that chemicals having insufficient evidence to waive experimental testing, based on any single physico-chemical rule, might have sufficient evidence when multiple physico-chemical parameters are combined. In our example the overall probability for substance A combining three rules could be as high as 95%. This methodology can be extended to include information from any other source (e.g. *in vitro* testing) giving information on the skin irritation potential of a substance.

ID ABS: 81

Optimization of an intelligent testing strategy to assess fish toxicity under the REACH framework

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The aim of REACH is to improve the protection of human health and the environment through a better and earlier identification of the intrinsic properties of chemical substances. REACH promotes the use of many methods, both to save animals, and to increase the information robustness. The so-called alternative methods, which include testing (*in vitro*) and non testing ones (*in silico*, like QSAR and read-across), have an important role within REACH to better investigate the effects of chemical substances.

In this framework a scheme has been set to optimize testing decision for fish toxicity according to REACH requirements following official guidelines. Both the tonnage and the objectives of information requirements under REACH have been taken into account. In the workflow, existing information, reasoning for waiving the test, acute/chronic toxicity test priority and adequacy of possible alternative methods are addressed. This work will be the basis for the subsequent development of an intelligent testing strategy (ITS) for aquatic toxicity and its implementation into a web-based informatics tool.

Financial support to OSIRIS project (GOCE-CT-2007-037017) is gratefully acknowledged.

ID ABS: 92

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

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The toxicological safety assessment nowadays is performed as a sequential process (hazard identification – hazard assessment – exposure assessment – risk assessment), which is usually time consuming, expensive and uses many animals. Improvements to obtain a more efficient process remain limited as long as we stick to a sequential approach. New concepts would be needed to achieve real innovations in risk assessment. The Threshold of Toxicological Concern (TTC) potentially is such a new concept that has already existed for many years but has recently been developed further. Yet, application of TTC in food safety assessment still is limited.

We drafted a framework for application of TTC in safety assessment of chemically complex matrices (CCM). The safety of CCM is difficult to assess, as there are too many and mostly unknown substances present (often referred to as Forest of Peaks). Usually, for the evaluation of CCM a full safety assessment approach involving animal studies is needed. However, in case exposure to most substances through food would occur only in low quantities, TTC might be applicable.

TTC is designed to assess substances of which toxicological information is lacking. The assessment is based on the molecular structure. To apply the concept efficiently to CCM, a strategy is needed to deal with large numbers of unidentified substances. The framework we drafted addresses this issue and proposes a stepwise approach for the application of TTC in safety assessment of CCM. The framework and needed analytical and test strategy innovations that are under development will be presented.

ID ABS: 106

Comparative ultrastructural study on changes of platelets and fibrin networks in human and mouse animal model asthma

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Platelets and fibrin networks play an important role in asthma and the BALB/c asthmatic mouse model has previously been successfully used to study platelet ultrastructure. In control BALB/c mice major, thick fibers, minor, thin fibers and tight, platelet aggregates with typical pseudopodia formation, are present. Minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates. The question that now arose is whether platelets and fibrin networks of humans with asthma will have the same ultrastructure as seen in the BALB/c asthmatic model. In order to answer this question, ultrastructure of platelets and fibrin networks from 2 subjects (controlled asthma and uncontrolled, chronic asthma) were studied and compared to that of human controls and BALB/c asthmatic mice. Peak flow measurements of the controls and patients were also assessed. Results showed that similar platelet and fibrin network ultrastructure is found in uncontrolled, human subjects and BALB/c asthmatic animals. The challenge when using animal models is always whether the model adequately mimics the human disease; the current research therefore shows morphological support for the use of this model in the study of asthma. These morphological results may also provide additional information to plan treatment regimes for sufferers of this very debilitating disease.

ID ABS: 172

Strategies for the rational integration of ADME information into predictions of biological activity

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The ability of a xenobiotic to elicit a therapeutic or toxic response *in vivo* is determined by two key factors. The compound must possess inherent activity (or be abiotically/biotically transformed into a product which does) and be able to reach the target site in sufficient concentration; the latter being dependent on the absorption, distribution, metabolism and excretion (ADME) properties of the compound. In recent years there has been much progress in the development of models to predict the concentration-time profile of xenobiotics in the fields of both pharmacology (pharmacokinetics) and toxicology (toxicokinetics). Information is available from a range of sources, including *in silico* (e.g. screening rules, (Q)SAR, molecular modelling, expert systems) *in vitro* (artificial membrane permeation assays, binding assays, cellular models) and *in vivo* (inter-species correlations, allometric scaling) studies. Here we present a proposed workflow for the rational integration of ADME information from these diverse sources into the overall prediction of *in vivo* activity of a xenobiotic. Examples will be provided as to how this methodology can be applied within an Integrated Testing Strategy (ITS) to enhance the accuracy of predictions for *in vivo* activity. This is of use to both the areas of drug development and prediction of toxicity for risk assessment purposes.

ID ABS: 539

Application of an intelligent testing strategy to the US EPA endocrine disruptor screening program

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The current version of the proposed US EPA Endocrine Disruptor Screening Program is organized in two tiered batteries of tests. The first tier consists of *in vivo* and *in vitro* screens intended to identify chemicals capable of interacting with the estrogen, androgen and thyroid endocrine systems; and the second tier consists of animal-intensive developmental and reproductive screens in several species. Some as yet to be defined set of positive results in the Tier 1 battery will trigger Tier 2

testing. The proposed screening battery has been criticized for its cost, the low specificity of some of the animal assays, and the large numbers of animals consumed per chemical. Using highly conservative estimates, the Tier 1 battery will use a minimum of 518-536 animals and cost between $ 324,000 to more than $ 938,000 per chemical. We present an alternative, intelligent testing strategy that would save animals and resources and result in more efficient screening and characterization of the endocrine-disrupting potential of manufactured chemicals. The preliminary tier includes physical and chemical data, existing toxicological data, and *in vitro* and (Q)SAR methods that are either validated or nearly validated. The results of this alternative Tier 1 can be used in a weight-of-evidence approach to 1) identify priority chemicals and 2) design an intelligent, chemical-specific strategy for further screening or testing. Such a strategy would greatly reduce the use of animal testing for both Tier 1 and 2, while maintaining a high level of sensitivity for identification and classification of endocrine disrupting chemicals.

ID ABS: 545

Integrated testing strategies for acute inhalation toxicity assessment

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Progress has been made to reduce the numbers of animals used in acute inhalation toxicity tests, particularly for classification and labeling of respiratory hazards. In addition, there exist a plethora of respiratory-tissue specific cell and tissue models, encompassing different cell types, model architectures, and exposure conditions, as well as double and triple co-culture models. However, the development of non-animal models that could assess acute inhalation toxicity has not been pursued as comprehensively as models or strategies for acute oral toxicity, despite the scientific and animal welfare implications of *in vivo* acute inhalation testing. This presentation proposes multiple potential Intelligent Testing Strategies using existing models with the aim of providing coverage of the full potential toxicological sequelae of acute airborne agent exposure. This would include the breadth of different regions of the respiratory system, the diversity of potential toxicities, such as tissue damage or respiratory sensitization, and the consequences of systemic migration of the molecule or particle. We will present several test case chemicals for each of the potential strategies, using existing data and other chemical-specific information. Finally, this presentation provides a forward-looking analysis on the most efficient ways to move ahead, taking into account immediate regulatory and research needs as well as the explosion of recent work in cell and tissue models, computational toxicology, virtual lung and airway modeling, systems biology, and pathway analysis as it relates to inhalation toxicology.

PO2: Chemical and physical methods

ID ABS: 182

Potential of the direct peptide reactivity assay (DPRA) for the *in chemico* detection/discrimination of chemical respiratory vs skin sensitizers

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Low molecular weight chemicals require *in vivo* binding to macromolecules to become immunogenic. For many years it has been considered that the main difference between skin and respiratory sensitizers was the exposure route (skin vs respiratory track). Since, it has been shown that skin sensitizers can activate a Th1 and respiratory sensitizers can activate a Th2 immune response irrespective of the exposure route. Therefore it has been hypothesized that some differences in the modification

of amino acids might happen at the protein level. To investigate that point we have looked into the reaction mechanism and amino acid specificity of exclusive respiratory sensitizers (anhydrides), exclusive skin sensitizers (isothiazolones) and mix skin/respiratory sensitizers (isocyanates). On the one hand, we found that trimellitic anhydride, a pure respiratory sensitizer, was specifically and exclusively reacting with lysine and peptides containing lysine, while 2-methyl-2H-isothiazol-3-one (MI), a pure skin sensitizer, was specifically and exclusively reacting with cysteine and cysteine containing peptides. On the other hand, we found that arylisocyanates, respiratory and skin sensitizers, were reacting with cysteine and cysteine containing peptides but also significantly with lysine and lysine containing peptides. These qualitative results were quantitatively confirmed by the DPRA with a high lysine peptide depletion for anhydrides, a high cysteine peptide depletion for isothiazolones and a mix cysteine/lysine peptide depletion for isocyanate derivatives. Therefore, the DPRA is able to identify chemical respiratory sensitizers, as it includes not only a cysteine peptide but also a lysine peptide. Moreover, the depletion ratio lysine vs cystein peptide could be a good approach to identify potential chemical respiratory sensitizers.

This work is funded in part by The European Cosmetics Association, Brussels, Belgium and by the Sens-It-Iv project (grant European Commission 018681).

ID ABS: 344

Viscosity studies of water-soluble nonstarch polysaccharides extracted from feeds with different wheat proportions

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Introduction: Wheat increases the viscosity of contents in the digestive tract of birds, impairing nutrient utilization. Extract viscosity values of grains could be used as predictors of anti-nutritional properties of NSP in cereals. The viscous properties of NSP depend on several factors (chemical composition, molecular size, composition of the extraction media). Experiments were carried out to investigate the relationship between extract viscosity and different feed wheat contents.

Methods: Feed samples were milled by a laboratory grinder in a 600 micron sieve. Two extraction procedures were tested, without and with inactivation of endogenous enzymes, by incubation with 80% ethanol at 80°C. The dynamic viscosity was determined using a cone/plate viscometer (Brookfield Model DVIII Cone CP-40).

Results: The main soluble NSP in wheat are arabinoxylans. Wheat soluble NSP give rise to highly viscous aqueous solutions even at low concentrations. The viscosity values of the water extracts obtained after endogenous enzyme inactivation (procedure 1) are higher than those obtained without enzyme inactivation (procedure 2), where soluble NSP were hydrolyzed and their molecular mass reduced. The elevated temperatures affect polymer solubility by increasing enzymatic degradation of water-insoluble pentosans to water-soluble forms via transarabinosylation. In procedure 1 the viscosity increase is up to 133.33% while in procedure 2 only 120%, for 40% wheat concentration. Aqueous extract viscosities correlated well with the wheat concentrations of the feeds. We observed a higher positive correlation (r=0.9827) between the dynamic viscosity and the wheat content in procedure 1 than in procedure 2 (r=0.9288).

ID ABS: 345

Influence of extraction conditions and barley soluble beta-glucan content on water extract viscosity

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Introduction: Most of the anti-nutritive activities of non-starch polysaccharides (NSP) which affect broiler performance have been attributed to soluble polysaccharides. Most polysaccharides give viscous aqueous solutions. The viscosity of NSP depends on their solubility and molecular weights. The study had in view the influence of extraction conditions and barley content on the water extract viscosity.

Methods: Feed samples with different barley contents were milled by a laboratory grinder in a 600 micron sieve. Two extraction procedures were tested. In procedure 1 we incubated the sample with 80% (v/v) ethanol at 80°C for endogenous enzyme inactivation, and then added water to the pellet and incubated at 40°C for 2 h with constant stirring. In procedure 2 we skipped the incubation step with ethanol. The dynamic viscosity

of aqueous extracts was determined using a cone/plate viscometer (Brookfield Model DVIII Cone CP-40).

Results: Barley contains substantial amounts of both soluble and insoluble NSP. The main water soluble NSP in barley are highly viscous beta-glucans. The viscosity values of the water extracts obtained in procedure 1 are higher than those obtained in procedure 2. In procedure 1 the dynamic viscosity increases up to 142.62% while in procedure 2 only to 132.38 for 40% barley concentration, due to depolymerisation of polysaccharides which decreases the viscosity of the aqueous solutions. Aqueous extract viscosities correlated well with the barley concentrations of the feeds. There is a higher correlation between viscosity and barley content in procedure 1 (r=0.8988) than in procedure 2 (r=0.6961).

ID ABS: 421

Use of chemical reactivity to refine *in vitro* eye irritation prediction with the SkinEthic™ reconstructed human corneal epithelium

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Prediction of eye irritation potential of new compounds by means of *in vitro* methods is a crucial point for cosmetic industries in the current context of the Cosmetic Directive and REACh legislation. For this purpose, a biological test method, using the SkinEthic™ Reconstructed Human Corneal Epithelium and based on two exposure times, has been developed and optimized to predict the irritating versus non irritating potential of chemicals. This work demonstrated, on a set of 90 chemicals, that the test method performances can be appreciably increased by measuring the chemical reactivity of test compounds and orientating them toward the appropriate exposure time of the SkinEthic™ test method according to this reactivity flag. Chemical reactivity was determined by measuring the depletion of three peptides, such as glutathione (GSH) and two synthetic peptides containing a cysteine or lysine residue (Gerberick et al., 2007). HPLC-MS and HPLC-UV analytical methods were developed for assaying reduced & oxidized glutathiones (GSH & GSSG) and cysteine and lysine peptides, respectively. Depletion rates for the sum of GSH & GSSG and the cysteine or lysine peptide were measured to estimate the reactivity status of chemicals. In conclusion, chemical reactivity measurement was helpful to decide what exposure time to use in the SkinEthic™ test method. It could be a prerequisite to define the molecular behavior of compounds in the same way as other physical-chemical parameters.

ID ABS: 537

Chemical alternatives to animal tests

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Animal tests are based on the principle of biochemical reactions in a living organism integrated in a complete immune system. Depending on the research, use of animals may vary, but the chemical reaction of the animal test may be modelled as a chemical reaction in a cell culture. *In vitro* procedures that conduct animal experiments at microscopic level are emerging as alternatives to *in vivo* procedures, which use the whole animal. *In vitro* studies offer more scope for controlled studies, they are very economical, and use lower numbers of animals than whole animal tests. However, a lack of sound validation methods to assess these studies raises concern over human welfare, and that animal welfare should be balanced against human safety. Proper scientific principles should be followed to ensure that these studies protect the animal welfare while ensuring human safety.

PO3: High throughput technologies

ID ABS: 29

HTS for selective muscarinic cholinergic receptor antagonists

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In experiments on rats we developed a methodological approach to evaluate the selectivity of muscarinic cholinergic receptor (M-ChR) antagonist action in whole organism conditions. According to the results obtained in the investigation, the protective effect of M cholinolytics in acute poisonings with organophosphates (DDVP, DFP, etc.) depends on M1 subtype ChR occupation. The efficiency of antagonists in the inhibition of the tremor reaction caused by the M-ChR agonist arecoline depends on M2 subtype ChR interaction. It was established by linear regression that there is a high degree of correlation (r=0.99) for different M cholinolytics between the ratios of ED_{50} of M antagonists in the tests with arecoline and organophosphates and the ratios of dissociation constants of antagonist complexes with M-ChR from the homogenates of rat cerebral cortex and heart containing M1 and M2 ChR subtypes, respectively. Thus, the ratio of ED_{50} arecoline/ED_{50} DDVP serves as a measure of the selectivity of drug action. In experiments on *Daphnia magna* the effects of some non-selective, mainly M1 and M2 ChR antagonists on the toxicity of DDVP and arecoline were studied. There was a strong correlation between the ED_{50} of antagonists in the tests with arecoline and DDVP in the experiments on rats and the EC_{50} of antagonists in experiments on *Daphnia magna*. The principal similarity in action of muscarinic antagonists in *Daphnia magna* and rats allows the recommendation of *Daphnia* for screening for selective muscarinic receptor antagonists.

ID ABS: 76

Miniaturized three-dimensional cell culture and metabolic enzyme arrays for high-throughput toxicity assays

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Demand for *in vitro* toxicity tests as replacements for animal testing has been heightened due to the implementation of REACH and the Seventh Amendment regulations. Solidus Biosciences has developed microarray platforms that support a variety of cell-based toxicity screens that enable measurements of both acute toxicity and metabolism-induced toxicity of chemical compounds. The DataChip (the Data Analysis Toxicology Assay Chip) is a miniaturized 3D cell-culture array that can support high throughput toxicity screening of a wide range of cell types to assess organ-specific toxicity. A single DataChip contains 1,080 individual cell cultures comprising a spatially addressable array of targets. The Metabolizing Enzyme Toxicology Assay Chip (MetaChip) is a similarly configured array of immobilized metabolic enzymes designed to emulate effects of Phase I and II human metabolism on cytotoxicity. The MetaChip is flexible and can accommodate liver microsomes, s9 fractions, and many combinations of recombinant metabolic enzymes.

This new platform can be used to screen new chemical entities developed by the pharmaceutical, cosmetic, and fine chemical industries during all stages of development. Used alone or in combination with additional assays, the DataChip/MetaChip can quickly evaluate the systemic and acute toxicity of chemical compounds. As an *in vitro* alternative to animal testing, the DataChip/MetaChip platform will help companies meet the increasing market demand for humane alternatives to the use of animals in toxicity screening. Recent results will be presented, comparing toxicity profiles determined using the DataChip and MetaChip with available *in vivo* data for compounds published by ICCVAM/NICEATM.

ID ABS: 511

Could artificial life replace animals in the laboratory in the future?

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Artificial life is quite young and essentially interdisciplinary. Artificial life is fundamentally directed towards both the origins of biology and its future, the scope and complexity of its subject require interdisciplinary cooperation and collaboration. It can guide how we use new technologies to extend life and create new forms of it, including drugs, and this science could lead to elimination of experiments on animals in the laboratory.

The aim of this study was to investigate the possibilities to eliminate animal testing and to review the use of models of artificial life.

It was found that some models require little work and perfection to be used in laboratory experiments.

PO4: Omics and systems biology

ID ABS: 12

A mechanistic comparison of *in vitro* and *in vivo* studies of lung toxicity using transcriptomic analysis

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In the development of an integrated risk assessment approach for aerosol ingredients, inhalation toxicology is an important safety endpoint for the consumer. Our strategy aims to maximise consideration of pragmatic approaches such as exposure-based waiving for ingredients. However, these approaches are not applicable for all classes of ingredient and the current hazard identification requires an *in vivo*, sub chronic inhalation study. The development of suitable *in vitro* alternatives poses significant challenges, including comparative dosimetry and the heterogeneous structure and cellular composition of the lung. Global expression profiling has enabled a holistic overview to further our mechanistic understanding of the molecular alterations implicated following exposure to specific polymers. The *in vivo* findings show clear dose and adaptive responses across the 22 week recovery period assessed, which, amongst other changes show a move from an acute inflammatory state to one of significant tissue remodelling. In order to further the development of *in vitro* assays we have undertaken a comparison of several *in vitro* cell systems (e.g. monolayer, multi-differentiated and co-culture methods) using transcriptomics to define the functional capacity of the cell systems in terms of molecular toxicity pathways. Here we provide a comparison of the transcriptomic data from both *in vitro* and *in vivo* studies. The applicability and limitations of the test systems are discussed in relation to the molecular pathways identified and their biological relevance

ID ABS: 145

Gene expression profiles as endpoints in hazard identification of environmental exposure

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Transcriptomic technology represents an innovative approach to monitor the exposure to toxicants, to evaluate their effects in terms of hazard and mechanisms of action, to assess cellular responses to different doses and to classify toxicants.

We use either commercial or custom Agilent oligo-microarrays to identify genes that are transcriptionally regulated by different kinds of exposures, ranging from environmental contaminants, especially those eliciting endocrine modulation, to low-dose ionizing radiation, in appropriate cell targets, including both eukaryotic *in vitro* cell culture systems and *ex vivo* samples. At the same time microarray technologies have been implemented for expression analysis in prokaryotic cells. All our microarray experiments are performed according to Minimum Information About a Microarray Experiment (MIAME) guidelines, and the data are reported in ArrayExpress, the MIAME compliant-database of microarray expression data of the European Bioinformatics Institute (EBI). The statistical analysis is performed by GeneSpring GX software or with the different tools provided by the Bioconductor open-source project. EASE High-Throughput GoMiner, MappFinder, Pathway-Express, Gene Set Enrichment Analysis (GSEA) are the tools usually applied to the biological interpretation and functional analysis of expression microarray data. The results that we have obtained in the last seven years clearly show that transcript profiles offer a great opportunity in hazard identification and hopefully in improving risk assessment.

ID ABS: 274

Gene ontology categorization and bioinformatic processing in human cell culture phenotyping

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Computational analysis of gene expression data provides a means of reviewing the molecular capabilities of cell lines. Experimentally transformed or tumor-derived immortal cell lines are common tools for *in vitro* studies, e.g., toxicity testing. Problematic to such research, permanent cell lines are considered to only partially reflect the full phenotypic characteristics of their normal origin. To analyze the retention of normality states in permanent cell lines, the current study utilized a reversed genetics approach (from protein to RNA) to analyze gene expression data within a standardized systems biology model for normal and transformed human oral epithelium. This model consisted of normal oral keratinocytes (NOK), the SV40T antigen-immortalized cell line SVpgC2a and the squamous cell carcinoma-derived cell line SqCC/Y1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) identified 19 abundant proteins that were differentially expressed in one or both of the transformed lines relative to NOK. Analysis of the differentially expressed proteins in the bioinformatics program Gene Ontology Tree Machine (GOTM) indicated significant changes in eight and three gene ontologies (GOs) in the transformed states. Analysis of transcripts within the GOs enriched on the basis of protein expression using the bioinformatics program AffyAnnotator and Ingenuity Pathway Analysis (IPA) indicated changes in 10 and 6 molecular networks in SVpgC2a and SqCC/Y1, respectively. Key gene alterations, being centrally located in networks with minimally three interactions to deregulated genes, included 18 transcription factors. The results demonstrate the utility of bioinformatics processing of gene expression data for gaining a comprehensive overview of the complete actions and competency of cell culture models.

ID ABS: 352

Systems biology PBPK modelling of metabolic interactions

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We demonstrate the capabilities of a new integrated approach and software tools to facilitate the development of systems biology models of interactions in metabolic networks.

We proceed by automatically merging and coupling metabolic pathways SBML models for individual chemicals to a template physiologically based pharmacokinetic (PBPK) model, using GNU MCSim. The reaction network and transport model generated is very efficient and can simulate the interactions between a theoretically unlimited number of substances, at the body and organ or tissue levels. By using a fine-grain description of reactions, development time increases only linearly with the number of substances considered, while the number of possible interactions increases exponentially. In contrast, traditional approaches using Ki values consider only first order interactions, and their complexity increases quadratically with the number of substances.

An example of application to the prediction of the joint kinetics of a set of 100 arbitrary chemicals or drugs (and their metabolites) is given. The qualitative and quantitative behaviour of the pathway network is analysed using Monte-Carlo simulations.

The integrative approach to interaction modelling is efficient and can be extended beyond metabolic interactions. It applies to drug-drug interactions or to generic chemical substances. It relies on the availability of specific data on the rate constants of individual reactions. Such data can be obtained through specifically designed enzyme kinetics experiments, or by computational chemistry modelling of enzymatic reactions. We are currently exploring both approaches.

ID ABS: 405

Toxicogenomics, another technological "hype" or a true revolution? How to further the societal acceptance and embedding of emerging technologies

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Animal welfare is a "hot topic" in Western societies, including the Netherlands. Whereas much attention focuses on animal wellbeing in relation to the bio-industry or household animals, several surveys also demonstrate that many people are concerned about animal welfare within research facilities and doubt whether the use of animals is legitimate or necessary at all. On the other hand, today's risk society has a high demand for safety. A new approach in toxicology to obtain more accurate data on safety without the use of animal models would therefore be much welcomed.

Toxicogenomics promises to cover it all: more reliable and accurate data (and hence safer products) with a substantial reduction of the total number of animals used in the assessment thereof. However, toxicogenomics cannot be embedded without public and political support. But how to cope with an increasingly critical public and rigid policy practices towards the acceptance of a new technology in our contemporary society? Many studies have already indicated that dissemination of scientific information will not automatically lead to acceptance of a technology. Science's credibility and the public's trust are no longer self-evident.

By combining discourse analysis, interviews and participant observation, this project addresses the question of how conditions for political and public acceptance of genomics-based non-animal alternatives can be improved and how the process of embedding toxicogenomics in current practices can be stimulated. Notably, we present our ideas on how the communication between toxicogenomics researchers, policymakers and the public can be improved in a more interactive manner (upstream innovation).

ID ABS: 479

Tox-Profiler: a web-application for interpretation and storage of toxicogenomics based gene-expression data

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Toxicogenomics may aid the development of *in vitro* models, especially when transcriptional responses of toxic compounds between *in vitro* and *in vivo* experiments can be compared. We addressed this issue by developing Tox-Profiler, a web-application for the analysis and storage of toxicogenomics data. Tox-Profiler contains a method that quantifies the expression of a gene-group within a gene expression profile. This has the benefit of easy biological interpretation and allows for cross comparison of gene expression profiles. In addition, Tox-Profiler contains a database of tox-related gene expression profiles that are analyzed on the gene-group level. The main content of the database is filled with two major datasets. First, the ICONIX dataset, which contains 1700 expression profiles from 344 distinct compounds tested in rat livers. Secondly, the cMAP dataset, which contains the expression profiles of about 1300 small compounds, which are tested in 4 different cell lines. The transcriptional response of these experiments can be assessed on the gene-group level. We illustrate the utility of our application by showing gene specific responses for compound classes and dose dependent responses for model compounds. We also performed correlation analysis between t-values of gene groups and physiological parameters; this revealed mostly significant results for immunology related gene groups. Finally, we used cluster analysis to visualize the relation between the gene groups over all experiments. We think that comparison at pathway level of *in vitro* toxicogenomics datasets with already published *in vivo* toxicogenomics datasets will further contribute to the 3Rs.

ID ABS: 480

Transcript profiling of the induction of terminal differentiation of keratinocytes and its potential relevance in toxicity testing

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Expression *in vitro* of differentiated properties typical to the cell of origin *in vivo* greatly enhances the applicability of cell culture models in alternative methods research. In keratinocytes regularly cultured under serum-free conditions, serum exposure orchestrates an induction of a multifaceted wound-healing program that includes the onset of terminal differentiation (TD). Simultaneously, accelerated TD represents an under-explored toxicity mechanism. To investigate mechanisms underlying TD, a serum-free systems biology model consisting of normal oral keratinocytes (NOK) and two malignant oral carcinoma lines (SqCC/Y1 and LK0412) was exposed to serum (5% fetal bovine serum for 4 days) and subjected to microarray analysis. Around 5 and 10-fold higher numbers of serum-regulated genes with increased and decreased expression, respectively, were noted in NOK compared to the carcinoma lines. Bioinformatics analysis of transcripts altered > two-fold using the Gene Ontology (GO) Tree Machine enriched multiple GO-categories after serum exposure in NOK, while the carcinoma cell lines enriched fewer categories with only some overlap to the normal state response. Ingenuity Pathway Analysis generated 13 significant networks with 19 key regulator genes in NOK following serum exposure. Differently, the carcinoma lines generated 3 networks respectively, with 4 key regulators in SqCC/Y1 and 8 in LK0412. Overall, and useful to alternative methods research, our findings provide novel GO-categories, networks and key regulator signatures applicable for elucidating the genetic basis underlying resistance to TD and delineating to what extent cell line models reflect processes of normal tissue turnover. Future studies are needed to assess their applicability to toxicity testing.

ID ABS: 494

Towards an *in vitro* systems biology approach to assess (developmental) neurotoxicity

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There is an urgent need for cheaper, faster and more scientific approaches to assess (developmental) neurotoxicity. Current OECD guidelines are based on animal models representing a timely and costly process (estimated 1.4 million US dollars per compound). Toxicologists recommend the development of alternative *in vitro* methods for initial screening and characterisation. Systems biology, aiming to understand how biological systems operate, represents a promising approach. It uses genomics, proteomics and metabolomics technologies and integrates their data to identify cellular interactions and pathways including those of toxicity.

An *in vitro* metabolomics approach was applied to investigate its significance for neurotoxicity assessment. Primary re-aggregating brain cell cultures exhibiting relevant morphological and functional CNS processes were treated with methyl mercury chloride and caffeine. To identify treatment induced metabolic alterations, cellular metabolic profiles were acquired by mass spectrometry and analysed using bioinformatics. Results revealed concentration dependent metabolic alterations for methyl mercury chloride but not for caffeine at sub-cytotoxic concentrations. The most significant altered metabolites by methyl mercury chloride treatment were identified as γ-aminobutyric acid, choline, glutamine, creatine and spermine. Additionally, eight compounds having target organ toxicities for brain, liver or kidney were tested. Results showed that compounds targeting the brain could be identified based on the induced metabolic alterations including those identified earlier.

Overall, results show the significance of *in vitro* metabolomics to detect neurotoxicity and discover its biomarkers. Further study includes screening of reference compounds and integration with genomics and proteomics data to create an *in vitro* systems biology approach to assess (developmental) neurotoxicity.

ID ABS: 531

Transcriptomic analysis of adipose tissue metabolic variation in human subjects

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The increase in obesity and diabetes to epidemic levels of over the past decade has lead to a marked increase in the use of animal models for metabolic research. While inbred animal models provide useful information, they intrinsically ignore the variation in phenotype observed in human subjects.

In this study, 648 obese female subjects (age 20-50 yr, BMI ≥ 30 kg/m^2) were tested for postprandial fat oxidising capacity. 35 individuals were then selected as being within the highest 10% (HX) or lowest 10% (LX) in terms of fat oxidation (17 HX, 3.9±1.88 g min^{-1}, 18 LX, 0.27±1.66 g min-1). RNA was extracted from adipose tissue biopsies and hybridised to the Affymetrix Human Exon 1.0ST array. Analysis was carried out using GeneSpring GX 10 and IPA 7.

Results: After discarding the lowest 20% raw intensity signal values, 15715 transcripts passed the filter for expression analysis, with 2927 satisfying a t-test cut-off of $P < 0.05$ and 837 for $P < 0.001$ (Benjamini-Hochberg multiple testing correction); 179 transcripts demonstrated a fold change of 1.3 or greater ($P<0.05$). Principle Component Analysis revealed that HFX and LFX were distinct populations. Filtering gene expression identified the genes in fatty acid synthase, SREBP1c and GLUT4 as being most highly differentially expressed. Pathway analysis highlighted the central role of GLUT4 in the phenotypes.

The results demonstrate that fat oxidising capacity can be discriminated on the basis of gene expression. Analysis of metabolic and transcriptomic variation within human populations may be key to the development of new drug and dietary interventions.

ID ABS: 541

The use of *in vitro* toxicogenomics for screening estrogenic activity: what is the added value to classical estrogenicity screens?

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Due to its high throughput nature, toxicogenomics can provide a broad picture of a toxic response, offering the ideal functional platform for grouping of chemicals according to mechanistic similarity. In this way, toxicogenomics has found its way into predictive toxicology. So far, the predictive potential of toxicogenomics in an endocrine disruptive screening context has not been extensively examined. However, since the majority of endocrine disruptive compounds mediate their effects by interaction with nuclear receptors and other transcription factors, gene expression analysis might form the ideal basis for elucidating mode of action. The aim of this study was to evaluate the potential of a toxicogenomics approach in combination with an estrogen-sensitive MCF-7 cell system in an estrogenicity screening context. In total, 18 compounds with endocrine disruptive potential were selected, of which 11 are listed as ICCVAM reference compounds for validation of estrogen receptor (ER) binding and transactivation assays. In this way, the grouping and classification potential of the gene expression profiles could be directly compared to the more classical estrogenicity screens, such as ER binding, ER transactivation and MCF-7 cell proliferation assays. Results indicated that this toxicogenomics approach was clearly capable of grouping compounds into strong, weak and non estrogenic groups, thereby reflecting interesting classifier genes from different regulated pathways (PGR, ERBB2, CXCL12, AREG, EGR3). These results might open the discussion on the added value of a broader endpoint evaluation in estrogenicity screens provided by omics-techniques compared to the classical single endpoint based screens.

PO5: Non-invasive technologies

ID ABS: 110

Non-invasive endocrine monitoring in urine samples: a preferential method for assessing chinchillas' (*Ch. lanigera*) reproductive physiology and welfare

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The chinchilla, a South American hystricomorph rodent, possesses one of the most valuable pelts in the world. Intensive hunting for fur placed the species at the brink of extinction, and today it is included in the Appendix I of CITES. Although native chinchilla are extremely rare, a hybrid produced by crossbreeding the two chinchilla taxa has been domesticated and selected for superior fur production for more than 80 years.

Despite its biological and economic importance, little scientific information is available about this species' reproductive physiology and welfare in captive conditions. Physiological measures of these aspects have typically relied upon the evaluation of steroid hormones in serum or plasma. However, attempts to obtain repeated blood samples from chinchilla were unsuccessful because of small vein size and their stress-susceptible nature. Non-invasive techniques could permit long-term endocrine monitoring while avoiding animal suffering and the stress-evoking stimuli of restraint, translocation and repeated venipuncture. With this in mind, the objectives of our

studies were to demonstrate the validity and accuracy of urinary steroid metabolite quantifications for studying different aspects of the chinchilla reproductive biology and welfare in captive conditions.

Using those methods, we were able to establish the endocrine profile of female pregnancy and post-partum oestrus as well as the relationship between abnormal repetitive behaviours (fur-chewing) and physiological stress developed in captive populations. An improved understanding of these aspects will undoubtedly help animal managers to develop more effective captive breeding programs for both domestic and wild chinchillas.

ID ABS: 294

Longitudinal measurements of breathing frequency in sephadex challenged rats. Non-invasive method to monitor lung inflammation

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Sephadex induced lung inflammation and oedema in rat is a model used for studying anti-inflammatory drugs with invasive readouts. The aim of the present study was to evaluate non-invasive longitudinal measurements by monitoring lung inflammation/oedema as an alternative to invasive bronchoalveolar lavage fluid (BALF) and lung weight readouts in sephadex challenged rats.

Male SD rats were instilled i.t. with sephadex (G100 superfine). Breathing frequency (f) and tidal volume (TV), were monitored non-invasively using whole body plethysmography (EMMS, UK) prior to and 4, 8, 24, 48, 72 and 96 h post sephadex challenge. The changes in lung mechanics (FlexiVent, SCIREQ, Canada) and inflammatory cells in BALF were analysed at each time point. At termination (96 h), the left lung lobe weights were recorded.

Non-invasive measurements showed significant changes in f and TV that were detectable already 4 h post sephadex challenge. These changes peaked at 24 h and remained throughout the experiment in longitudinal measurements. In sephadex challenged rats, a significant decrease in dynamic lung compliance and increase in dynamic lung resistance together with a significant influx of leukocytes in BALF and increase in lung weights were also present at all time points.

Taken together, in sephadex rats, the changes in breathing patterns (f and TV) correlate with changes in lung mechanics, inflammatory cells in BALF and lung weight. These data suggest that non-invasive measurements of f and TV can be used to longitudinally monitor the treatment effects of anti-inflammatory drugs in sephadex induced lung inflammation in rats.

ID ABS: 336

Measurement of fecal glucocorticoid metabolites as a tool to monitor stress during mating and pregnancy

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In this study we applied a non-invasive method to monitor activity of the hypothalamic-pituitary-adrenal axis on the days before mating and during pregnancy in the female Syrian hamster. We first performed an ACTH challenge test to demonstrate that changes in adrenocortical function are well reflected in the concentrations of fecal glucocorticoid metabolites (FGM) measured by our enzyme immunoassay. We observed a three-fold increase in FGM concentration above basal level about 28 h after the injection. We then mated 33 adult females with sexually experienced males. Fecal samples were collected daily from Day -6 before mating until the day before delivery. A total of 22 females became pregnant (P) and continued gestation until term, whereas 11 did not get pregnant or suffered embryo reabsorption (NP). From Day -6 until the day of mating, FGM concentrations were similar in both groups (P group: 143 ± 33 ng/g feces, NP group: 150 ± 27 ng/g feces). Interestingly, however on Days 1, 2 and 3 post-mating NP females had higher FGM levels than P ones. From Day 6 of gestation onwards, FCM concentrations started to increase in the P group and remained significantly higher than in NP females from Day 8 throughout gestation. Our results are in accordance with serum cortisol changes during the pregnancy of hamsters reported in literature and confirm the inhibitory effect of high glucocorticoid levels on reproductive function.

ID ABS: 427

In vivo confocal microscopic grading system for non-invasive standardized corneal evaluation: application to toxic-induced damage in rat

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Purpose: To propose a non-invasive *in vivo* confocal microscopic scoring system for evaluation of irritant-induced corneal changes.

Materials and methods: Rat corneas were instilled with 0.01-0.5% benzalkonium chloride (BAC) for 11 days and examined using non-invasive, high-resolution *in vivo* confocal microscopy (HRT-II) to measure corneal thickness and characterize corneal damage patterns in the superficial epithelial, basal epithelial, stromal and endothelial layers from Day 1 to Day 31. Severity scores were given for each predefined evaluation parameter (presence of the layer, shape/size of the cells, reflectivity patterns, inflammation and neovascularization) and then totalled.

Results: The scoring system revealed a dose-dependent effect of BAC and discriminated between high- and low-dose treatments. The highest 0.25% and 0.5% concentrations caused epithelial denudation, stromal inflammation and neovascularization, loss of endothelial visibility and fibrosis. The low 0.01% and 0.1% BAC concentrations induced damage mainly restricted to the epithelium extending from cell border loss (0.01%) to epithelial erosion (0.1%).

Conclusions: HRT is a new tool for ocular surface toxicity evaluation in animal models, which avoids killing large numbers of animals and is so in growing use. This HRT-II scoring standardizes damage evaluation at the cellular level, even when assessing irritating compounds at low concentrations, where classical clinical evaluations fail to be discriminating.

PO6: Non-vertebrate models

ID ABS: 350

A *Drosophila melanogaster* model for identifying interventions for treating Alzheimer's disease

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Accumulation of Amyloid-β (Aβ) peptides in the brain of aging individuals has been suggested to be the priming event in the progress of AD.

Drosophila melanogaster is a powerful genetics tool that has recently emerged as a model for AD. *Drosophila* can be utilized as a rapid model system to test the efficacy of putative neuroprotective compounds and can even be utilized in screens for therapeutic discovery.

Here we report the successful utilization of transgenic flies that express Aβ42 in the nervous system to develop an assay for identifying therapeutic strategies that can improve the functional deterioration observed in untreated flies. Our transgenic flies

also co-express a fluorescent reporter gene in Aβ targeted neurons allowing us to assess neuropathology leading up to shortened lifespan, behavioral deterioration and reduced cholinergic neuron function. Survival and locomotor function assays along with FACS analysis all successfully identified the AD phenotype in these transgenic flies.

Interventions such as rapamycin and reservatrol treatments were utilized to act as proof of principle for this *Drosophila melanogaster* model.

Proof of principle studies can identify therapies and illustrate a rapid and cost effective paradigm for testing and optimizing therapies for patients with AD. As such, the potential for using Drosophila as a pre-screen to identify therapies that most likely can prove effective in animal studies is rather high.

ID ABS: 470

A new application of the slug mucosal irritation (SMI) assay: detecting nasal stinging, itching and burning (SIB)

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Some marketed nasal formulations are known to cause stinging, itching, and/or burning (SIB) sensations. In general, these discomforts are often not detected until the clinical trial phase, since neither animal tests nor *in vitro* models were able to detect these effects in the past. Slugs however, react on these stimuli by an increase in mucus production (MP).

The aim of this study was to optimize and refine a new variant of the SMI-assay by conducting a comparative study of the existing 5-day local tolerance SMI-test and the newly developed 1-day protocol. In this new test, there are 3 contact periods of 15 min, which is sufficient to detect burning, since it is an immediate reaction. After each CP, the MP was measured.

Five marketed nasal sprays (with oxymetazoline HCl, mometasone furoate, fluticasone propionate, flunisolide hemihydrate and azelastine as active ingredient) were tested. All tested sprays show an elevated MP (ranging from 3.62 ±1.27% to 9.85 ±1.36%) compared with phosphate buffered saline (-0.01 ±0.09%), indicating the occurrence of SIB. Osmolarity measurements showed that in the case of flunisolide hemihydrate, it is primarily the hypertonicity which causes the reaction (1580 mOsm). In the other formulations (280-340 mOsm), however, the reaction is due to the active ingredient itself. In clinical studies, SIB were rarely observed for the formulations with the lowest MP, while for the hypertonic formulation 13-44% of the patients reported burning sensations.

The new 1-day protocol is able to detect clinical discomfort caused by nasal sprays.

PO7: *In silico* models

ID ABS: 30

Virtual uterus: a computational tool for myometrium function and development during pregnancy

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The mechanisms that initiate premature and full term labour in the human are poorly understood, and because of species differences animal models are misleading. Premature labour is associated with a higher rate of neo-natal mortality and post-natal morbidity, and so there is a clinical need for understanding the mechanisms of premature labour. There is a resource management need for accurate identification of the onset of full term labour. We approach these issues by applying virtual tissue en-

gineering – the construction of biophysically, histologically and anatomically detailed models of the spatio-temporal patterns of electrical activity of the myometrium that control, via intracellular Ca^{++}, the mechanical activity.

We have constructed models of myometrial cell electrophysiology and calcium dynamics based on cell and tissue electrophysiological data from the literature. These models quantitatively reproduce the bursting activity seen in uterine tissue obtained at full term by Caesarean section, and the effects of oestradiol and oxytocin.

Myometrial tissue architecture is visualised histologically in 2D, and quantitatively reconstructed in 3D by diffusion-tensor magnetic resonance imaging of lower and upper (fundal) segment biopsies obtained during Caesarian delivery.

The geometry of the full term uterus is reconstructed from in vivo MRI and *ex vivo* full term hysterectomy. Computed spatio-temporal patterns of excitation are used to interpret recordings of uterine electrical activity obtained by an array of electrodes on the abdominal surface.

This research was supported by the Dr Hadwen Trust.

ID ABS: 31

Use of chemoinformatics in predictive toxicology

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The field of chemoinformatics deals with computational prediction of biological properties of small molecules. Although the main use of the tools is in the development of pharmaceuticals, the same toolset can be applied in predicting toxic effects of compounds.

The tools can be further divided into structure and ligand-based methods. In the former approach physical properties of protein active sites are analyzed to predict binding affinity of a small molecule to the site. Ligand-based methods compare small molecules directly against each other and therefore do not require any structural information of macromolecular binding partners.

The focus of the current presentation is on direct comparison of small molecules. This can be done by two principal techniques: 3D overlay and fingerprints. A query compound whose toxicology one wants to study can be compared to a collection of bioactive compounds whose binding target profiles are known. Knowledge of the targets of the top-ranking bioactives speeds up the whole process by highlighting targets most likely to bind the query molecule. An example of such a finding are two topologically dissimilar campthothecin and tyrphostin AG-825, which both are known to bind DNA topoisomerase one and whose chemical similarity is revealed with 3D overlay tools.

ID ABS: 71

Categorization of chemicals for repeated dose toxicity

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Recently, the category approach was proposed as a most promising method to fill a data gap in hazard assessment of chemical substances. The development of a reliable category requires evidence based on toxicity mechanisms. Especially in case of the complex endpoints with many pathways leading to one toxicological effect, categorization based on the toxicological pathway is very effective. In this presentation, we show examples for categorization of chemicals for the repeat-dose toxicity test.

Categorization was conducted for about 300 chemicals with a published repeat-dose toxicity test report. The target organs and the kinds of toxicological effects were specified for each chemical based on the analyses of toxicity test data including blood chemical examination and histopathological examination. Toxicological pathways from a trigger molecular event to a resulting effect in the repeat-dose toxicity test were constructed for each chemical based on the available knowledge of distribution, metabolism and toxicological mechanism for the target chemicals. Some relationships between chemical structure and toxicological effect were found in the group of chemicals with similar toxicological pathways. As a result, we have successfully developed categories for some types of effects on liver, kidney, erythrocyte, testes, etc.

ID ABS: 87

In silico models to predict rodent carcinogenicity of naturally-occurring chemicals: comparative study and first insights into modes of action

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Current regulatory constraints and increasing concern for animal welfare have made the industry develop alternative strategies including *in silico* methods to sustain innovation.
There is a growing interest for products of natural origin in both the cosmetics and the food industries. Yet many of these chemicals contain structural features known as toxicophores causing chronic effects such as carcinogenicity. Since few naturally-occurring chemical entities have been tested in long-term rodent bioassays, and carcinogenicity is a critical toxicological endpoint for hazard and risk assessment purposes, there is an urgent need for efficient and reliable testing strategies. Computational approaches could be integrated in those strategies, bringing obvious advantages in terms of time, cost and animal protection.

A number of *in silico* models are available to predict rodent carcinogenicity. In the present study we used a dataset of 50 natural chemicals (Valerio et al., 2007) to evaluate (i) the applicability domain and (ii) the predictive performance of some statistical (MC4PC and Lazar) and mechanistic models (Derek for Windows, Oncologic, Toxtree). Oncologic appeared to be the most sensitive but with a limited applicability domain, while MC4PC and Toxtree displayed a high specificity but rather low sensitivity. Lazar and DfW performances were average but balanced.

Compliance with the 5 OECD principles was assessed, more particularly the transparency with regards to mechanistic insights. A comparison was made between the structural alerts predicted by DfW, Oncologic and Toxtree.

ID ABS: 88

Building a decision tree algorithm to predict skin irritation severity effects

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Over the past years great efforts have been made to develop alternative strategies including *in silico* methods that would comply with regulatory constraints (OECD Principles). A ban on animal testing for chemicals to be used in cosmetics came into effect in the EU in March 2009 for acute toxicity, genotoxicity, and skin or eye irritation.

With regards to skin irritation, an *in vitro* assay has been validated for regulatory use: it categorizes chemicals into 2 classes based on the EU risk phrase (R38). Besides, there are a number of *in silico* models (e.g. Toxtree & Derek for Windows) that provide a binary classification distinguishing irritants and non-irritants. Still there is a need for models enabling the prediction of irritancy potency for risk assessment purposes.

In the present study, a decision tree algorithm was developed to predict severity effects for diverse chemical classes.
The algorithm is made of regression models encoded using the MatLab software; those models are based on a set of structural features exported from the Leadscope platform and other physico-chemical descriptors relevant to skin irritation. The pool of physico-chemical descriptors was selected on the basis of the underlying mechanisms of action of skin irritancy (parameters such as bioavailability, chemical reactivity, interactions with cell membranes).

The training set contained chemicals representative of a cosmetic industry portfolio (>150). Performances using datasets compiled from public domain sources will be discussed.

ID ABS: 90

Redefining physico-chemical boundaries for skin irritation and corrosion potential

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A set of QSAR rules was evaluated for predicting the absence of skin irritation and/or corrosion potential of the general form: *If* Phys-chem. property *exceeds* threshold *then* substance is not a skin irritant and/or corrosive.

The mechanistical interpretation is that substances fulfilling one or more of these exclusion rules are not able to penetrate the epidermis, and therefore cannot exert their (potential) toxic effect. External validation of the rules using 201 (confidential) EU New Substances gave 99.3% correct predictions of non-corrosivity and 96.6% correct predictions of non-irritancy. Predictions using these rules would have allowed waiving of skin irritation tests for an estimated 40% of all EU New Substance notifications. However, regulatory and practical acceptance of these rules seems to be hampered by a) the yes/no character of the predictions, b) the absence of an indication of individual prediction uncertainty and c) the (un)availability of measured physico-chemical data. Therefore the published rules have been redefined using computer estimated physico-chemical properties. Instead of establishing a (visual) threshold value, the likelihood ratio of the probability density functions is calculated. This makes it possible to express a prediction as the probability that a substance belongs to the group of non-skin irritants. This probability is based on the actual distribution of the EU New Substance skin irritation data (>1600 substances) over the physico-chemical parameter domain. The redefined probabilistic physico-chemical rules together with an external validation of these rules using literature data will be discussed.

ID ABS: 120

Skin sensitisation and aquatic toxicity prediction using structural alerts and octanol-water partition coefficients in a knowledge-based system

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Skin sensitisation potential and fish acute toxicity both show a substantial dependence on molecular lipophilicity and chemical reactivity (before or after metabolism) (Schultz et al., 2006). For skin sensitisation, reactivity is generally a requirement for antigen formation, while in aquatic toxicity it usually provides an additional mode of action that overlays non-specific narcosis, producing so-called excess toxicity. Excess toxicity may also result from specific receptor-mediated mechanisms. In this work, aquatic toxicity data from a variety of sources have been analysed to generate structural alerts associated with reactivity, such as alpha-halo carbonyl and activated haloaromatic groups, which add to those already recognised (von der Ohe et al., 2005). The alerts have been incorporated into an existing knowledge-based toxicity prediction system. The lipophilicity and reactivity dependence of the aquatic toxicity have been considered and included in a reasoning model that informs the predictions. The capabilities and limitations of the approach are illustrated and discussed. Comparisons are made with skin sensitisation prediction using related structural alerts. In conclusion, such methods can make a significant contribution to answering the challenge to predictive toxicology presented by the hazard assessment of the large number of existing chemicals considered by the REACH regulations.

This work was performed as part of a project sponsored by Defra through the Sustainable Arable LINK Programme.

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ID ABS: 131

Knowledge base of basic active structures from twenty-eight-day repeated dose toxicity test data in rats

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Chemical compounds affecting a bioactivity can usually be classified into several groups, each of which shares a characteristic substructure. We call these substructures "basic active structures" or BASs. Data mining technology has enabled the systematic elaboration of BASs. We applied the method to 141 low-molecular-weight organic compounds selected from twenty-eight-day repeated dose toxicity test data in the NEDO SAR project database. The results are now disclosed on the knowledge BASiC.

The process of BAS extraction is as follows. (i) Linear fragments were first extracted from the molecules. (ii) Cascade model, a data mining method, was applied to create characteristic rules for each activity. (iii) Each rule was then examined using a structural refinement system, in which an initial core substructure appearing in the rule incorporates the surrounding atoms and bonds, and increases the discriminating capability between active and inactive compounds. (iv) Experienced users easily recognize BAS candidates in the supporting structures, as the structural diversity and the number of compounds become relatively limited. Finally, a BAS candidate is run through the refinement system to confirm its ability to perform the desired activity. Steps (iii) to (iv) are repeated until the extracted BASs are those found in most of the active compounds.

Application to hemolytic anemia found glycol ether as a BAS in addition to a well known aromatic amine. An independent survey of toxicity mechanisms in the project has also found this substructure to be the potential cause of toxicity and approved the usefulness of the method.

ID ABS: 247

The role of category formation in the read-across prediction of effects relating to reproductive toxicity

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Given the large number of chemicals requiring toxicological evaluation under REACH and the associated cost and animal usage (especially for reproductive toxicity), numerous alternative in silico methods are being explored. However, such methods are often limited by the scarcity of toxicological data. A method currently gaining acceptance to fill these data gaps is chemical grouping (or category formation) and read-across. Read-across exploits existing data from similar compounds to make a prediction via interpolation. Grouping similar compounds and forming chemical categories, according to structural/mechanistic similarity, is a prerequisite. In this study, the freely available Toxmatch software was investigated as a tool to form chemical categories suitable for read-across. This is based on the hypothesis that structurally similar compounds will act via a common mechanism. With no prior mechanistic knowledge, Toxmatch was used to form chemical categories for 57 query chemicals from a database of 233 compounds which had been assigned to US FDA classes for teratogenicity. Read-across predictions were then made for each query compound. The results showed that mechanistic categories could be formed for 17 of the 57 query chemicals, and within these categories read across predictions were made correctly. For the remaining query compounds, suitable categories could not be made due to structural analogues being absent. It was concluded that 2D similarity methods are a useful tool in building chemical categories for read-across approaches in which a priori mechanistic knowledge is limited. The funding of the European Union 6th Framework OSIRIS Integrated Project (GOCE-037017-OSIRIS) is gratefully acknowledged.

ID ABS: 256

Development of a support system for evaluating the repeat-dose toxicity of untested chemicals

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In order to evaluate the hazard of untested chemicals, various *in silico* methods have been developed for many endpoints such as genotoxicity and skin-sensitization, etc. However, a reliable *in silico* method for evaluating the repeat-dose toxicity of chemicals still remains undeveloped. Since the mechanism of repeat dose toxicity is very complicated, it is difficult to directly relate chemical structure to the toxicity. In order to evaluate repeat-dose toxicity based on chemical structure, expert judgment is essential, and the evaluation support system is required to evaluate properly and efficiently.

Recently we started a project to develop a system called "Hazard Evaluation Support System (HESS) Integrated Platform" for supporting toxicological experts to evaluate effectively the repeat-dose toxicity of untested chemicals based on known knowledge of their analog chemicals. This system incorporates a database, in which repeat-dose toxicity test data for more than 500 chemicals including detailed test data such as blood biochemical examination and histopathological findings were compiled. In addition to the repeat-dose toxicity test data above, the database includes the toxicity mechanism information such as *in vitro* test results and signal transfer pathways, and the metabolism information.

Based on the knowledge from this database, we are developing a category library for repeat-dose toxicity and a Bayesian net prediction model for repeat-dose toxicity. In this presentation, we will give an overview and introduce the recent achievements in the project.

ID ABS: 302

Optimising *in silico* models to facilitate a reduction in *in vivo* toxicity testing

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It is important that valid alternative toxicity testing methods are available if there is to be a reduction in the number of *in vivo* experiments which are conducted. This is especially true when considering the level of testing required under the REACH legislation. By using *in silico* modelling tools, such as (quantitative) structure-activity relationships ((Q)SARs), it is possible to screen compounds for obvious toxic properties, thus reducing the number of *in vivo* tests required. A widely used *in silico* tool is Derek for Windows (DfW). For the output of models, such as DfW, to be considered trustworthy it is important that certain standards are met. These standards are summarised in the OECD Principles for the Validation of QSARs. DfW conforms to a high degree with these principles but lacks a strictly defined domain of applicability. The aim of this work is to highlight how structural fragments may be used to define the applicability domain for structural alert models such as DfW. The results from this study show that fragments of molecules can be used to define the domain of structural alerts and negative activity space. Strategies for the use of these tools will be illustrated for skin sensitisation and mutagenicity.

ID ABS: 322

Use of non-test information from chemical structure and *in chemico* reactivity to identify potentially toxic compounds

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A deeper understanding of modes and mechanisms of toxic action will assist in the replacement and reduction of animal tests for toxicity endpoints. A key question with regard to toxicity is whether or not a compound will be able to undergo electrophilic interactions with biological macromolecules forming permanent covalent bonds. The aim of this investigation is to develop strategies to identify, through experimental and computational means, compounds that are "reactive" and associate

this reactivity to different toxicological events. The underlying reaction chemistry behind a number of interactions e.g. protein binding, DNA binding has been defined from organic chemistry reaction mechanisms. This knowledge is supplemented by experimentally determined, *in chemico* reactivity through a simple assay with glutathione. In addition, experimentally determined electrophilicity data have been supplemented by those retrieved from the literature to form a searchable database. These data have been used to develop models to predict intrinsic reactivity. Computational approaches, using Density Functional Theory level calculations, indicate that local models perform better to identify trends within the data and thus may be better suited to the identification of toxic compounds and ultimately their potency. The utility of these reactivity-led approaches in integrated testing strategies for toxicity will be illustrated.

This project was sponsored by Defra through the Sustainable Arable Link Programme. The funding of the European Union 6th Framework OSIRIS Integrated Project (GOCE-037017-OSIRIS), CAESAR Specific Targeted Project (SSPI-022674-CAESAR) and InSilicoTox Marie Curie Project (MTKD-CT-2006-42328) is also gratefully acknowledged.

ID ABS: 364

ORCHESTRA: a new EC project to link the research of *in silico* models with users' needs

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The project ORCHESTRA (Organising dissemination on Results of projects on Chemical Evaluation, Spreading Techniques for Risk Assessment) is a new, EC funded project dedicated to disseminate and exploit the activities of nine EU research projects dealing with *in silico* models for toxicity prediction of chemicals. The project, coordinated by E. Benfenati, involves seven partners, including end-users, scientists and specialists in dissemination and exploitation.

ORCHESTRA wants to facilitate the acceptance and use of *in silico* models. There are many *in silico* models available, both free and commercial. There are several tools available which allow development of new models, or propose already optimised ones. However, several barriers exist in their use.

Through direct interviews and questionnaires to stakeholders, workshops and seminars, a web portal, written materials, and films, the transfer from developers to final users will be investigated. We will evaluate barriers, needs, interests, and benefits relative to the use of computer models for environmental assessment.

Specific requirements for the specific sectors will be addressed, taking as case studies the experience within a series of case studies. Thus, special attention will be given to end-users, both from industry and regulators. In this way, ORCHESTRA will promote in silico models.

We acknowledge the ORCHESTRA project.

ID ABS: 365

Additive SMILES-based carcinogenicity models: a new approach to increase robustness and prediction

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In silico models can contribute to the assessment of chemical toxicity. Today the use of the simplified molecular input line entry system (SMILES) to characterise the chemicals is very broad, and can be easily and freely obtained through the web. Starting from SMILES, we built up *in silico* models for carcinogenicity potency. The SMILES format has been used to obtain simple chemical descriptors. Optimal descriptors calculated by the Monte Carlo method were utilized in modeling of carcinogenicity as a continuous value (logTD50). Some attributes, derived from SMILES, were rejected, if their occurrence in the molecules we used was too rare; for this we introduced an algorithm to define the value of the threshold. To validate the model we split the chemicals into three: a subtraining, calibration, and test set. Two systems of modeling were compared: 1) model based on the correlation balance, i.e. construction of a model separately for subtraining and calibration sets, and validation with an external test set; and 2) "classic" construction of a model, i.e., construction of model using united training set (that contains both the subtraining and calibration sets) with validation with the external test set. The correlation balance gave a more robust prediction of the carcinogenicity.

We acknowledge the EC project CHEMPREDICT

ID ABS: 399

Improvement of skin penetration prediction for volatile compounds from cosmetic and dermatological formulations

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The accurate prediction of dermal uptake and exposure of topically applied chemicals is relevant for formulation development and risk assessment. Refinement of the usual predictive model was completed recently. This model is able to predict the amount of chemical in the skin and in the receptor fluid after the topical application of cosmetic or dermatological formulations. The aim of this study was to take into consideration the vapor pressure of volatile chemicals to predict the cumulative amount.

In the *in silico* model developed by Kasting, the volatility of chemicals was considered. Recently, the impact of evaporation on skin uptake of volatile chemicals was evaluated. The model of L'Oréal was recently refined according to the chemical volatility. Measurements of skin bioavailability of 6 volatile chemicals were compared to the predictive data from the L'Oréal model. We demonstrated that the consideration of the vapor pressure improves the *in silico* prediction of dermal uptake for such chemicals.

Many cosmetic ingredients are highly volatile, like fragrance components. Without correction for evaporation, a great overestimation of skin exposure can occur. When evaporation is taken into account, the cumulative amount penetrated into the skin can decreases by a factor of two for most volatile chemicals. Thus, using this improvement gives more relevant and consistent results with a typical exposure scenario.

ID ABS: 508

An industrial platform as an alternative to the use of animal experimentation in the preclinical setting

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An emerging possibility to bridge the requirements of cost-efficiency and ethical issues in research involving animals is offered by "Virtual Animal" models. Optimata Virtual Patient® (OVP) is a set of proprietary mathematical algorithms illustrating the dynamics of key physiological, pathological and pharmacological processes involved in human diseases. The Virtual Animals is a subset platform aimed at conducting virtual preclinical trials. These algorithms, in conjunction with pharmacokinetic (PK) and pharmacodynamic (PD) models, enable to "virtualize" a drug and test its effects on a subject and on a population, *in silico*. The technology is preclinically and the clinically validated for the accuracy of its efficacy and toxicity predictions. Thus, the Virtual Mouse module was used to retrieve *in silico* a preclinical trial in which mice were xenografted with solid cancer, glioblastoma multiforme (GBM), and treated with paclitaxel. Tumor growth inhibition was calculated. Comparable experiments were performed using the Virtual Mouse module of the OVP. Input information included public PK/PD data and the applied paclitaxel regimen. Predictions were compared to the experimental results, showing high accuracy (r=0.88). Given initial information from a reduced dose escalation process in a small number of animals, the mathematical algorithms could replace a larger preclinical study by simulating a complete dose escalation process in a "synthetic" animal population. This combined *in vivo*/*in silico* trial can cut time and cost by 50% or more.

ID ABS: 510

The use of *in vitro* toxicity data and physiologically based kinetic modeling to predict *in vivo* embryotoxic dose levels

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At present, regulatory assessment of systemic toxic effects is almost solely carried out using animal models. The EU legislation REACH stimulates the use and acceptance of *in vitro* and in silico approaches to obtain information on the toxicity of chemicals. *In vitro* toxicity tests define concentration-response curves and EC_{50} values for specific target cells. These tests have only been used qualitatively, because so far the *in vitro* effect concentrations could not be linked to external dose levels *in vivo* to establish dose-response relationships for safety assessment. In the present project Physiologically Based Kinetic (PBK) models are used to translate *in vitro* concentrations to dose levels *in vivo*. The Embryonic Stem cell Test (EST), which uses the inhibition of embryonic stem cell differentiation as a measure of embryotoxic potency, was used to determine the *in vitro* embryotoxic effect concentrations of the proximate embryotoxic metabolites of four glycol ethers. ID_{50} values (50% inhibition of differentiation) of the glycol ether metabolites methoxy-, ethoxy-, butoxy-, and phenoxyacetic acid were 2.5, 3.9, 5.9 and 7.8 mM, respectively. Differences in potencies of compounds within the chemical class were small in the EST compared to what has been found *in vivo* as reported in literature, probably because the EST does not take *in vivo* kinetics into account. The use of PBK models describing these differences in *in vivo* kinetics provides a platform to translate *in vitro* toxicity data to *in vivo* dose levels for safety assessment.

ID ABS: 512

In silico models as an alternative to animal experimentation in the process of drug development

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Research involving animals has been a subject of intense debate in recent decades. Concerns as to the predictability, ethical aspects and cost have raised the need for complimentary tools that will enable better prediction of potential adverse side effects utilizing the absolute minimum number of animals.

An integrative use of biomathematical models may allow realizing this noble objective, while not compromising clinical relevance of the preclinical phase. Optimata Virtual Patient® (OVP) is a set of mathematical algorithms illustrating the dynamics of key physiological and pathological processes involved in human diseases, which allows conducting virtual clinical and preclinical trials for predicting short- and long-term drug effects.

PharmaSeed Bioservices is a fully integrated preclinical CRO specializing in unique safety and efficacy in the field of CNS, oncology and metabolic disorders. Often times PharmaSeed Bioservices is required to compile and execute a resource-tight preclinical program that would suffice the stringent regulatory barriers for a new drug/biotech compound. In order to generate more information from a decreasing number of individual animals, PharmaSeed Bioservices is therefore required to employ new, low-invasive, imaging and biomarker modalities that can generate more data points from each experiment and set the stage for their clinical equivalence.

Optimata's proprietary *in silico* models, enable to "virtualize" a drug, or a combination of drugs, and test its effects on a subject and on populations, *in silico*. Combining this with state-of-the-art *in vitro* and *in vivo* methodologies should allow for a higher accuracy and overall significantly increased cost-effectiveness of preclinical programs, coupled with meaningful ethical benefits.

ID ABS: 536

QSAR modeling using the ATSDR database of health guidance values

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The Agency for Toxic Substances and Disease Registry (ATSDR) develops minimal risk levels (MRLs) to be used as health guidance values (HGVs) for populations exposed to toxic chemicals at hazardous waste sites. There are over 100,000 chemical substances that can be legally manufactured or imported into the United States, and HGVs are available for only a small percentage of these compounds. Because the potential exists for long-term exposure to chemicals at or near hazardous waste sites, ATSDR has initiated a program to develop new QSAR methods to obtain HGVs that are protective of long-term human exposure for substances lacking experimental data. To date, oral HGVs for 411 chemicals were collected. Regression of these data against a large library of fragment descriptors suggested that (1) despite differences in modes of action HGVs are prone to QSAR modeling; (2) accuracy of the model log-linearly increases with the increasing size of training set; (3) models of 90% and above accuracy can be developed by using a ten-fold expansion of the database. The more high quality HGV-related data are collected, the fewer animals are needed to provide public health guidance concerning exposures to hazardous substances, potentially completely substituting computational risk assessment for animal testing.

PO8: Databases

ID ABS: 8

All information is alternatives information

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Advances in omics and systems biology, new developments in non-invasive and *in vitro* technologies, and identification of new non-vertebrate models allow for the introduction of alternatives and for new improved procedures in research. In order for those working in the laboratory to learn about new options, in order to effectively share new and valuable information, the studies must be published, the research results need to be indexed, and the publications readily and easily accessible.

New access policies of the United States National Institutes of Health (NIH), together with the power and depth of the National Library of Medicine (NLM), have improved public access to current research. While some policy requirements are limited to NIH funded research results, other related databases are much larger and continue to grow. These permit those with little financial support to access the latest information previously available only to those with significant wherewithal.

The NIH Public Access Policy requires that publications resulting from NIH-funded research be submitted to and made available via PubMed Central, the free digital archive of biomedical and life sciences journal literature. Additional databases include PubMed, NLM Gateway, NCBI Entrez (National Center for Biotechnology Information), and SIS (Specialized Information Services). These free NIH resources offer access to many scientific publications in full text, thereby providing immediate and authoritative research information, all of which is relevant to animal research and welfare.

ID ABS: 50

E-SovTox – a web-database on selected publicly available (eco)toxicity data published in the Russian language

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The REACH directive (Annexes VII-X) states, "all available *in vitro* data, *in vivo* data, historical human data, data from valid (Q)SARs and data from structurally related substances shall be assessed first". However, a considerable amount of information from Russian language data sources that can be beneficial for implementation of REACH remains unexplored, as the access to this information is limited due to the language barrier as well as the low level of digitalization of respective materials. Those hidden data may be used for the development of integrated testing strategies that enable a significant increase in the use of non-testing information for regulatory decision making, thus minimizing the need for animal testing (3Rs).

In the EC FP6 Integrated Project OSIRIS, we collected those materials using our knowledge of the Russian language and access to the corresponding literature and created a web-database "E-SovTox", which includes eco- and toxicological data collected mainly from Russian scientific journals published since 1957 and archived in Estonian libraries. Altogether data from ~500 articles and ~650 substances are presented. The database contains numerical (eco)toxicity data, literature references, abstracts in Russian and English and pdf-files of the scanned and partially OCR-treated articles. The goal of the creation of this database is to provide original data with associated literature references (and not to validate the data). The terms of use of this database will depend on user and purpose and remain to be determined. The search engine works in Roman and Cyrillic characters. The database will be finalized in 2010.

ID ABS: 156

ASPCA animal poison control center uses its databases to study the efficacy and safety of three emetics in dogs and cats utilizing 3R principles

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The ASPCA Animal Poison Control Center (APCC) provides a 24-hour consulting service to pet owners and veterinary staff on management of animal poisoning throughout the United States and Canada. With each case, history including signalment, exposure, treatment and outcome is collected, entered, and stored in a relational database. Previously, this information has been used to identify species-specific toxicoses including *Lilium* sp., xylitol, macadamia nuts, and raisins and grapes. The present study was initiated to evaluate the use of three different emetics in dogs and cats. Emetics are often used in certain situations to induce vomiting after a toxicant has been ingested. In dogs and cats, several emetics are used although little data are available on safety and efficacy. Three commonly used emetics, apomorphine, xylazine, and 3% hydrogen peroxide, were selected. Data involving the use of these emetics was retrieved from ASPCA databases. Information collected included effectiveness, adverse effects, frequency of vomiting, and the estimated amount of agent recovered.

Preliminary data suggests hydrogen peroxide and apomorphine are effective emetics in dogs without report of significant adverse reactions. Xylazine was the most effective emetic in cats, whereas, apomorphine was completely ineffective. When successfully induced, most patients vomited some portion of toxicant.

The results show that the APCC database can be used to study clinical interventions, and in this instance the information can help veterinary staff decide the best emetic to use in dogs and cats. This investigation indicates that retrieving information from databases can replace the need for animals in laboratory studies.

ID ABS: 214

Use of historical local lymph node data in the development of alternative test methods for skin sensitization – collection of new data

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In developing new alternative test methods, the availability of high quality, relevant and reliable *in vivo* data for the endpoint of interest is essential. To assist with the work associated with the development and validation of alternative methods for skin sensitization testing we have previously published a database comprising LLNA data on 211 individual chemicals. This encompassed diverse chemical classes, a substantial range of physicochemical properties as well as allergenic potencies. Here, we have collated an additional database of results which is complementary to the first. This comprises some 109 substances which both add to and extend the spectrum of chemistry covered. In combination, the datasets represent a very comprehensive package of LLNA results, including potency estimations as well as an indication of being pre or pro hapten for some 320 chemicals. The first database also contained a small number of inaccuracies (affecting eight chemicals) which are herein corrected. It is anticipated that this extended database will represent the primary resource facilitating the development, evaluation and eventual validation of new approaches to skin sensitization assessment.

ID ABS: 304

Enhancing the value of carcinogenicity data

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Databases are an important tool through which to share data, thereby reducing the need for toxicity testing. As carcinogenicity studies are low throughput, expensive and time consuming there is a clear benefit to organisations in capturing the data from these studies in a structure-searchable format. To this end we have proposed a database schema for collating carcinogenicity data.

The proposed schema includes key experimental conditions such as species, strain, sex, doses and route of administration. To allow assessments to be made on the quality of the data, fields have been added to capture information on whether the study was conducted according to international accepted guidelines, to GLP and the level of mortality observed. A sub-table has also been added to capture tumour incidence data. The vocabulary used for tissue and tumour type has been standardised to ensure compatibility with other important data sources such as the Carcinogenic Potency Database (CPDB). Control data is displayed alongside the tumour incidence data for each dose group in order to facilitate assessments of the significance of any findings reported.

Feedback is being sought to determine if the proposed schema will meet the needs of organisations wishing to interrogate the data for structure-activity relationships. The schema has also been used to extract data on the carcinogenicity of pesticides obtained through a CRADA between Lhasa and the U.S. FDA ICSAS as part of a data sharing initiative between the U.S. EPA OPP and FDA ICSAS.

ID ABS: 325

Progress in the sharing of toxicity data between organizations in order to reduce the number of animals used for testing

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Test substances within the pharmaceutical industry are often formulated in a vehicle (i.e. as a solution or suspension) or include one or more excipient(s) in order to increase solubility and promote absorption. The vehicle/excipient *per se* should evoke no effect or little effect in the animals they are administered to. Data regarding the biological effects in the absence

and presence of the vehicles/excipients is important for both the planning and interpretation of toxicological and pharmacological studies. In order to reduce the amount of animal testing required in this area, a group of major European pharmaceutical companies together with the U. K. charities RSPCA (The Royal Society for the Prevention of Cruelty to Animals) and FRAME (Fund for the Replacement of Animals in Medical Experiments) decided to share excipient/vehicle toxicity data with each other. They contacted the not-for-profit company and charity Lhasa Limited of the UK in order to develop and host an excipient/vehicles database. The project is well underway, and the first release of the full database is due in May 2009. This project has allowed the sharing of data to take place between pharmaceutical organizations and in addition has provided a central repository for excipient/vehicle data, allowing much easier retrieval of the information.

ID ABS: 349

The serum-free media interactive online database

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Fetal bovine serum (FBS) is a ubiquitously used essential supplement in cell culture media. However, there are serious scientific and ethical concerns about the widespread use of FBS concerning its harvest and production. During the last three decades, FBS could be substituted through other supplements or by the use of defined chemical components in serum-free cell culture. A number of serum-free media formulations have been described for continuous mammalian and insect cell lines as well as for primary cultures. However, switching to serum-free media still needs a time-consuming literature survey and manufacturer search for appropriate media formulations, respectively. Here we present the second collection of commercially available serum-free media in an updated freely accessible interactive online database. Serum-free media and continuous cell lines already adapted to serum-free culture can be searched for by means of different criteria. Searchable criteria are the degree of chemical definition, e.g. serum-free, animal-derived component-free or chemically defined, and the kind of medium, e.g. basal media, media supplements, or full replacement media. In order to specify the cell lines that are adapted for serum-free media, search terms like organism, organ, tissue, cell type and disease can be used. Almost all commercially available serum-free media and adapted/non-adapted cell lines currently available from main distributors (e.g. ATCC, ECACC and DMSZ) are included in the database. Despite extensive search for serum-free media and adapted cell lines, there is still a lack of detailed information from companies and suppliers, which is specifically highlighted. The database will be accessible at http://www.zet.or.at.

ID ABS: 434

Information at your fingertips... but people make the difference

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In the early '90s, Bill Gates gave a keynote address entitled "Information At Your Fingertips", in which he laid out his vision of personal computing. Today, we have truly arrived at his vision in many, many ways. We not only have computing on our desktops, but we now have it in our hands with ever more powerful means to access information. Information is truly at the research community's fingertips, but many people do not hold the knowledge of how to access that information to best meet their research needs or to address the 3Rs of Alternatives – Reduction, Refinement and Replacement. Information has become overwhelming and knowing where and how to access that information is the key. It takes PEOPLE to make the DIFFERENCE. It is only through the combined knowledge and communication of information and research professionals that the depth and breadth of the 3Rs can fully be addressed. In this poster we will explore the DIFFERENCE that people can make on discovery, interpretation and application of the 3Rs.

ID ABS: 546

AltTox.org: an interactive platform for advancing non-animal methods of toxicity testing

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AltTox.org is an interactive website devoted exclusively to the transition in toxicity testing from animal-based to non-animal methods. This transition is being spurred by animal welfare, human health, and other factors, and made possible by advances in biology and technology. The 2007 National Research Council report on Toxicity Testing in the 21st Century called for nothing less than a paradigm shift in favor of mechanism-based assessment instead of outcome-driven assessment; non-animal methods are likely to become the preferred models for mechanism-driven assessments. This paradigm shift will take coordinated efforts by stakeholders in government, industry, academia, and NGOs. AltTox is designed to provide a common platform for these diverse stakeholders to exchange information and perspectives on the relevant science and policy issues. The website consists of two interconnected components: a series of message boards, or forums, and an informational resource center. The forums act as bulletin boards for rapid exchange of information and perspectives. The resource center features concisely summarized and comprehensive information on toxicity testing and alternative test methods. A highlight of the resource center is a series of opinion pieces on "The Way Forward", written by invited experts in relevant subfields. Launched in December, 2007, AltTox receives 6,000 visits per month and 48,000 forum message views per month. The site is managed by The Humane Society of the United States and The Procter & Gamble Company, is funded by these and other organizations, and its content is overseen by international experts serving as editorial board members and forum moderators.

ID ABS: 555

Building an information database for developmental neurotoxicity testing: a systematic approach

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Existing guidelines for developmental neurotoxicity (DNT) assessment are based on an *in vivo* approach requiring neuropathological and behavioural tests on around 4,000 animals per substance. These tests are not always sensitive and specific enough to detect toxicity to the developing human brain.

In vitro systems can reproduce several key cellular processes of brain development. These cell models can be the basis for test batteries, which through multiple endpoints can analyse compounds with diverse mechanisms of action. To efficiently establish these testing strategies, mechanistic knowledge is needed to match the system/methods properties and the chemical's mode of action (MoA). This will help to know in advance, and possibly overcome, the limitations of the *in vitro* test system.

We are developing a tool that uses simple search strategies and a public database (NIH-NLM-Toxnet) to retrieve, organise, analyse information about chemicals. This database tool has a dual aim: first, to prioritise compounds for DNT testing according to existing indications of their toxicity to the developing nervous system and second, to retrieve information on MoA, biotransformation and other biochemical features relevant for the testing strategy.

Future work will focus on a deeper investigation of the MoA of a list of substances possibly affecting the developing brain and subsequently identify those compounds whose MoA can be potentially identified by one or a battery of *in vitro* methods. For risk assessment these test methods should be evaluated as early warning signs. Additionally, links should be sought between short-term tests and long-term low-dose exposures as more realistic scenarios.

ID ABS: 563

From guinea pig to computer mouse: the evolution and impact of an alternatives book and database

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The alternatives book and database "From Guinea Pig to Computer Mouse: Alternative Methods for a Progressive, Humane Education" (2nd ed.) was published by InterNICHE in 2003, updated and launched on-line in 2006, and updated again in 2009. Part A of the resource addresses humane education and replacement alternatives, curricular design, assessment of knowledge and skills acquisition, and conscientious objection. Part B, written by contributing teachers, provides international case studies addressing the development and implementation of alternatives. Part C is the database of over 500 alternatives, presenting the results of primary research by InterNICHE. The database is ordered according to discipline (anatomy, clinical skills and surgery, physiology, etc); according to medium (software, video, model/simulator, web-based resource); and according to individual alternative product. For each product there is a full description, detailed specifications, price and source. Part D details web and other resources, libraries of alternatives, organisations and producers, and the Appendix presents the InterNICHE Policy on the Use of Animals and Alternatives in Education. The database provides concise information to teachers, students, campaigners and others to facilitate informed choices about learning tools and approaches, and is a required reference resource for some university ethics committees. The resource has been translated whole or in part into 14 languages, and over 12,000 copies in book form or on CD/DVD have been distributed in over 70 countries. On-line use of the resource, and downloads, have also been numerous and global.

PO9: *In vitro* technologies

ID ABS: 11

Cell based assay for label-free, multiparametric, long-term monitoring of cellular vitality

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As an alternative method for animal experiments in the field of toxicodynamics the cell based assays developed at the Heinz Nixdorf-Lehrstuhl für Medizinische Elektronik of Technische Universität München are suitable. These are system solutions for online analysis of living cells. The cell based assays monitor different parameters directly in living cells. These parameters are extracellular acidification (pH), cellular respiration (pO_2) and changes in morphology (impedance) of the living cells. The measurement is label-free, parallel, continuous and in real-time. With this cell based assays it is possible to determine the toxicity of a drug *in vitro* (without the use of animals) using living cells as test organism. Measurements can be performed over weeks without the use of labels. Unique data from drug/cell interaction can be determined. Advantageous properties of the system are: multiparametric (determination of vitality and morphology), long-term and label-free measurements, high sensitivity and an optimized fluidic system. Results using 3T3 fibroblast in a toxicodynamic experiment are shown. Other fields of applications are pharmacology, basic biological research, individualized chemotherapy, environmental monitoring or quality testing of transplant cells. Different cell types (e.g. 3T3, MCF-7, MDA, yeast, algae, primary human cells) and substances (chemotherapeutics, toxins, stimuli) have been tested with the system.

ID ABS: 13

In vitro evaluation of 3D skin models for congenital ichthyosis following standardized procedures

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For the testing of skin absorption, the OECD approved guidelines for *in vivo* and *in vitro* testing, legitimating the replacement of animal experiments by alternative tests. As a member of a German research consortium we have previously contributed to the validation of a new test protocol using commercially available reconstructed human epidermis to quantify skin permeation and penetration. The aim of the present study was to assess a recently established 3D model for congenital ichthyosis, representing a model disease for hereditary severe epidermal barrier function defects. We generated control models, models from patient cells and models mimicking congenital ichthyosis through RNAi-mediated gene knock down. The tissues were evaluated for permeation of the OECD standard substances caffeine and testosterone by applying validated experimental procedures. Major and disease typical differences in barrier permeability and function were observed, as for both test compounds the permeation coefficients were markedly increased in patient and gene knock down models when compared with control samples. In addition, the models are being evaluated for therapeutic approaches using innovative nanocarrier systems. Effects on skin penetration were studied with solid lipid nanoparticles and dendritic core-multishell nanotransporters. Both nanoparticles, loaded with nile red as a model dye, demonstrated strongly enhanced penetration into control models as compared to conventional cream. The penetration was even more pronounced in models created from patient samples, thus reflecting the defective epidermal barrier. In conclusion, standardized procedures were successfully applied to characterize an *in vitro* disease model, which renders future investigations on potential new drug targets feasible.

ID ABS: 15

Dynamic culture in *quasi vivo* TM multi compartment bioreactors upregulates cytochrome expression in human hepatocytes

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In vitro liver models for toxicity testing suffer from a number of drawbacks, including short term viability, and phenotypic changes mainly associated with huge drops in P450 expression of hepatocytes. This has been generally attributed to the fact that the complexity of the physiological environment is not replicated in petri dishes or microplates. In fact, all cells are exquisitely sensitive to their microenvironment, which is rich with cues from other cells, and from mechanical stimuli due to flow, perfusion and movement. Current methods for investigating cellular responses *in vitro* are inadequate in this sense, since the complex interplay of mechanical and biochemical factors is absent. To address these issues we have developed a "system on a plate" modular multicompartmental bioreactor (MCB) which enables microwell protocols to be transferred directly to the bioreactor modules, without redesign of cell culture experiments. The new system offers mechanical stimuli from flow, and biochemical stimuli from cells placed in connected modules. Human hepatocytes were cultured in the MCB system by connecting eight modules in series. The cells were subject to a flow rate of 180 microliter/min, and gene expression was quantified with respect to freshly isolated hepatocytes from the same liver sample, as well as cells in control (multiwell) conditions. The results show that even after 4 weeks, most P450 enzymes (CYP 3A4, 2B6, 2C9, 1A2, 3A7,1A1) and several phase II conjugating enzymes are upregulated in the MCB as compared with the static controls, reaching the same levels as found in freshly isolated hepatocytes.

ID ABS: 17

ISOCYP-TOX: a new ready-to-use concept for *in vitro* evaluation of biotransformation-mediated toxicity

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The toxicity of a compound may be caused not only by the parent molecule but also by its metabolites. Metabolism, indeed, can result in a bioactivation phenomenon rather than in a detoxification process, leading to metabolism-dependent toxicity that cannot be assessed by cell lines currently used for basal cytotoxicity screening, as they lack biotransforming enzymes. Toxicity is responsible of 30% of the attrition rate in drug development. Thus there is an urgent need to develop methods to evaluate compound toxicity at early stages.

ADVANCELL has developed a new technology platform based on adenoviral transduction for transient expression of biotransformation enzymes that allows: a) to modulate the CYP450 enzymatic activity and reach comparable levels to those obtained in subcellular fractions or fresh hepatocytes, b) to perform studies either with each of the CYP isoforms individually, or customized combinations, and c) to reproduce human metabolic idiosyncrasy.

Based on this concept, ADVANCELL has generated a new cell-based and ready-to-use reagent, ISOCYP-TOX, for *in vitro* screening of CYP450 biotransformation-mediated toxicity in 96-well plate format amenable to HTS platforms. The reagent consists of a HepG2 cell line transiently transduced with individual CYPs, making these cells metabolically competent. ISOCYP-TOX represents a cost-effective method for the early detection and screening of bioactivation-dependent acute toxicity of compounds through rapid analysis for curve fitting and IC_{50} generation, as well as identification of CYP isoforms involved in the production of toxic metabolites. A short prevalidation study with 21 compounds was performed in collaboration with pharma companies. Results will be presented and discussed.

ID ABS: 18

In vitro basal cytotoxicity assay applied to estimating acute oral systemic toxicity of grandisin and its major metabolite

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Preclinical investigations can start with preliminary *in vitro* studies before using animal models. Following this approach, the number of animals used in preclinical acute toxicity testing can be reduced. In this study we employed an in-house validated *in vitro* cytotoxicity test based on the Spielmann approach to toxicity evaluation of the lignan grandisin, a candidate anticancer agent, and its major metabolite, 4-O-demethylgrandisin, by neutral red uptake (NRU) assay on mouse fibroblasts Balb/c 3T3 cell line. Using different concentrations of grandisin and its major metabolite (2.31 μmol/ml; 1.16 μmol/ml; 0.58 μmol/ml; 0.29 μmol/ml; 0.14 μmol/ml; 0.07 μmol/ml; 0.04 μmol/ml; 0.002 μmol/ml) in the Balb/c 3T3-A31 NRU cytotoxicity assay, after incubation for 48 h, we obtained IC_{50} values for grandisin and its metabolite of 0.078 μM/ml and 0.043 μM/ml, respectively. The computed LD50 of grandisin and 4-O-demethylgrandisin were 617.72 mg/kg and 429.95 mg/kg, respectively. Both were classified under the Globally Harmonized System as category 4. Since pharmacological and toxicological data are crucial in the developmental stages of drug discovery, using an *in vitro* assay we demonstrated that grandisin and its metabolite do not possess quite equal toxicity profiles. Furthermore, the data presented herein can contribute to reducing the number of animals required in subsequent studies.

ID ABS: 19

THP-1 monocytes but not macrophages as a potential alternative for CD34+ dendritic cells to identify chemical skin sensitizers

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Early detection of the sensitizing potential of chemicals is an emerging issue for chemical, pharmaceutical and cosmetic industries. In our institute, an *in vitro* classification model for prediction of chemical-induced skin sensitization based on gene expression signatures in human CD34+ dendritic cells (DC) has been developed. This primary cell model is able to closely mimic the induction phase of sensitization by Langerhans cells in the skin, but it has drawbacks, such as the availability of cord blood.

The aim of this study was to investigate whether human THP-1 monocytes or macrophages display a similar expression profile for 13 predictive gene markers previously identified in DC and whether they also possess a discriminating capacity towards skin sensitizers and non-sensitizers based on these marker genes. To this end, the cell models were exposed to 5 skin sensitizers (ammonium hexachloroplatinate IV, 1 chloro 2,4 dinitrobenzene, eugenol, para-phenylenediamine, and tetramethylthiuram disulfide) and 5 non-sensitizers (L glutamic acid, methyl salicylate, sodium dodecyl sulfate, tributyltin chloride, and zinc sulfate) for 6, 10, and 24 hours, and mRNA expression of the 13 genes was analyzed using real-time RT-PCR.

The transcriptional response of 7 out of 13 genes in THP 1 monocytes was significantly correlated with DC, whereas only 2 out of 13 genes in THP-1 macrophages were. After cross-validation of a discriminant analysis of the gene expression profiles in THP-1 monocytes, this cell model demonstrated to also have the capacity to distinguish skin sensitizers from non-sensitizers. However, the CD34-DC model was superior to THP-1 monocytes for discrimination of (non-)sensitizing chemicals.

ID ABS: 20

Gene profiles of BEAS-2B cells after *in vitro* exposure to respiratory (non-)sensitizing chemicals

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Respiratory sensitization is a concern for occupational and environmental health in consumer product development. Despite international regulatory requirements there is no established protocol for the identification of chemical respiratory sensitizers. New tests should be based on mechanistic understanding and should be preferentially restricted to *in vitro* assays. The major goal of this study was to investigate the alterations in gene expression of human bronchial epithelial (BEAS-2B) cells after exposure to respiratory sensitizers and respiratory non-sensitizing chemicals and to identify genes that are able to discriminate between both groups of chemicals. BEAS-2B cells were exposed for 6, 10, and 24 h to the respiratory sensitizers ammonium hexachloroplatinate IV, hexamethylene diisocyanate, and trimellitic anhydride, the irritants acrolein and methyl salicylate, and the skin sensitizer 1-chloro-2,4-dinitrobenzene. Overall changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide arrays. Fisher Linear Discriminant Analysis was used to obtain a ranking of genes that reflects their potential to discriminate between respiratory sensitizing and respiratory non-sensitizing chemicals. The 10 most discriminative genes were BC042064, A_24_P229834, DOCK11, THC2544911, DLGAP4, NINJ1, PFKM, FLJ10986, IL28RA, and CASP9. Based on the differentially expressed genes, pathway analysis was used to identify possible underlying mechanisms of respiratory sensitization. We demonstrated that in bronchial epithelial cells the canonical PTEN signaling pathway is probably the most specific pathway in the context of respiratory sensitization. Results are indicative that the BEAS-2B cell line can be used as an alternative cell model to screen chemical compounds for their respiratory sensitizing potential.

ID ABS: 32

Assessment of sensitivity pattern – three different *in vitro* bioactivity assays

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Metabolic products of many living organisms in general and their secondary metabolites in particular are biologically active. Nature promises a wealth of biologically active compounds. Finding out the active principle of a compound is a difficult task. Bioactivity of a natural compound is assessed by various methods. The methods include *in vitro*, *in vivo* and mechanism based assays. Of these, the *in vitro* test is an alternative to the *in vivo* test, as the *in vivo* test has problems like ethical issues. Throughout the world people are looking for easy and reliable techniques to assess bioactivity. An attempt was made in this study to look for the sensitivity of three different *in vitro* methods, i.e. the Dye Exclusion Method (DEM), Sulphoradamine B (SRB) and 3-(4,5 dimethyl thiazol-2-Yl)2, 5-diphenyl tetrazolium bromide (MTT) assays. The protocols for these assays were standardized. A total of 135 marine flora and fauna extracts (crude) were collected from a marine source. All collected samples were used as study material to look for their bioactivity using the three selected *in vitro* methods. From the results obtained the sensitivity was determined. Of the three assays used, the MTT assay was found to be the best. This assay can be used in a laboratory in which the thymidine uptake assay cannot be performed.

ID ABS: 44

Isolation, characterization and hepatic differentiation of adult stem/progenitor cells from easily accessible human sources

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In our previous research, mesenchymal stem/progenitor cells (MSC) derived from adult human bone marrow were successfully triggered to differentiate into a homogeneous population of functional hepatocyte-like cells by exposing them to hepatogenic factors in a gradual and sequential time-dependent manner. However, human bone marrow is only available to a limited extent, yields a low number of multipotent cells upon processing and carries a risk for the donor. Therefore, in the context of industrial needs, human bone marrow as a source of MSC might not be ideal. It was therefore decided to search for alternative - readily available and easily accessible - sources of adult human MSC. Both human skin and human adipose tissue are candidate sources. They contain a relatively high number of MSC and can be easily derived from plastic surgical waste material. These sources have the additional advantage that they may be obtained from volunteers of all ages, being in good health and of both sexes.

In the present study, the general geno- and phenotype of human skin-derived precursors (hSKP) and human adipose tissue stem cells (hADSC) are explored by means of microarray analysis and immunocytochemistry, respectively. Special attention is paid to the variability in gene and protein expression profiles of the cells among different donors (age, gender, health condition) and application of different culture conditions (various passages and cell densities). In addition, the potency of hSKP to differentiate into hepatocyte-like cells, when cultured under the same conditions as optimised for MSC from postnatal bone marrow, is investigated.

ID ABS: 45

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

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The increasing demand for assessing inhalation toxicity hazards calls for new testing strategies comprising both *in vitro* and *in vivo* assays. For this purpose, we are currently evaluating a direct exposure strategy, in which cells are exposed to toxic gases at the air/liquid interface. The human carcinoma alveolar epithelial cell line A549, grown on microporous membranes, is exposed to test atmospheres in a system enabling at the same time steady state nutrification, humidification and direct gas exposure. Under coordination of the Fraunhofer Institute, we are assessing the intra- and interlaboratory reproducibility and predictive capacity of the method by characterizing the toxicity of four gases, i.e. NO_2, SO_2, formaldehyde, and ozone. The aims of this study are: optimisation and refinement of experimental protocols; generation of standard operating procedures; assessment of reproducibility within and between laboratories; establishment of test acceptance criteria; determination of the *in vitro* vs. *in vivo* dose-response relationships. After transfer of the method, optimization of protocols and experimental procedures the four partners started definite testing of the gases. Each gas, together with an online analytical monitoring system, is passed from one lab to the next after six weeks of experimentation. The test design comprised one hour gas exposure followed by direct determination of cytotoxicity (electrical current exclusion method, CASY®, Innovatis) and genotoxicity (COMET assay). So far, the project has proven satisfying transferability of the test system, depending on the laboratory being practiced in this complex methodology.

ID ABS: 46

An *ex vivo* murine mandible culture model for inflammatory bone destruction

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Inflammatory bone destruction is central to the pathogenesis of diseases such as periodontitis, and rheumatoid arthritis. To reduce the number of animals used for research in inflammatory mediated bone destruction a murine mandible slice culture model has been developed and the responsiveness of the model examined.

Methods: Hemi-mandibles dissected from 10-12 week old male CD1 mice were sliced into 1mm transverse sections and maintained in Trowel-type cultures at 37°C in 5% CO_2 in air for up to 14 days, in the presence or absence of 100 ng/ml LPS. Viability was assessed by histomorphometry and resident osteoclastic response to stimulation measured by quantification of TRAP positive multinuclear cells. Sections were immunolabelled for LPS receptor (TLR4), and markers of proliferation (PCNA) and bone matrix (BSP). To develop local bone resorption, labelled preosteoclasts were microinjected into the periodontal ligament of mandible slices prior to culture.

Results: LPS stimulation significantly increased the number of ligament cell nuclei after 7 days, while longer durations resulted in significantly reduced numbers. PCNA immunopositivity suggested no increase in cell proliferation in treated slices. Loss of ligament tissue architecture and reduction in BSP immunolabelling was observed in response to LPS and increases in both TLR4 expression and TRAP positive osteoclasts noted. Tracer fluorescence indicated microinjected preosteoclasts remained at the injection site, while slice histomorphometry was maintained.

Conclusions: This method provides suitable conditions for the culture of murine mandible slices and a viable model for the inflammatory mediated bone pathophysiology and novel treatment modalities.

(Supported by NC3Rs grant 77844)

ID ABS: 53

Monitoring of cardiac cytotoxicity in real-time with the xCELLigence system

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Cell-based assays are a valuable tool for the investigation of compound-induced cytotoxicity, e.g. in the early phase of compound screenings. Most commonly used *in vitro* assays for the analysis of cell viability and cell death are invasive or destructive end-point assays, providing relatively limited information to the researcher. The new impedance-based xCELLigence System, co-developed by Roche and ACEA, allows a label-free and continuous monitoring of cell cultures in real-time. The electric impedance is generated by the interaction of adherent cells with a microelectrode biosensor, reflecting cellular parameters, such as proliferation, cell death, adhesion, spreading, and morphological alterations. Here we demonstrate that compound-induced cardiotoxicity can be continuously monitored and quantified by the xCELLigence System. To this end, ES cell derived mouse Cor.At® cardiomyocytes, developed by Axiogenesis, were cultured on E-plates 96 and treated with known cardiotoxic compounds. Cell cultures were continuously monitored by the xCELLigence System, yielding a comprehensive compound-specific profiling, which could be used to calculate a time-resolved IC_{50}. Interestingly, fetal-like and adult-like Cor.At® cells responded differently to the tested compounds, accounting for an altered sensitivity of the two different cardiac phenotypes. A putative accumulation of cytotoxic effects was evaluated by long-term experiments with repeated compound treatment, which could be continuously monitored during the whole period of the experiment. The herein presented data describe a powerful new cell-based assay for the *in vitro* investigation of compound-mediated cardiotoxicity by combining the standardized and pure Cor.At® cardiomyocyte cell culture conditions with the xCELLigence System.

ID ABS: 56

Human skin explants as an *in vitro* alternative to animal testing

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Skin is composed of the epidermis, dermis, subcutaneous fat, blood vessels, nerves, and appendages. The currently available *in vitro* model systems for skin research are not equally representative of these different compartments. Our group is developing a portfolio of alternative *in vitro* model systems for skin research, to enable gaining new biological insights without animal testing.

2D- and 3D cell-based systems are more promising for mechanistic understanding; however, they do not represent the full complexity of the skin. The skin explants model system provides the highest *in vitro* physiological complexity possible today, however current skin explant systems are optimized for epidermal functions, but not for the optimal metabolic activity of the dermal and adipose compartments.

Using full-thickness human skin biopsies obtained from healthy donors undergoing abdominal surgeries with informed consent, we are developing a system that will enable the evaluation of dermatological agents for their biological activities via both systemic and topical treatments. We continue to optimize culture conditions for viability, metabolic activity and integrity of all three compartments. Multiple molecular and biochemical methods are being used to identify biomarkers for tissue functions and for responsiveness to test agents. While the system is still under development, we are gaining knowledge on the abilities and the limitations of this system while using agents of known clinical or *in vitro* activities.

ID ABS: 58

MEA technology for *in vitro* neurotoxicity testing in the context of regulatory requirements

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Prediction of neurotoxic effects is a key feature in the toxicological profile of many compounds and is therefore required by regulatory testing schemes. Currently, neurotoxicity assessment required by the OECD and EC test guidelines is based solely on *in vivo* testing, evaluating mainly effects on neurobehavior and neuropathology. *In vitro* recordings from cultured neuronal networks coupled to microelectrode arrays (MEAs) represent a simplified model to study the electrophysiological behaviour that could serve as a neuronal functional endpoint for *in vitro* neurotoxicity evaluation. Indeed, this technique is a powerful tool to routinely evaluate the dynamics of the spontaneous and evoked activity behavior in response to external chemical exposure. Neurons of various origins can be grown coupled to such an MEA device. Electrophysiological activity can be monitored starting from a few days *in vitro* for up to several months. Neurons exchange information by firing action potential spikes and bursts that can be easily extracted from MEA recordings. It has been shown that these spatial and temporal patterns of activity are highly sensitive to neuroactive and neurotoxic agents at very low concentrations. Neuronal electrophysiology responds to transmitters, their blockers, agonists and many other pharmaceuticals in a histiotypic manner similar to *in vivo* situation. The extracellular nature of the recording system enables the evaluation of both acute manipulation and long term exposure (developmental neurotoxicity). This represents a functional neuronal endpoint that can be integrated together with other standard available cell-specific endpoints (e.g. cytoskeleton damage or energy metabolism) into a reliable *in vitro* neurotoxicity testing strategy

ID ABS: 59

Protein biomarkers for *in vitro* testing of embryotoxicity

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New challenges for hazard and risk assessment in the chemical industry with regard to REACH legislation in Europe and related activities in the US and Japan require the development of novel in vitro models for toxicity tests. Developmental toxicity is regulated by guidelines, including assessments of brain morphology, behaviour, development of young animals, measurements of biomarkers for gliosis and cytotoxicity and more, requiring huge numbers of test animals. In the frame of a European FP6 project on reproductive toxicology (www.reprotect.eu) we used protein lysates from the validated embryonic stem cell test (EST) protocol and related ES models in a differential quantitative proteomic study to identify novel surrogate protein biomarkers for embryo toxicity.

The combination of quantitative proteomic differences and functional data from appropriate *in vitro* models has generated a data set on a set of model substances assigned to four categories of embryotoxicity, i.e. “strong”, “moderate”, “mild”, or “non-embryotoxic” based on *in vivo* data (selected by independent experts for the Reprotect consortium). Substance-dependent cardiomyocyte protein extracts were subjected to systematic differential proteomic profiling. Dual radioisotope labelling of proteins provided the rigorous quantitative pattern control necessary to obtain statistical significance. Moreover human and mouse embryonic stem cell models for neuronal differentiation and further substances were included to understand the general significance of our findings.

The aim is to provide molecular content for novel and fast *in vitro* strategies for safety tests for developmental toxicity and the potential to substantially reduce animal experiments according to the 3Rs concept (Reduce/Refine/Replace).

ID ABS: 61

In vitro assessments of hepatic toxicity using the xCELLigence real-time cell analyzer

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Cell-based *in vitro* assays are a key tool for the assessment of compound-induced hepatotoxicity in early drug development before animal testing. In order to improve the predictive value of *in vitro* tests and to support the 3Rs concept of Reducing, Refining and Replacing animal experimentation, there is a need for new, innovative techniques. Most commonly used conventional *in vitro* assays for cell viability are end-point assays, which eventually also require lysis of cells. A non-invasive and label-free tool allowing continuous measurements is provided by the xCELLigence real-time Cell Analyzer system, co-developed by Roche and ACEA Biosciences. The method is based on measuring the impedance of adherent cells, which interact with a microelectrode biosensor. This allows dynamic monitoring of cellular events, such as proliferation, apoptosis, adhesion, spreading and morphological alterations, in a 96well format over an extended period of time. This study demonstrates that the xCELLigence System, combined with molecular expression profiling analysis, may be a suitable *in vitro* method to identify toxic effects of compounds. By using primary rat and human hepatocytes, development of liver toxicity can be continuously monitored over time and quantified by the xCELLigence System, yielding a comprehensive, compound-specific impedance profiling, which may be used to calculate a time-resolved IC_{50} and helps characterization of compounds and compound series at an early phase of drug development.

For life science research only.

ID ABS: 63

Development of an *in vitro* sensitization assay based on monocyte-derived dendritic cells

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Dendritic cells, including Langerhans cells, forming a sentinel network for pathogen detection are the most abundant antigen presenting cells in the skin. Through their ability of hapten uptake, processing and presentation to T-cells they play a critical role in the induction of contact allergies. In this process dendritic cells undergo fundamental changes, e.g. surface marker expression. Their observance marks a potential endpoint in the experimental set-up of a predictive *in vitro* skin sensitization assay. Thus, CD1a-/CD14+ peripheral blood monocytes from donors where purified by density centrifugation and positive selection of anti-CD14-Ig coupled magnetic microbeads. CD1a-/CD14+ monocytes were differentiated into immature dendritic cells by 5 day culture in the presence of IL-4 and GM-CSF. Substance treatment for 48 h was followed by FACS analysis of HLA-DR, CD86, CD80, CD14, CD1a and CD83.

In this assay all strong sensitizers tested as well as non-sensitizers where identified correctly referring to their allergic potential in the LLNA, whereas moderate sensitizers (according to the LLNA) showed surface marker changes only close to cytotoxic concentrations. Limitations, e.g. donor variability and work intensiveness, are widely discussed. However, this assay leads to results that reflect the reaction of a healthy donor population in contrast to single individuals of cell lines. Moreover, after interpretation of comprehensive investigations we assume that a classification of the sensitizing potential of substances may be possible, marking a clear advantage over an all-or-none interpretation of cell line based assays already established.

Therefore this assay provides a basic application in assessing the allergic potential of active components.

ID ABS: 65

A vascularised liver cell module as an alternative to animal experiments

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The liver is the largest internal organ responsible for metabolism and therefore an interesting analysis system for substances. Existing *in vitro* liver test systems show only a small part of biological reactions of the liver *in vivo*. Essential for hepatocyte (HC) vitality and function *in vitro* are a vascularisation to guarantee cell supply and metabolite evacuation, extracellular matrix contact and the co-culture with endothelial cells (EC), which build up a filtration barrier between blood and HC, and are involved in regeneration processes.

We created a vascularized matrix for long-term co-culture of human and porcine HC and EC. Basis is a decellularized porcine jejunal segment with an obtained vascular system. The vascular system could be reseeded with EC whereas the former intestinal lumen provides a large surface for the co-cultivation of HC. We furthermore developed a computer controlled bioreactor system for the perfusion of the scaffold simulating blood flow conditions. Media and tissue samples allow analyses of cell viability, differentiation and function during the cultivation.

The culture of HC on the vascularized matrix shows good results for cell growth and conservation of liver specific functions. The HC synthesize albumin and urea during the whole cultivation period. Phase I and II metabolism of dextrometorphan could be shown over 21 days. Immunohistochemical staining demonstrates HC proliferation, receipt of differentiation, and the formation of tight and adhering junctions between the cells. The seeded EC remained differentiated.

The new vascularised liver module thereby could represent an interesting alternative to animal experiments for substance analysis.

ID ABS: 66

Development of a 3D-liver cell culture model using polystyrene (PS) scaffolds

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Introduction: The aim of the study was to develop a reliable and powerful cellular model for studying drug metabolism. Therefore, HepaRG cells, a human progenitor cell line that is able to differentiate into hepatocyte-like cells, were cultured in a three-dimensional (3D), porous matrix (activated polystyrene) to reach more physiological culture conditions than in 2D cell culture.

Methods: HepaRG cell morphology and settling were investigated using haematoxylin staining, formazan formation, light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM) analysis. Cell proliferation (MTT assay) and cell cytotoxicity (LDH assay) were determined. Differentiation of HepaRG cells was induced by dimethyl sulfoxide. Thereafter, CYP450 expression was analyzed by qRT-PCR and activity by fluorescence based assays. Metabolic functionalities (albumin synthesis, urea formation) were measured over time.

Results: Cell staining and formazan formation revealed a uniform distribution of HepaRG cells in the PS matrix. Ultrastructure analysis showed HepaRG cells in monolayers adhering tightly to the PS matrix. Analysis of cell viability, cytotoxicity and cell proliferation proved that PS scaffolds are well tolerated by HepaRG cells. Differentiation of HepaRG cells within PS scaffolds resulted in significantly higher mRNA expression and activity levels of important biotransformation phase I CYP450 genes compared to conventional 2D cell cultures.

Discussion: The cultivation of differentiated HepaRG cells in the porous PS matrix might maintain hepatocyte-like morphology and functionality for an appropriate cultivation time. The development of 3D-liver cell culture models might therefore be a promising tool to reduce the number of animals used in drug development.

ID ABS: 67

Optimization of culture conditions for human intestinal Caco-2 cells to improve functional differentiation: serum-free medium and substrate effects

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Although representing the best and most extensively used cell culture model of absorptive enterocytes, the human Caco-2 cell line displays a high degree of heterogeneity in the expression of differentiated functions that is largely due to differences in culture procedures. Addition of fetal bovine serum in the culture medium represents a source of variability in the performance of this differentiated cell culture model. Traditional plastic cultureware or permeable cell culture inserts are commonly used for Caco-2 cells and can further increase the variability in the expression of differentiated functions. Caco-2 cells (parental cell line obtained from INSERM, Paris) were grown and differentiated for up 21 days on plastic or permeable polycarbonate cell culture inserts, and development of polarity and expression of intestinal functions were assessed by immunofluorescence and confocal microscopy, quantitative RT-PCR and *in situ* enzyme assays. Cells seeded on permeable inserts were also maintained in different defined media containing insulin, transferrin, selenium, a lipid mixture of oleate, palmitate and cholesterol, and a defined mixture of growth factors and hormones (MITO+ serum extender). The effects of the different serum substitutes on cell differentiation were assessed by permeability assays, gene expression studies, and apical intestinal enzyme assays. The results of this study will determine conditions for best performance of Caco-2 cells as a differentiated intestinal-specific cell culture model.

Work supported by EU FP6 Project "LIINTOP" Contract number STREP-037499

ID ABS: 77

The *in vitro* BALB/c 3T3 cell transformation assay to profile the carcinogenic activity of environmental mixtures

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The *in vitro* BALB/c 3T3 transformation assay resembles the *in vivo* multistep tumor process and for many years has been widely used to screen the cancer potential of both genotoxic and non-genotoxic chemicals: It shows good predictability and high sensitivity and specificity and is time-saving and inexpensive compared to animal studies.

The transformation test has also been employed in mechanistic studies to elucidate the possible mechanism of action of carcinogens and was improved in order to provide a suitable model to discriminate the initiating and promoting activity of carcinogens.

Environmentally polluted sample extracts represent complex mixtures whose toxic behavior, due to possible additive or synergistic effects, cannot be predicted by the concentration of each individual component. The assessment of the carcinogenic potential of these complex mixtures requires the use of reliable and sensitive assays measuring relevant endpoints.

The effects associated with environmental samples were evaluated in the BALB/c 3T3 model in order to analyze real mixtures extracted from environmental matrices, to highlight the dose-response relationship and to identify the biomolecular markers directly related to exposure.

This *in vitro* approach could support resource-efficient environmental monitoring and the reduction in the use of animals in toxicology.

Supported by "Fondazione del Monte di Bologna e Ravenna", Bologna, Italy

ID ABS: 82

Human neurospheres can identify neurotoxicants *in vitro*

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Current developmental neurotoxicity (DNT) testing guidelines propose investigations in rodents, which require huge numbers of animals. With regards to the 3Rs and the European Regulation of Chemicals (REACH), alternative testing strategies are needed, which refine and reduce animal experiments by allowing faster and cheaper screening. We have established a 3D test system for DNT screening based on primary human fetal neural progenitor cells, which is now embedded in the BMBF joint project "Development of predictive *in vitro* test for developmental neurotoxicity testing". Within this project, different cell models are compared with regard to their DNT predictability by employing a battery of test compounds. In our system first results indicate that the well known developmental neurotoxicant methylmercury affects proliferation, migration and differentiation of neurospheres in a nanomolar range, while a negative test substance, the liver toxicant paracetamol, showed interference with these processes in millimolar concentrations. Furthermore, the DNT compounds MAM, valproic acid and lead also affect these endpoints, while glutamate, which is not developmentally neurotoxic, is well distinguishable. After shorter exposure times, specific effects on those DNT endpoints are observed at concentrations which do not cause cytotoxicity.

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

ID ABS: 91

Development of an *in vitro* assay for the assessment of photosensitizers

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Photosensitivity is a delayed type IV hypersensitivity induced by a broad spectrum of active components and concurrent exposure of skin to ultraviolet radiation. Due to a high frequency of applied substances in toiletry and clinical therapy there is need for predictive methods in order to determine/classify potential photosensitizers before applying them to cosmetics or pharmaceuticals. Up to now, no adequate *in vitro* alternatives are available. Our intention was to provide an *in vitro* photosensitivity assay for assessing photosensitizers. Since dendritic cells play a key role in the induction of contact allergies, this *in vitro* assay is based on the established monocyte derived dendritic cell (MoDC) assay.

For this purpose, CD1a-/CD14+ monocytes are positively selected from human peripheral blood and differentiated by IL-4 and GM-CSF supplement for 5 days. Test substances are pre-incubated with MoDCs prior to UVA radiation followed by 48h incubation. CD86, HLA-DR and CD83 are measured by FACS.

Known chemicals were chosen for valuation including chlorpromazine, olaquindox (phototoxic and photoallergen), muskambrette (photoallergen and allergen), 2,4-dinitrochlorobenzene and nickelsulfate (allergen only). Results obtained from the in vitro assay were consistent with previously described photosensitizing potentials for all chemicals tested.

Thus, this assay allows the evaluation of the photoallergic potential of substances. Moreover the assessment of their allergic, phototoxic and toxic potential in this single assay is an additional benefit. To improve validity additional substances are tested at present.

The method presented here provides a promising assay for assessing photoallergic and in addition phototoxic potential of relevant substances.

ID ABS: 96

Development and optimization of a DC-based assay for sensitizer identification: the learning process involved in setting-up an inter-laboratory study

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The European Sens-it-iv project aims to develop predictive *in vitro* assays able to discriminate between sensitizing and non-sensitizing compounds. In this study protocols for dendritic-like cell lines (THP-1, U937 and MUTZ-3 progenitor) were compared in an inter-laboratory evaluation involving seven European laboratories. This extensive study aimed to assess three main factors: i) transferability of technology; ii) intra- and inter-laboratory variability and iii) comparison of the results obtained for the three cell lines. The well-known biomarkers CD86 and CXCL8 were used as read-outs.

Cells were exposed to increasing concentrations of 2 sensitizers (DNCB and cinnamaldehyde) and 2 non-sensitizers (SLS and salicylic acid) for 24 hours. Cell survival, CD86 protein expression (flow cytometry) and CXCL8 secretion (ELISA) were analysed.

In general, low intra- and inter-laboratory variability was observed for the cell viability, CD86 and CXCL8 results. All three cell lines correctly discriminated sensitizers from non-sensitizers: the % CD86 positive cells and CXCL8 secretion increased with increasing sensitizer concentrations, but not with increasing non-sensitizer concentrations.

An extensive process of standardization and harmonization of the protocols took place before the start of the inter-laboratory evaluation and resulted in the successful transfer of knowledge and technology between the laboratories and in an overall low intra- and inter-laboratory variability. The consortium is ready for testing an extended panel of test chemicals with the same standardised protocols and for evaluating the performance of the assays with novel biomarkers.

ID ABS: 107

Analysis of the corneal surface with ophthalmologist tool examination and confocal

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While relevant and validated *in vitro* and/or *ex vivo* alternatives exist for ocular corrosivity potential testing, reliable methods for ocular irritative potential assessment are still missing. The aim of this work is to develop an innovative model of isolated cornea, taking into account the tear dynamics and allowing an ophthalmological examination of the cornea. The pathway of cosmetic products on the human ocular surface depends strictly on physiological variables, such as tear parameters (volume, turnover, reflex and dynamics) and corneal characteristics (verticality, blinking and sensitivity). Right after entry into the eye, products increase the tear flow and are washed away within a time depending on their intrinsic viscosity, hydrolipidic affinity and irritative potential. In order to be close to human ophthalmological investigation practices, we developed a model composed of a core system in polypropylene coupled with a medical grade hydrodynamic perfusion apparatus that simulates tear movement and turnover. The entire system is autonomous and can be easily moved for standard ophthalmological investigation without perturbing the tear flow. The tear turnover rate is easily adjustable and reproducible. The system presented can be maintained during several hours.

ID ABS: 119

Development of an *in vitro* human diabetic wound bioassay

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Diabetes sufferers already number approximately 2 million in the UK and 200 million worldwide and the treatment of non-healing wounds in these patients is placing a huge burden on healthcare budgets. Therapeutic research in this area is hampered by a lack of suitable animal models which accurately replicate this dysfunctional healing. The aim of this project therefore, is to develop an *in vitro* bioassay for the study of diabetic wound healing in humans. Cultures (n=3) of diabetic wound fibroblasts (DF) and patient-matched normal dermal fibroblasts (NF) were immortalized, using human telomerase (hTERT), to create cell lines. Growth and wound healing characteristics of these cell lines mirror those of primary cells demonstrating that immortalisation maintains the disease phenotype. Microarray gene expression analysis identified 4005 genes that were differentially expressed between NF-hTERT and DF-hTERT cells; 106 of these genes showed a differential wound/serum response. The upstream regulatory sequences of several (QRT-PCR validated) candidate genes were amplified and cloned into promoterless fluorescent reporter vectors. Transient transfection of reporter vectors into disease and normal cell lines has allowed expression of disease-specific markers to be monitored. Currently, lentiviral systems are being utilised to stably transfer these promoter-reporter constructs into the cell lines which will form an inexpensive, highly reproducible cell-based fluorescent reporter bioassay to screen novel therapeutics capable of treating impaired diabetic wound healing and, as a result, replace unnecessary animal experimentation. Acknowledgments: the National Centre for the Replacement, Refinement and Reduction of Animals in Research, UK.

ID ABS: 132

Development of high-performance prediction system for chemical toxicity in the cell using tricolor bioluminescence probes

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New chemicals must be screened for harmful effects to protect human health. In recent times, screening systems based on cells are preferred over systems based on animals. This project aims at creating a high-performance prediction system for the toxicity of harmful chemicals on cells using multiple bioluminescence probes. In this tricolor reporter *in vitro* assay system the expression of three genes that may be modulated by toxic chemicals is monitored simultaneously using green-, orange- and red-emitting beetle luciferases. In this paper, we introduce the basis of the prediction system for immunotoxicity using the tricolor reporter assay. We constructed stable transformant cell lines expressing the tricolor reporter assay systems. We selected three reporter genes, INF-γ and IL1-β as effective responsive promoters, and G3PDH as stable control promoter. We identified the stable transformant lines constructed by tricolor reporter assay systems based on the above genes. These cell lines could be used to estimate the immunotoxicity of chemicals.

This system was supported by NEDO Toxicological Project in Japan.

ID ABS: 141

Effects of cell line maintenance protocols on growth and differentiation of Caco-2 cells

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The human intestinal Caco-2 cell line has been extensively used as a model of the intestinal barrier. However, it is widely reported in literature that culture-related conditions, as well as the different Caco-2 cell lines utilized in different laboratories, often lead to problems of reproducibility, making it difficult to compare results.

We developed a new protocol in which Caco-2 cells were subcultured at 50% of confluence (Low Density, LD) instead of 90% of confluence, as usually reported in literature (High Density, HD). Using this new protocol, Caco-2 cells preserved a higher proliferation potential resulting in a cell population, which, upon reaching confluence, was able to differentiate almost synchronously, forming a more homogeneous and perfectly polarized cell monolayer, as compared to that obtained with the HD cells.

We investigated if these structural differences could also influence the effects of toxicants on 21-day-differentiated LD and HD cells. We analyzed the acute toxicity of Fe(II)/ascorbate; the HD cells showed a much higher resistance to the treatment in terms of monolayer disruption and gene expression, suggesting a strong effect of the maintenance protocol on the physiological behaviour of the cells.

Our results suggest that different cell line maintenance protocols confer to Caco-2 cells a number of morphological and physiological differences that must be taken into account when these cells are used as an intestinal model.

Work supported by EU FP6 Project "LIINTOP" Contract number LSHB-CT-2006-037499

ID ABS: 159

Identifying respiratory toxicity using the EpiAirway™ human 3-D model combined with multiple endpoint analysis

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The registration of new chemicals under REACH and Amendment VII to the Cosmetics Directive in Europe requires the development, validation, and utilization of new *in vitro* methodologies to replace costly and time consuming animal toxicity testing. An *in vitro* human cell model combined with multiple endpoint analysis may provide a robust model for evaluating many types of respiratory toxicity. The objective of this study was to assess the toxicity of known respiratory toxicants in the three-dimensional *in vitro* human EpiAirway™ system. This model contains highly differentiated human tracheal/bronchial epithelial cells cultured at the liquid-air interface. The cells were apically treated with respiratory toxicants bleomycin and doxorubicin over a broad range of exposure concentrations. Cells and media were collected after 24 and 72 hour exposures. Toxicity was assessed by measuring cell viability (MTT), cellular glutathione levels, TNF-α, IL-1α and IL-6 cytokine expression levels, and histology. Doxorubicin reduced cell viability and GSH levels in a dose dependant manner after 72 hours. At 24 hours, qRT-PCR results showed doxorubicin induced expression (>5-fold) of TNF-α and IL-6 while histology showed significant structural breakdown. Bleomycin reduced cell viability to 90% of control levels by 72 hours, and GSH levels were reduced to 75-80% of control after only 24 hours, both in a dose dependant manner. qRT-PCR results showed bleomycin induced expression (>5-fold) of IL-1α and IL-6 at 24 hours while histology showed significant structural breakdown. In conclusion, the 3D EpiAirway™ system combined with multiple endpoint analysis provided toxicity data consistent with those observed *in vivo*.

ID ABS: 165

Enhanced development of a bile canaliculi network in hepatocyte sandwich culture with direct oxygen supply through polydimethylsiloxane membranes

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Collagen (CN) gel sandwich culture has widely been used for drug metabolism/toxicity testing, because it allows hepatocytes to reestablish normal liver tissue polarity in terms of bile canalicular membrane formation. However, conventional sandwich culture on polystyrene induces small bile canaliculi and takes 4~5 days to reestablish the functional bile canaliculi, leading to low efficiency and sensitivity to test drug metabolism and excretion. To develop a more efficient hepatocyte culture system based on the collagen gel sandwich, we utilized a highly oxygen-permeable polydimethylsiloxane (PDMS) membrane whose surface was covalently-modified with CN molecules, with the expectation that the PDMS-based direct oxygenation would enhance the reestablishment of the bile canaliculi network. Freshly isolated rat hepatocytes were seeded on CN-modified PDMS membranes set in a 24-well plate format and CN gel was overlaid on the hepatocyte layer 24 h after seeding. Imaging analysis of the bile canalicular network development using 5-(and-6)-carboxy-2',7'-dichlo-

ro-fluorescein (CDF) accumulated in bile canaliculi showed that functional bile canalicular networks emerged quickly and increased from day 2 compared to those in a CN-sandwich on polystyrene. Furthermore, areas of the CDF accumulated more widely spread than in the CN-sandwich on polystyrene. We therefore conclude that the improved CN sandwich culture system using PDMS membranes gives high prediction of drug transport in the liver through the integration of the proper arrangement of matrix and the improved oxygen supply to hepatocytes.

ID ABS: 166

Cytotoxicity evaluation of papain using human keratinocytes

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The value of papain in cleansing tissue wounds has been known for several hundred years. This cysteine protease is extracted from the latex of the immature papaya leaves and fruits. Due to a trypsin-like action, papain cytotoxicity evaluation is difficult. This study intends to show how papain acts in human keratinocytes (HK) using a feeder layer formed by murine fibroblasts (ATCC CCL 92). Papain was tested using several different concentrations (from 0.005 to 0.75% (w/v)) for 24 and 48 h of contact at 37°C, 97% humidity and 5% CO_2 in cell culture flasks. In the attempt to observe a reverse mechanism, the cells were also maintained for 7 days after 24 and 48 h of contact. The viable cells were measured by MTS/PMS, where the active component is a tetrazolium compound and the living cells reduce it to a colored formazan product that is quantified at 490 nm. The different inhibitory concentrations estimated to affect the endpoint in question by 50% were IC_{50} (24h) = 0.000662 mmol/l, IC_{50} (48h) = 0.000397 mmol/l; after 7 days IC_{50} (24h) = 0.000664 mmol/l and IC50 (48h) = 0.000390 mmol/l. Basal cytotoxicity can be used in combination with other information for many purposes in the process of safety or risk evaluation, e.g. to predict starting doses for *in vivo* acute oral LD_{50} values in rodents. HK provides good conditions for analyzing new proteolytic enzyme preparations that may be used to supplement normal digestive activity, or to confer upon an individual a new digestive capability.

ID ABS: 179

In vitro models of biological barriers: towards automation

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The transport of molecules between the different compartments of the body is a key issue in both pharmacology and toxicology. Existing *in vitro* approaches to study transport through biological barriers, such as the lung and intestinal epithelia, remain limited both by the simplicity of the biological models used and by the labor-intensive nature of the experiments themselves.

We are investigating the use of microfluidics and microfabrication to automate these experiments. Using microfabrication, cell culture chips have been fabricated. The chips contain five wells in which epithelial cells can be seeded and cultured. The base of each well consists of a porous membrane in order to allow the formation of polarised cell layers and study the movement of toxins or drugs through the membrane. Integrated platinum electrodes allow trans-epithelial electrical resistance (TEER) measurements in parallel across all five wells to determine the "tightness" of the cell layers. The necessary liquids, such as culture medium or buffers, are transported to and from the cells by a microfluidics system. A microfluidics circuit above the wells allows the automatic preparation of a series of dilutions of the molecule to be tested while a similar circuit below the cell layer is used to collect molecules that may have crossed the cell layer.

First results from this system are very promising, and we are now investigating the extension of this approach to include other cell lines as models of organs and tissues, such as the liver and the vascular endothelium.

ID ABS: 184

Development of alternative to animal methods for reproductive toxicity studies: an *in vitro* blood-testis-barrier

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Alternative methods for reprotoxicity studies are of high interest in the scope of regulatory requests. Scientists agreed on the fact that the rational design of toxicological test batteries represents the most effective solution for the adequate replacement of *in vivo* testing conducted according to OECD guidelines. Our research contributes at this scientific effort thanks to an original *in vitro* Blood-Testis-Barrier model originally developed in our laboratory. The model is aimed at reproducing the *in vivo* organization of rat testis seminiferous epithelium. The concept is based on a 3D culture in a bicameral chamber: on the bottom of the insert, peritubular cells are cultured and on the top, a mixture of Sertoli and germ cells are coated within an artificial extracellular matrix. We have checked both the barrier and the spermatogenesis functions.

We successfully obtain a cord-like organization with a polarization of Sertoli cells and germ cells in the center of the structure. The degree of organization depends on the morphogenetic gradients and the matrix composition. A physical compartment with an apical and basal space is created, confirmed by the presence of tight junctions between Sertoli and peritubular cells. Mitotic activity, DNA-condensation and difference in cell morphology were also observed together with a cellular activity linked to the spermatogenesis. More studies are planned to further characterize the functionality of the junctions and germ cell differentiation within the culture.

Using this model, validation studies with Methoxychlor or Vinclozolin, two endocrine disruptors, were started to assess their effects on the barrier and/or spermatogenesis.

ID ABS: 196

Influence of modes of action and physicochemical properties on the correlation between *in vitro* and acute fish toxicity data

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New EU legislation is providing an impetus for research aimed at replacing acute fish toxicity testing to assess the ecotoxicity of a chemical with *in vitro* alternatives. In line with such research, the objective of this study was to determine what factors influence the correlation between *in vitro* and *in vivo* fish toxicity data. Basal cytotoxicity (IC_{50}) and acute toxicity data from fathead minnow (LC_{50}) of 82 chemicals were obtained from the Halle Registry of Cytotoxicity (v. 2006-02-27) and the EPA fathead minnow database (EPA Mid-continent Ecology Division, Duluth, MN), respectively. When comparing IC_{50} with LC_{50} data, a high correlation coefficient of 0.84 was found. However IC_{50} data was less sensitive than LC_{50} data by an order of magnitude. This lower sensitivity was significantly explained by the octanol-water partition coefficient, (Kow), the Henry's Law Constant (H), and the mode of action of the compound. These results support the notions that a) the bioavailability of hydrophobic (i.e. high Kow) and volatile (i.e. high H) chemicals is significantly lower in *in vitro* assays than in the fish test set-up and b) a single cell assay with cell death as its only endpoint cannot mimic all modes of toxic action possible in the whole organism. It is therefore suggested to use a battery of cell assays and endpoints and use free instead of nominal concentrations to determine effect concentrations to improve *in vitro-in vivo* fish acute toxicity extrapolations. Free concentrations may be estimated using Kow and H.

ID ABS: 198

What is the minimum size of hepatic tissue to obtain physiologically-relevant responses?

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To miniaturize cell-based biochips and devices, micro-tissues, which exhibit physiologically-relevant responses identical to their original tissues/organs, should be embedded. However, the minimally-required size of such micro-tissues has not consistently been determined. We therefore determined the minimally-required size for two-dimensional micro-tissues of human hepatocarcinoma Hep G2 cells and evaluated their responses to several model chemicals as the first step, because Hep G2 preserves various hepatic functions as well as growth capacity.

To control the size of the micro-tissues, we employed a convenient micro-patterning technique, photocatalytic lithography. Cells stably and densely adhered only on the micro-patterned cell-adhesive region formed by the photocatalytic lithography for at least 6 days. Using the 3-day cultured micro-tissue, cytotoxicity tests were performed after a further 48 h exposure to aflatoxin B1 and adriamycin as typical examples of indirect and direct mutagens, respectively. The observed cytotoxicity was compared with that obtained in the conventional 96-well plate-based assay (ca. 6 mm in diameter and hundred-thousands cells). The cytotoxicity of aflatoxin B1 significantly increased in the micro-tissues that were 630 μm in diameter (ca. 1000 cells) or larger and reached the level observed in the plate-based assay. This agreed well with the enhancement of their cytochrome P450 1A1/2 capacities. In contrast, the cytotoxicity of adriamycin was independent of the size of micro-tissues examined and was equal to that observed in the plate-based assay. This is presumably due to the fact that all micro-tissues exhibited the same growth capacity. The present evaluation is necessary for miniaturizing cell-based biochips and devices retaining physiologically-relevant responses.

ID ABS: 208

The security in utilizing *in vitro* reconstituted human oral epithelium. An oncogenetic pathway study

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Introduction: Human oral epithelium cells derived from primary cultures allow the establishment of an *in vitro* restored epithelium which, when returned to the same local origin, allows the occlusion of several oral defects.

Objective: Concern about the activation of some oncogenic pathways justifies the investigation of their cell mechanisms. The aim of this work is to detect any possible cell function abnormalities that are not compatible with normal tissue mechanisms.

Material and Methods: This project was approved by the Research Ethical Committee of the Institute of Nuclear and Energetic Research, under the License N° 087/CEP-IPEN/SP. Normal human oral keratinocytes from primary cultures were seeded in cell culture dishes with special keratinocyte culture medium. The cells were maintained in a humid incubator at 37°C containing 5% CO2. When the cells reached confluence they were removed from the dish, fixed (formaldehyde 10%), embedded (paraffin) and sectioned for immunohistochemistry, and the protein from the cell lysate was utilized for Western blotting. The utilized antibodies were: p53, PTEN, pAkt, β-Catenin, Metallothionein.

Results: The results confirm the normal structure of the cultivated *in vitro* epithelium and did not show any oncogenic pathways that would compromise their utilization in oral reconstructions.

Conclusion: We conclude that these *in vitro* cultivated epithelia established from cells harvested from the oral mucosa are totally safe and biocompatible for use in tissue repair and can accuracy reproduce normal cell structures.

ID ABS: 213

In vitro effects of volatile chemical mixtures on human derived cells

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The traditional approach for setting acceptable exposure limits to Volatile Organic Compounds (VOCs) by regulatory bodies consists in correlating toxicity data from animal models with humans and assuming an additive effect when there are chemical mixtures present. Therefore there is a need to develop techniques for assessing overall toxicity of VOC exposure on human health. In this research we have developed a static technique for assessing the toxicity of individual VOCs (benzene, toluene, xylene and formaldehyde) and their mixtures on human cells derived from key target organs such as lung (A549) and liver (HepG2). The cells were grown on porous snapwell membrane inserts and exposed to the air interface for 1 hour. The toxicity resulting from VOC exposure was evaluated 24 h post exposure using an *in vitro* toxicity assay i.e. MTS (Tetrazolium salt, Promega) assay. Cytotoxicity of formaldehyde (IC_{50} = 3305 ±500 ppm) in A549 cells was found higher than benzene (IC_{50} = 31835 ±1750 ppm), toluene and xylene. The findings also suggest that exposure to individual and mixtures of volatile compounds can yield different toxicity profiles. The Isobole model revealed a synergistic effect (IC_{50} = 41259 ±2500) for Benzene-Formaldehyde binary mixture, while antagonistic effects were quantified using binary (benzene-toluene, benzene-xylene and toluene-xylene) and ternary combinations of Benzene-Toluene-Xylene (IC_{50} = 32449 ±1510) on A549 cells. It is hoped that the reported IC_{50} will attract the atention of regulatory bodies, as this method represents a more efficient technique for setting safe levels of exposure to VOC mixtures.

ID ABS: 215

3D skin model to simulate the herpes infection

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Approximately 90% of the world population has been infected during their life with the herpes simplex virus (HSV). The viral infection in the host causes the outgrowth of fever blisters. After the infection itself the virus stays for a long time in latency in the host nerve cells.

The aim of this work was to establish an integration of a nerve cell line into the already established skin model. We built up a 3D complex skin based model to better simulate the herpes infection *in vivo*, which allows us also to test for the effect of new drugs. The nerve cell line pheochromocytoma (PC-12) from the rat was used for this purpose. The pre-treatment of the PC-12 cell line with NGF is important for the HSV infection. In this work, we investigated the optimal requirements for the infection.

PC-12 cells are cultivated in different media than that used for the skin models. In this work we could demonstrate that the nerve cell line can be successfully cultivated in the medium used primarily for the skin equivalent.

ID ABS: 221

In vitro toxicity assessment of diesel exhaust using a direct dynamic exposure method

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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 lung cells (A549) directly to diesel exhaust at a range of exposure of 0.25-2 hrs and 0-24 hrs post-incubation periods. In summary, cells were grown on porous membranes and placed inside dynamic exposure chambers connected to a diesel engine exhaust. The cyto-

toxicity of exhaust was analysed using ATP, MTS and NRU *in vitro* assays. The exhaust was also analysed for pollutants such as CO, CO_2, NOx, HC and diesel particulate.

Results of this study indicated that human lung cells (A549) were sensitive to diesel exhaust pollutants at all exposure times. The MTS assay showed greater reproducibility at 1 hr exposure and 0 hr post-incubation (M ±SE: 28 ±4%) compared to 24 hr post-incubation (M ±SE: 55 ±10%). The method from this study can potentially also be used to assess human exposure to exhaust from diesel engines using petroleum diesel or biodiesel.

ID ABS: 226

Choice of cellular source for species crossover assays in drug development – implications from a 3Rs perspective

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Drug development projects often use several species for their analysis. Human proteins and cells are used as far as possible, but when animal testing is required for identification of broader effects on multiple mechanisms, rodents are often the species of choice. To justify the animal experiments, it is important to know that your candidate drug is a potent modulator also of the *in vivo* species of choice, e.g. rat or mouse. Species crossover can sometimes be established using recombinant protein based screening assays, but for some applications, there is also a need to understand relative potency in cellular systems.

For anti-inflammatory targets assays using human peripheral blood cells or whole blood are often employed in the screening cascade. These assays also allow for good counter-screening tools to be established in e.g. rodent species. We have been employing human and rat peripheral blood assays to minimize the number of compounds for *in vivo* testing. However, the rat peripheral blood assay requires a fairly large number of animals. For mouse, peripheral blood based assays are not even feasible. Hence, we have turned to using cells from spleen to substitute the peripheral blood cells. The two different cellular sources have been validated by use of a range of anti-inflammatory reference compounds, showing good correlation in terms of both absolute and relative potencies. By the shift in cellular source we estimate a yearly reduction of >90% animals used. This also allows for cross-species testing in mouse, which was not possible using the old method.

ID ABS: 230

Real-time dynamic monitoring of *in vitro* toxicity as applied to botulinum neurotoxin: potential for the replacement of animal testing

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We have explored the use of the xCELLigence system for testing the activity of *Clostridium botulinum* neurotoxins. The sale of these toxins for medical and cosmetic use (Botox®; Myobloc®) requires LD_{50} testing on a large number of mice at multiple stages of production. Development of a cheap and reliable cell-based assay is hampered by the fact that the toxin undergoes numerous processing and transport steps before exerting its toxic effect, all of which are difficult to model *in vitro*. We have shown that the xCELLigence system can detect morphological changes elicited by botulinum toxin in cultured neuronal cell lines in a dose-dependent manner, exhibiting kinetics similar to the predicted toxicity time course. Furthermore, these dynamic morphological changes can be blocked by employing neutralizing antibodies against the toxin. We will show data demonstrating the effect of botulinum toxin on several neuronal cell lines and primary neuronal cells.

The xCELLigence system, co-developed by Roche and ACEA Biosciences, allows for sensitive and robust assessment of cellular changes in real time. Cells are seeded onto plates containing microelectrodes, allowing for precise measurement of changes in electrical impedance, which corresponds to cell number, shape and degree of substrate attachment. In addition, subtle changes in cytoskeletal structure and cellular contraction, such as those induced by botulinum toxin, can be detected. We believe that with further characterization of the botulinum toxin response, the xCELLigence system has the potential to replace animal testing for botulinum toxin and other pharmaceutical agents.

ID ABS: 231

An attempt to include the human metabolic factor in to the embryotoxicity test by mouse Es-D3 cells

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The embryonic stem cell test (EST) is a validated *in vitro* assay that has been established to classify compounds with respect to their embryotoxic potential. When the influence of dental biomaterial on human embryotoxicity is evaluated, the influence of human metabolic revitalization factor cannot be disregarded; however, in the EST protocol, which used mouse cell lines, evaluation including the influence of a human metabolism factor was impossible. We therefore examined the influence of embryotoxicity on thalidomide, and the dental alloy's composition of silver, copper, palladium, and zinc ions. Each chemical was cultured with TEST LIVER TM (TOYOBO) that could maintain human CYP3A4 and ammonia metabolizing ability for a long period *in vitro*. On day 10 of the assay, differentiation into contracting myocardial cells was determined under a light microscope.

The rate of wells in contracting cells in the control group was 66.7% compared with the non-metabolizing control group. The contracting cell rate was decreased intentionally by silver and thalidomide in the non-metabolizing group; however, copper and zinc seemed have a slightly higher late than the non-metabolizing group. Neither showed a significant difference from other metal ions.

As for lowering of the differentiation rate further by thalidomide, a result using more clinical data was obtained. These metals are already used by dental clinics as composition elements of dental alloys for patients; therefore, not only these data but also more diversified data are necessary. In addition, assessment the use of human metabolic activation of the metal requires further analysis.

ID ABS: 232

Effects of cell recovery factor in cell differentiation culture with the embryonic stem cell test

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The experimental protocol of embryonic stem cell test (EST) using ES-D3 cells and 3T3 cells as an *in vitro* embryotoxicity test for biomaterials and medicine was established by H. Spielmann of Germany in 1997. In 1988, Imai proposed the cell recovery test as a variation of the cytotoxicity test. By exposing the material to cells, the transitory influence on recovery of the cell proliferation level is assessed. The cell recovery test was also developed similarly allowing the examination of not only recovery of cell proliferation but also recovery from chemical disruption of cell differentiation. After exposing both cells to NaF and SnF2, used to prevent tooth decay, we compared the results of each culture in fresh medium with the findings obtained by the EST.

For 5-day recovery and 7-day recovery, each value increased slightly. NaF and SnF2 both showed non-embryotoxicity under all conditions.

Based on these findings, no influence of cellular recovery was seen with demonstrated in NaF and SnF2. These chemicals are both fluorine compounds, and the embryotoxicity level of SnF2 is presumed to be slightly stronger than that of NaF.

No influence of cellular recovery was demonstrated with NaF and SnF2. Stronger embryotoxicity is a concern when the recovery culture shows that the toxicity level of a chemical remains constant; therefore, it is necessary to consider the utility of examining recovery from embryotoxicity.

ID ABS: 237

Towards a delineation of applicability domain of the VITOSENS skin sensitization assay

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The VITOSENS® assay is a predictive in vitro alternative for the identification of chemicals with a skin-sensitizing potential in humans. The method relies on the specific response that human dendritic cells exhibit when exposed to a (non)-sensitizing compound. In our approach the responses to *in vitro* exposure of human CD34+ progenitor-derived dendritic cells are assessed at gene-expression level. In a preceding microarray study, a set of genes was identified that showed discriminative response to (non)-sensitizers (Schoeters et al., 2007, *Mol. Immunol.* 44). In developing the current assay, the differential expression of these genes was measured by real-time RT-PCR and translated into a classification model. VITOSENS® was evaluated by a cross-validation on 21 chemicals and showed a concordance close to 90% (Hooyberghs et al., 2008, *Toxicol. Appl. Pharmacol.* 231).

During the past year the assay has been challenged by different chemicals which were chosen for guidance in the delineation of the applicability domain. The selection was aimed at a good coverage of weak to extreme potency classes, pre/pro-haptens and reaction mechanisms. This presentation summarizes the assay's performance on the extended set of chemicals, the observed strengths and weaknesses, and the road we will follow towards its use in the replacement of animal tests.

ID ABS: 243

Artificial vascularized human skin equivalent

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The main disadvantage of present skin models is the lack of a blood supply, which is essential for the survival of a graft as well as for the resorption of a test substance.

Therefore, in this study the combination of a biological vascularized matrix (BioVaSc) with a skin equivalent was examined. To facilitate the migration of vessels from a repopulated BioVaSc into the skin model, the conditions to build up a monolayer of microvascular endothelial cells (mvEC) at the interface of the skin equivalent and the BioVaSc were established. The optimal cell concentration was ascertained to be 500,000 mvEC per cm2 BioVaSc, resulting in a cell monolayer after 7 days of cultivation. The cells expressed the specific endothelial cell markers CD31 and vWF. The combination of a mvEC-seeded BioVaSc with the dermal component of the skin equivalent showed stable bonding. At day 7, a monolayer of mvECs and in some regions the formation of round cell clusters were observed. However, at day 21 only a few isolated cells could be detected. In contrast, upon combination of the whole skin equivalent, including an epidermal component, with the mvEC-seeded BioVaSc, a cell monolayer without cell clusters was observed at day 21. The cells expressed the specific endothelial cell markers CD31 and vWF.

In conclusion, in this study the fabrication of a human skin equivalent on a vascularized matrix with an intermediate functional monolayer of mvECs could be demonstrated. This provides a basis for further approaches inducing angiogenesis in the skin equivalent.

ID ABS: 252

Establishment of a rat hepatic *in vitro* system that provides a good *in vitro-in vivo* correlation of carcinogen-induced gene expression alterations

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At present, toxicogenomics for predictive toxicology is mostly used *in vivo* in order to obtain comprehensive information about the involved mode and mechanism of action of toxicants. Recent studies have presented evidence that alterations of gene expression patterns in rat liver can be used to detect and categorize liver carcinogens. We could recently demonstrate that cultivation of primary rat hepatocytes between two layers of collagen (sandwich culture) is most appropriate to reproducibly detect gene expression alterations induced by the non-genotoxic carcinogen methapyrilene. In order to demonstrate the potential of the established *in vitro* system to detect gene expression signatures related to carcinogenicity, we investigated whether an *in vitro-in vivo* correlation exists for the deregulation of selected genes upon exposure with genotoxic and non-genotoxic agents. Primary hepatocytes were treated for 24 h with non-genotoxic carcinogens methapyrilene and piperonylbutoxide and genotoxic carcinogens 2-nitrofluorene and aflatoxin B1. The expression of up to 24 selected genes was analyzed using real-time RT-PCR and the observed gene expression alterations were compared with existing data of *in vivo* short-term studies. We could demonstrate that treatment of hepatocytes with different concentrations of the four carcinogens *in vitro* resulted in qualitatively identical deregulations of the majority of analyzed genes as observed *in vivo*. In conclusion, a good *in vitro-in vivo* correlation of carcinogen-induced gene expression alterations could be detected in the established hepatic *in vitro* system, which may eventually be used to reduce rodent long-term studies.

ID ABS: 253

A new pharmaceutical aerosol deposition device on cell cultures (PADDOCC) as alternative method for biocompatibility and ADME screening

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Development of new inhalative aerosol medicines requires both testing safety as well as efficacy, which are mostly done in animal experiments. Replacement of animal studies in the early phases of ADME with *in vitro* cell culture experiments is difficult due to the complexity of the processes involved: aerosol generation, deposition, drug release from the carrier and finally its absorption across the air blood barrier. As the alveolar epithelium is only covered by a thin film of lining fluid, submersed cell culture systems do not allow study of the effects of depositing aerosol particles or droplets on this delicate epithelial tissue.

We developed a new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) to simulate the inhalation of a single metered aerosol dose and its subsequent deposition on filter-grown pulmonary epithelial cell monolayers exposed to an air interface. The device is compatible with most available metered dose inhaler devices. In first experiments the reproducibility of deposition with commercially available dry powder inhalers containing salbutamol or budesonide could be demonstrated and subsequent transport studies across Calu-3 monolayers were performed.

PADDOCC appears to be an attractive alternative to animal testing in the early phases of developing aerosol medicines, allowing the investigation of drug permeability as well as the effects of formulation factors and excipients.

ID ABS: 267

Alternatives to the use of fetal bovine serum: platelet lysates as serum replacement in cell and tissue culture

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The search for alternatives to fetal bovine serum (FBS) in cell and tissue culture has become a major goal in terms of the 3R principle to reduce or avoid harvesting of FBS from bovine fetuses. FBS bears a number of disadvantages: (1) unknown composition, (2) high lot-to-lot variability, (3) ethical concerns about harvest from bovine fetuses, and (4) possible shortage in global supply. Several strategies have been developed to reduce or replace FBS in culture media. Here we report on the use of human platelet lysates (PL) as serum replacement. Lysates were prepared from outdated human donor thrombocyte concentrates by freeze-thawing in hypoosmolar saline. Release of platelet granule growth factors (GR) was determined by ELISA. Growth promoting and mitogenic capacity of PL was tested on renal cell lines. PL in DMEM support growth, proliferation and differentiation of proximal tubule-like LLC-PK1 (porcine kidney) and HK-2 (human kidney) cells, whereas distal tubule-like MDCK (dog kidney) cells grow well in serum-free DMEM/Ham-F-12 supplemented with PL. In addition to adherent cell lines, anchorage-independent Raji human lymphoma cells were investigated. PL fully supported growth and proliferation of Raji cells in suspension. In order to determine the proliferative potential of PL, stimulation of MAP kinases ERK1/2 was determined. Addition of PL to quiescent LLC-PK1 cultures resulted in specific phosphorylation and thus activation of ERK1/2 within minutes. The time course is identical with ERK1/2 activation upon addition of FBS. The data show the high potential of PL as a valuable substitute for FBS in cell and tissue culture.

ID ABS: 270

Simultaneous quantification of compounds via LC/MS by utilization of sample pooling technique

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Introduction: Conventional preclinical pharmacokinetic studies involve the administration of a single compound to several animals, which is followed by analysis of the plasma or tissue samples. These studies are time and material consuming because they require large amounts of compound(s) and many animals. Additionally, specific analytical methods must be developed for each compound. Recent approaches offer a significant increase in analytical throughput via a sample pooling technique: simultaneous quantification of multiple substances.

Objective: The main aim of our work was to develop and validate an analytical method to minimize material/time/cost and maximize output.

Method: A high throughput LC/MS method with electrospray ionization was developed and validated for quantification of permeability experiments of 7 compounds, i.e. known Pgp substrates which are investigated in MEMTRANS, an EU project. This method is based on on-line extraction turbulent HPLC coupled to mass spectrometer, in the single ion mode, enabling detection of multiple compounds in a single sample.

Result: Employment of cassette mode utilized analytical method presented high throughput by reducing time and material by up to 71%, besides introducing clean samples into the mass detector compared to individual sample introduction.

Discussion: If the drug-drug interactions are carefully taken into consideration, the same approach can be applied to quantify samples obtained by simultaneous introduction of compounds to one animal. This approach would also reduce the number of animals required in addition to reducing the time and material consumption.

ID ABS: 271

In vitro models of the epithelial barriers of the respiratory tract to study drug permeability and toxicity

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Objective: Cell-based *in vitro* models are useful to examine the characteristics of new drugs in advance of animal studies, thereby reducing the number of animals required. This study aims to model the epithelial barriers of the respiratory tract and the alveolar epithelium *in vitro* to enable permeability and toxicity studies.

Methods: The bronchial cell line Calu-3 and porcine alveolar epithelial cells (pAEpC) isolated from pigs were cultured as liquid-liquid cultures (LLC) on transwell filters. Furthermore, the cell line Calu-3 was cultured as air-interface culture (AIC). The integrity of the monolayers was qualified and characterized by measuring the transepithelial electrical resistance (TEER) and transport studies using a set of different drugs were performed.

Results: pAEpC and Calu-3 cells formed polarized cell monolayers *in vitro*. Transporter experiments with marker compounds revealed that pAEpC and Calu-3 distinguish between low and high permeable compounds. In contrast to experiments with pAEpC, P-glycoprotein (Pgp) activity was detectable in Calu-3 cells by using Pgp specific substrates. Differences in the permeability of tested drugs could be detected in the two models. Furthermore, Calu-3 cells were suitable for transport studies when cultured as AIC.

Discussion: Our data demonstrates that pAEpC and Calu-3 cells are appropriate for studying the permeability of drugs *in vitro*. Differences in the permeability of tested drugs and in the transport of Pgp specific substrates indicate tissue specific characteristics of the two models. In conclusion, we established *in vitro* models of the epithelial barriers of the respiratory tract that are useful for testing components in advance of animal studies.

ID ABS: 272

Validation of an *ex vivo* human cervical tissue model for the local delivery of nucleic acid drugs

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Objective: The aim of this study was to establish and validate an *ex vivo* human cervical tissue model for delivery and permeation studies in order to understand the barrier properties of the target tissue to which drugs, especially nucleic acid drugs for the treatment of cervical cancer, could be applied.

Methods: The system of choice to conduct the study was the static Franz cell that consists of donor and acceptor chamber. After defined periods of time samples were taken from the acceptor chamber and analysed for their content of the test compound. The compounds tested for their ability to permeate through cervical tissue were the hydrophilic mannitol, the small molecular propanolol and four dextrans of increasing molecular size (4, 10, 20 and 40 kDa).

Results: The three subgroups of molecules defined above showed a nearly equal distribution in patients of both menopausal statuses. The permeability decreased with increasing molecular weight and an upper permeability limit between 10 to 20 kDa seems to exist. The permeability varied significantly among different patient samples, whereas we could not ascertain an intraindividual variability.

Discussion and Conclusion: We may conclude that the human cervical tissue model, which is to our knowledge established here for the first time, has been well characterized and is therefore suitable for delivery and permeation studies and to support preclinical lead optimization of drugs acting locally at the cervical tissue. The model can also be used for the safety evaluation of drugs to be applied to the cervical tissue.

ID ABS: 273

In vitro release testing as rational tool in the development of topical corticosteroids

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Introduction: The use of animal (e.g. rat/porcine) and *ex vivo* human skin is widely employed as an approach to assess the performance of dermatological formulations in the product development phase. In the screening phase a wide range of test formulations is necessary, requiring the use and/or sacrifice of many animals. *In vitro* release tests using synthetic membranes can provide some predictive estimates of the formulation efficacy, thereby reducing the use of animals in the screening phase.

Objective: The aim was to develop and validate an *in vitro* release test for assessing triamcinolone acetonide release from novel dermatological formulations.

Methods: The Franz cell system fitted with synthetic membranes was used to assess the drug release of 10 formulations from the groups of hydrogels, o/w emulsions, w/o emulsions and water-free formulations containing 0.1% of triamcinolone acetonide. The test formulations were prepared according to DAB instructions. Commercial formulations were employed as references. The method was validated in term of its reproducibility and robustness.

Results: The test formulations based on carbomer gel and propanol-containing carbomer gel presented a higher release rate compared with the commercial o/w cream. The hydrophobic cream also presented slightly higher release than the commercial w/o emulsion. These 3 test products are strong candidates to take forward to the biocharacterization phase. Furthermore the method provided satisfactory reproducibility and robustness.

Discussion and Conclusion: The present *in vitro* release test narrowed the selection of test product candidates for the subsequent biocharacterization phase, representing a rational strategy in the screening phase and reducing the use of animals.

ID ABS: 280

Structured silicon surfaces: a tool to improve *in vitro* studies

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Cells in tissues and organs are surrounded by numerous micro and nano elements in all directions (3D). In contrast, in *in vitro* studies, cells grow on flat (2D) polystyrene surfaces. An important issue for *in vitro* studies is to find a way to reproduce the cell's natural environment while maintaining the simplicity and efficacy of cell culture. This would increase the relevance and credibility of *in vitro* studies, contributing to a reduction in animal experimentation.

Silicon surfaces, structured with micro pillars were fabricated, and the behavior of osteoblasts on these surfaces was studied using cell lines and primary cells. The results show that bone cells grown on structured surfaces have a 3D morphology, similar to their morphology in tissues. In contrast, cells grown on flat silicon surfaces show a spread 2D shape, similar to that obtained on polystyrene plates. Cell proliferation was measured to demonstrate the suitability of the surfaces for bone cell growth. Cytoskeletal morphology and focal adhesion distribution showed a strong reduction of cell adhesion and a less spread morphology on structured samples. Apoptosis and cell cycle blocks showed small differences between structured and control surfaces. Moreover the osteoblasts seem not to differentiate, maintaining their normal behavior.

In conclusion the structured silicon surfaces seem to offer an environment closer to the cell's *in vivo* environment. Next steps will include the analysis of cell adhesion and elasticity with AFM. This will give us information about the possibility to modify the cell behavior by changing the substrate microstructure.

ID ABS: 286

In vitro study of the toxicity induced by nickel compounds on human Calu-3 cells

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Epidemiological studies show that exposure to nickel compounds is associated with a variety of adverse health effects particularly of the respiratory system, such as chronic bronchitis, asthma and lung cancer. In the current study we used Calu-3, a human bronchial cell line, to investigate *in vitro* the effects of both water-soluble (NiCl2) and insoluble nickel forms (Ni μm and nm sized particles). Calu-3 cells were grown on transwells for 14 days and exposed to solutions of NiCl2 or nickel particles added to the apical compartment for up to 72 hours. The endpoints used were Trans-Epithelial Electrical Resistance (TEER) to assess the integrity of the barrier and Neutral Red Uptake (NRU) as a cytotoxicity assay. Moreover, the effect of nickel on oxidative stress was assessed by total reactive oxygen species (ROS) production and up-regulation of stress-related genes. Results showed a concentration-response effect on cell viability and TEER after treatment with the soluble form. However, with the particles (μm and nm size) no significant effect was recorded on the cell viability, whereas a concentration dependent increase of TEER was observed at non-cytotoxic concentrations after 72 hours. This effect on barrier integrity was higher with the μm sized than with the nm sized particles. In addition, an increase of ROS production and up-regulation of stress related genes were observed already at non-cytotoxic concentrations, with a more pronounced effect with $NiCl_2$ and μm sized nickel particles.

ID ABS: 292

In vitro testing of hemocompatibility and pyrogenicity

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Blood is one of the most heterogeneous systems in mammals. Its different components play complex roles in several physiological functions of life, including the blocking of invading pathogens and/or repair of damaged tissue. Since these interactions are critical for human health, the testing of medical devices that interact with blood must be hemocompatible.

In order to evaluate the hemocompatibility of new blood-contacting materials, we established an *in vitro* modified Chandler-loop model with fresh human whole blood. Hemostatic and/or immune parameters (complement) are recommended in order to assess material surfaces. It is important, however, to emphasize that contamination of the material may interact with the test system, causing false results.

Medical devices must be free of contamination with viable germs. Sterilization is a routine process that only kills living germs. Remnants of dead bacteria remain, e.g. endotoxins from Gram-negative bacteria. Such pyrogens induce an inflammatory immune response. Pyrogen tests on surfaces are generally performed either by determining endotoxins in rinsing solutions of samples by Limulus amoebocyte lysate (LAL) assay or by injecting the rinsing solutions into rabbits and measuring change of body temperature.

A recently validated *in vitro* pyrogen test (alternative to the rabbit test) based on human whole blood cytokine release, was adapted to assess pyrogenic contamination. Complement factors can be induced by bacteria and bacterial endotoxins. Therefore it is essential to remove all contaminants, regardless of whether they are alive or dead, before testing a material sample.

ID ABS: 299

Dynamic intestinal tissue model to evaluate the absorption behaviour of different substances

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Background: The 3R principle, first described by Russel and Burch in 1959, defines reduction, refinement and if possible the complete replacement of animal experiments. Animal experiments are ethically critical and their results cannot always be readily transferred to humans. For this reason more *in vitro* test systems must be developed, which must closely reflect cellular

processes in the human organism. By the use of a natural collagen scaffold and a dynamic bioreactor system an improved physiological intestinal test system was developed at the Fraunhofer IGB in Stuttgart.

Methods: Caco-2 cells and primary micro-vascular endothelial cells were co-cultivated in a 2-chamber bioreactor. The endothelial barrier simulates the *in vivo* gut-blood barrier for the systemic uptake of substances in the body. Contrary to static diffusion cells (e.g. Franz chamber), the bioreactor provides an apical and basolateral flow of culture media.

Results: Under dynamic culture conditions the Caco-2 cells grow in a high prismatic manner, similar to enterocytes *in vivo*. The 3D dynamic co-culture model could be validated so far with substances that simulate high permeable, low permeable and efflux transport.

Perspectives: Our test system should provide a basis for ADMET studies and the characterisation of different substances. The results should be validated for the prediction of systemic effects and for *in vivo* classification. Furthermore the cell line Caco-2 should be exchanged with primary enterocytes.

ID ABS: 305

Development of three-dimensional human mesenchymal stem cell tissue structures with targeted gene disruptions

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Whole animal targeted gene deletions, usually knockout mice, are commonly used to determine gene function and/or the genetic basis of human diseases. However, the generation of knockout animals is technically demanding, time consuming and the information obtained does not always translate well to human physiology. We have developed a novel three-dimensional (3D) spheroid culture model for human mesenchymal stem cells (MSCs), which could potentially be used to produce human 3D knockout tissues providing an alternative to animals. MSCs can be induced to differentiate down the osteogenic, chondrogenic and adipogenic lineages generating significant interest in using MSCs in cell-based therapies. Wnt signalling is a key regulator of MSC differentiation, and targeted gene disruption of the Wnt signalling pathways in MSC-spheroids will allow functional analysis of the mechanisms of MSC proliferation and differentiation. We have used RNA interference to knockdown the expression of the Wnt signalling component β-catenin in human cells. Expression vectors containing four different short-hairpin RNA (shRNA) sequences targeting β-catenin were co-transfected into 293FT cells with an enhanced yellow fluorescent protein tagged reporter plasmid. Significant knockdown of β-catenin expression by all four shRNA vectors was demonstrated by fluorescence microscopy, and verified using real-time PCR. All four shRNA expression vectors significantly reduced the gene expression of β-catenin ($p<0.001$): shRNA-1 by 4.5-fold, shRNA-2 by 3.6-fold, shRNA-3 by 5.0-fold and shRNA-4 by 5.6-fold. Human MSCs will now be transduced with the shRNA-4 β-catenin expression vector and induced to form 3D spheroid structures providing a model of β-catenin knockdown in a 3D culture system.

ID ABS: 324

The future of human cell culture media

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Human cell culture has become an essential tool for scientific research. Until now the most widely used media are supplemented with FCS (fetal calf serum), which contains most of the factors required for cell functionality and proliferation. However, fetal calf serum quality varies between batches and contains lots of undefined compounds, which carry the risk of contamination of the culture with undesired proteins or pathogens. Because of these risks, there is a worldwide interest in developing alternatives for such media using autologous proteins or chemically defined compounds. Studies suggest as possible substitutes for FCS: human serum, albumin, autologous/synthetic or recombinant hormones and proteins, presenting varying levels of success for different cell types.

The main concern of this paper is: Where is the boundary between optimal biosafety and the maximum efficiency of cell culture media? This review was based on research of publication indexes with the aim of following the developments in science and to discuss the bioethical, economic and scientific issues that still need to be worked on and the new perspective for the future, which appears to be a totally xenoprotein-free medium.

ID ABS: 333

Direct tissue profiling and imaging by mass spectrometry as a novel method for the investigation of skin sensitization

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All animal testing on whole cosmetic products was discontinued in September of 2004 following European legislation, Directive 76/768 EEC. In March 2009 animal testing of cosmetic ingredients was prohibited, although toxicity testing is permitted until 2013. An alternative to *in vivo* irritancy experiments already available uses a synthetic skin model and measures MTT and the release of IL-1a. There is currently however no available comparable *in vitro* test for sensitization. In part this is due to the fact immunocompetant synthetic skin models are not available as platforms for sensitization investigation.

In the work reported here human skin and the synthetic models; EpiVaginal FT (synthetic vaginal tissue which does contain dendritic cells) and EpiDerm FT (synthetic skin tissue which does not) (Mattek, Ashland, MA USA) have been treated with chemical irritants and sensitizers. Protein expression via direct tissue profiling has been carried out using Matrix assisted laser desorption ionisation (MALDI) Mass spectrometry imaging (MSI). MALDI-MSI analysis allows compound identification directly from a sample surface without unduly compromising the integrity of analyte molecules. Utilising imaging software it is also possible to determine distribution of analytes within samples sections. MALDI-MSI has been successfully implemented in previous studies of the skin. Subsequent to treatment of skin models with irritants and sensitizers it is proposed that protein biomarkers may be identified through on-tissue digests/tissue homogenisation followed by conventional proteomic analysis. Preliminary results include the comparative proteomic characterisation of the three untreated skin models.

This work is funded by COLIPA, The European Cosmetics Association, Brussels, Belgium.

ID ABS: 339

Assessing pulmonary toxicity of nickel, cobalt, and titanium using an *in vitro* human alveolar barrier model

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Particulate matters have adverse health effects through common pathways that produce inflammation and oxidative stress. Nickel, cobalt, and titanium are industrial and environmental pollutants. The lung epithelium represents the first barrier to be crossed by inhaled compounds. The main goal of the study was to evaluate the impact of selected compounds on the lung by assessing their toxicity on a reliable *in vitro* alveolar barrier model. The established *in vitro* alveolar type II monoculture (NCI H441) grown on trans-well inserts for 11-13 days was exposed for 48 h to nanoparticle forms of nickel, cobalt, and titanium oxide, and also to their soluble forms ($NiCl_2$, $CoCl_2$, $TiCl_4$) from the apical side. Following exposure, the effects of both particle and soluble forms on the alveolar barrier formation (TEER), cytotoxicity (NRU), inflammation (IL-8 production), and expression of stress related genes, Metalothionein 1X (MT1X), Heme oxygenase 1 (HMOX1), and Heat shock protein 70 (HSP70) using RT-PCR, were investigated. The soluble forms of selected compounds induced higher impact on barrier function and cell viability than the nanoparticle forms. The production of IL-8 and the expression of MT1X, HSP70, and HMOX1 were also increased by the soluble forms of the tested compounds compared to their particle forms, which induced at lower levels. Our preliminary results indicate that the NCI H441 *in vitro* alveolar barrier system may provide a suitable model for early screening for pulmonary toxicity, including particles.

ID ABS: 341

Characterization of an equine macrophage cell line: application to studies of EIAV infection

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EIAV is a monocyte/macrophage tropic virus. To date, even though EIAV has been under investigation for numerous years, very few details have been elucidated about EIAV/macrophage interactions. This is largely due to the absence of an equine macrophage cell line that would support viral replication. Herein we describe the spontaneous immortalization and generation of a clonal equine macrophage-like (EML) cell line with the functional and immunophenotype characteristics of differentiated equine monocyte derived macrophage(s) (eMDM(s)). These cells possess strong non-specific esterase (NSE) activity, are able to phagocytose fluorescent bioparticles, and produce nitrites in response to LPS. The EML-3C cell line expresses the EIAV receptor for cellular entry (ELR1) and supports replication of the virulent EIAV(PV) biological clone. Thus, EML-3C cells provide a useful cell line possessing equine macrophage related properties for the growth and study of EIAV infection as well as of other equine macrophage tropic viruses.

ID ABS: 347

Neural stem cells from human cord blood on bioengineered surfaces – novel approach to multiparameter bio-tests

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In order to investigate molecular mechanisms driving stem cell fate decisions and compound-induced adverse reactions, Human Umbilical Cord Blood-derived Neural Stem Cells (HUCB-NSCs) were cultured on bioengineered surfaces. Emerging nano/microtechnologies were used to create cell growth platforms with controlled content and spatial distribution of the bioactive and stem cell attractive areas. They were fabricated either by microcontact printing or piezoelectric spotting of polycationic biomolecules or extracellular matrix (ECM) proteins on cell-repellent surface. HUCB-NSCs were shown to adhere, differentiate and respond to MeHgCl on functional domains in a manner dependent on protein type and concentration, cell density and serum conditions. While receptor-mediated interactions with ECM proteins under absence of serum promote neuronal differentiation, non-specific adhesion to polycationic molecules maintains cells attached to the surface in non-differentiated stage. Tailoring the geometry of the bio-pattern enabled directing and monitoring of the neural stem cells' development. Functional domains were further engineered to create a "smart" microenvironment by immobilizing to the surface small signaling molecules together with ECM proteins. Stimulation of selected intracellular pathways by molecules of Wnt, Shh, CNTF, Jagged or Notch type resulted in differentiation of HUCB-NSC to either neuronal or astroglial lineage. Bioengineered protein microarrays provide a novel approach to the multiparameter bio-tests by adding important information on the sensitivity of certain molecular pathways to selected neurotoxins

ID ABS: 359

Animal models and alternatives for studying human aging

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Neurodegenerative diseases, cancer, metabolic diseases and cardiovascular diseases have been associated with the aging human population. These diseases have considerable societal, economic and healthcare implications.

Modelling age-related diseases in animals is problematic since these diseases are multifactor, with genetic predisposing factors and environmental and life style choices all playing important roles. These factors are almost impossible to simulate within a laboratory environment. Indeed, a recent study has shown that the immune naïve state of some rodent models is the reason why human-like disease pathologies are not observed.

Mouse models are used extensively in age-related disease research and provide more ethical alternative to studies in higher order vertebrates such as non-human primates. However, the average life span of a mouse is around 4 years which is considerably shorter than that of primates. Furthermore, DNA damage and repair mechanisms differ substantially between humans and mice. Alternative models include invertebrate species and eukaryotic cells. These models have proven informative regarding the genetic component of aging but cannot capture the clinical features of age-related diseases. The way in which human data and data from these studies can be used to validate vertebrate animal models of human aging will be discussed.

ID ABS: 363

HepaChip: a promising tool for *in vitro* assessment of liver toxicity

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In vitro toxicity tests used in drug development mostly rely on 2D-cell cultures which lack predictability with regard to the *in vivo* situation. Especially in liver toxicology, suitable models are missing. The major problem in culturing primary hepatocytes is their fast dedifferentiation in 2D cultures, which is accompanied by the loss of liver-specific functions. Thus, not all effects of drugs can be discovered in early drug development. Animal models also have limited significance because transfer of results to the human situation is restricted. Moreover, for ethical reasons animal studies should be avoided whenever possible. Consequentially, the pharmaceutical industry faces considerable risks regarding the safety of patients during clinical trials and the loss of huge R&D investments if a drug candidate fails in a late phase of drug development.

We are developing a new *in vitro* model for the assessment of liver toxicity, the so-called HepaChip. In this microfluidic chip cells can be assembled using dielectrophoretic and hydrodynamic forces. A 3D structure similar to a liver sinusoid is obtained by arranging hepatocytes and endothelial cells in an appropriate manner on an extracellular matrix coating. Medium perfusion within the co-culture further mimics the *in vivo* situation and should additionally support a differentiated hepatocyte phenotype. Compared to 2D cultures, hepatocytes cultured in the HepaChip should maintain their liver-specific functions. This is the prerequisite for a reliable *in vitro* screening of drug candidates. The HepaChip seems to be a promising tool to improve toxicity testing in drug development.

ID ABS: 388

An intelligent alternative: MCB connected culture *Quasi-Vivo* TM system

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Because *in vitro* assays cannot provide a complete picture of the whole body response, animal testing is considered to be of crucial importance in pharmaceutical testing. Current *in vitro* cytotoxicity models are limited by their incomplete modelling of cell-cell interactions. However, a number of drugs which pass through the battery of *in vitro* tests often get through animal testing and on to clinical trails only to reveal serious toxicity in humans. In what is a linear and strait-jacketed regime of testing, much time, money, and animal sacrifice could be spared by broadening the initial steps of drug trails, through intelligent *in vitro* tests that provide quality information.

The MCB *Quasi-Vivo* TM system enables whole body information to be obtained from *in vitro* tests and is an intelligent, effective way of reducing animal sacrifice. The system is composed of a series of modular bioreactor chambers designed to mimic crosstalk between cells or tissues. Cells are housed in the different culture chambers and are connected only by flow, such that cell-cell interaction is mediated by soluble ligands as occurs in the body. Using the MCB *Quasi-Vivo* TM system, hepatocytes were cultured with other cells in an *in vivo*-like environment. The IC_{50} value of diclofenac is reduced by an order of magnitude with respect to standard *in vitro* tests, thus correctly identifying it as a risk drug. Moreover CYP3A4 expression of hepatocytes is upregulated to *in vivo* levels in the MCB. By virtue of its *Quasi-Vivo* TM environment, the MCB system is more effective than existing *in vitro* assays.

ID ABS: 398

Development of *in silico* and *in vitro* tools to predict exposure of cosmetics ingredients

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REACH is the regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals. It places greater responsibility on industry to manage the risks that chemicals might cause to human health and environment.

Knowledge of dermal absorption is a key parameter to evaluate chemical exposure from topical applications for risk assessment. Animal experiments have to be avoided for ethical considerations and for scientific relevance. Currently, the OECD guideline n°428 recommends use of human skin or alternatively pig skin for *in vitro* cutaneous absorption studies. However the use of such test methods is limited by the number of skin explants available as well as tricky sample preparation leading to time consuming. To overcome these limitations, new *in silico* model and *in vitro* testing strategies have been recently developed.

Combining data obtained with QSAR tools and data obtained with reconstructed human epidermis (RHE) allows the evaluation of chemical permeation potential. Such information could be implemented in Integrated Testing Strategies and used for risk assessment. *In silico* as well as *in vitro* tools have been adapted to the physico-chemical properties of the chemicals. Moreover, these tools have been developed according to cosmetic conditions of use.

Examples will be given to illustrate the use of *in silico* and *in vitro* tools to predict topical exposure of cosmetic ingredients. Further developments are needed to enlarge these strategies and to refine the dermal absorption prediction

ID ABS: 425

Monocyte activation test as an alternative to the rabbit pyrogen test

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Pyrogenic contamination of medicines for parenteral use is a public health problem. Exogenous pyrogens are frequently components from Gram-negative bacterial cell walls (LPS, endotoxin) and rarely from Gram-positive bacterial cell walls (LTA, non-endotoxin). The European *Pharmacopoeia* lists two tests for pyrogenicity: the *in vivo* rabbit pyrogen test and the *in vitro* Bacterial Endotoxins Test.

In order to reduce animal testing for the pyrogen-free quality control of medicines for parenteral use, an *in vitro* method has been developed as Monocyte Activation Test (MAT) based on the human fever mechanism. This test is intended to detect endotoxin and non-endotoxin contaminants. The aim of this study concerns first, the applicability of the MAT to antibiotics and the determination of the most probable working dilution that eliminates interfering factors. A validated fresh human whole blood method is applied using a commercial ELISA kit for the detection of the cytokine IL-1β. Furthermore, a product can be contaminated with pyrogens from Gram-positive or Gram-negative bacteria but also with both. So, in the second part, the difference in the comportment of LPS, LTA and a combination of both has been studied using the same method. Same quantities of LPS or LTA, expressed in endotoxin equivalents, and the combination of both in equal quantities were added to the product. Then, we evaluated the pyrogenic contaminants and the results are expressed as recoveries. They are similar for the two first preparations, but it was different for the combination.

Our results show that the MAT is a useful method for the detection of pyrogenic contaminants.

ID ABS: 430

Hypoxia induced signalling in pulmonary epithelial and microvascular endothelial cells.

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Under hypoxic conditions eukaryotic cells modulate the expression of specific genes involved in adaptive physiological responses, including glucose metabolism, angiogenesis, vasoconstriction and inflammation. However, the underlying molecular mechanisms are cell type specific and are not yet fully elucidated. In the lung, epithelial cells and endothelial cells are highly sensitive to hypoxia and together orchestrate a rapid and sustained adaptive response. Cyclic AMP Response Element Binding Protein (CREB) has been shown in animal studies to be involved in the hypoxia responses in the lung. Therefore we examined the effect of hypoxia on CREB and CREB related pathways in an epithelial and microvascular endothelial *in vitro* model (A549 and HMEC-1 cells respectively). Hypoxia induced CREB activation in both cell types, although this effect was more pronounced in A549 cells. Activating Transcription Factor 3 (ATF-3), which belongs to the CREB family of transcription factors, was heavily induced by hypoxia in epithelial cells but not in microvascular endothelial cells. Both cell types demonstrated hypoxia induced secretion of Vascular Endothelial Growth Factor (VEGF) and interleukin 6 (IL-6). Furthermore, secretion of the vasoconstrictor endothelin-1 (ET1) was increased in HMEC-1 cells but decreased in A549 cells. ET-1 is a HIF-1 alpha (Hypoxia Inducible Factor alpha) target gene, but it also contains putative CREB binding sites. Thus, these results suggest that CREB plays an important role in the molecular responses to hypoxia in pulmonary epithelial and microvascular endothelial cells. However, some of these hypoxia induced responses are different or even opposite in epithelial and microvascular endothelial cells.

ID ABS: 460

Regulatory requirements for *in vitro* systems to meet performance standards during validation and over time

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A recent US National Research Council report envisions humane alternative toxicological tests that are faster, less expensive, and more accurate than their animal counterparts. *In vitro* tissue models have been or are in the process of being validated as alternatives to animal testing for safety evaluation of cosmetics, pharmaceuticals, and consumer products. Currently available organotypic human models include dermal (EpiDerm, EpiDerm-FT), ocular (EpiOcular), ectocervical (EpiVaginal) and airway (EpiAirway). As these models become validated, regulatory agencies and users need to be assured that the models will provide consistent, high quality data over time, not just during the validation process. Recommended guidelines include "full characterization of cells or tissues, sampling of each lot … for performance, and regular use of controls and benchmark chemicals to provide assurance of consistency of assay performance". The current poster summarizes the long-term reproducibility of EpiDerm and EpiOcular. Quality control testing of weekly production batches was performed using the MTT assay. The exposure time that reduced tissue viability to 50% (ET-50) for Triton X-100 was determined. For EpiOcular, the yearly average ET-50s have ranged from 22.0-27.3 minutes. The coefficients of variation (CV) for the negative control tissues have averaged <6% for every year since 1997; the yearly average CV has never exceeded 6.7%. For EpiDerm, the yearly average ET-50s have ranged from 5.9-7.5 hrs. The CV for the negative control has averaged <7.5% for every year since 1996. These results over the past 14 years of commercial production address regulatory concerns regarding performance standards over time.

ID ABS: 482

Microarray analyses indicate high genomic stability of the oral keratinocyte cell line SVpgC2a in short-term and long-term culture

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Genomic analyses are increasingly used in toxicity assessments. Unwanted experimental variation in gene expression originating from transfer protocols and continued passage of cells has so far received little attention. We investigated genomic consequences of continued culture of oral keratinocytes (SVpgC2a) from transcriptome and chromosome analysis. Transcript profiling by microarray of cultures transferred 3 to 8 times indicated small variations in transcript profiles. Moreover, cultures transferred 75 times showed similar variation as those transferred only 3 times. Correlation analysis indicated larger contribution to variation from the analysis itself rather than from long-term growth of the cells. Cells grown for 75 passages exhibited a change level of <1.7% among 8400 assessed transcripts vs. 4970 transcripts that were detected but unchanged. Bioinformatic analysis of the data implicated enrichment of random gene ontologies. Considering influences of short-term culture, expression profiles of replicate samplings over 48h confirmed the contribution only of technical variation. Differently, karyotyping analysis of cells transferred 53 times implicated changes in a majority of chromosomes. Thus, substantial chromosome changes did not correlate to minor transcript changes. Formaldehyde caused cytotoxicity in a dose-dependent manner in SVpgC2a, but the formaldehyde toxicity profile remained unaltered after >50 passages. Useful to toxicity testing, SVpgC2a seemed to exhibit high genotypic and phenotypic stability in long-term culture.

ID ABS: 488

A novel method for investigating vascular leakage in pressurised human vessels

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Measurements of vascular permeability have typically been conducted using *in vivo* animal models such as the Evans blue dye (EBD) model, where extravasation of the dye into the wall of blood vessels is used as a marker of vascular permeability. This model involves the injection of EBD into the blood stream before the animal is sacrificed and the EBD is extracted from the tissue for quantification. In many cases, a separate animal is used to evaluate each time point or dose in a curve, requiring large numbers of animals to be sacrificed. Such models also suffer from disadvantages including potential species differences and an inability to track changes in real-time. In addition, currently available cell-based assays, even if using human cells, might not reflect the true 3D architecture of human blood vessels and are not therefore entirely applicable. The use of fresh isolated human blood vessels overcomes many of these problems. The extravasation of albumin-bound EBD can be monitored continuously, and in real-time, from the lumen of the vessel into the vessel wall in human subcutaneous resistance arteries using Biopta's perfusion myograph and software. Intraluminal perfusion of thrombin (0.5 Units/ml), known to induce vascular leakage in arteries, caused a time-dependent accumulation of albumin bound dye in the vessel wall which was not observed in the vehicle control group.

This new *in vitro* method provides continuous real-time measurement of vascular permeability in isolated human tissues and represents a novel alternative to *in vivo* animal methods for measuring vascular permeability.

ID ABS: 501

DAP, a new fluorescent cell-based assay which predicts human acute toxicity to 82%

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We used the MEIC data base from the SSCT to establish a correlation between an innovative cell-based assay and human acute toxicity. The MEIC data base offers human LC_{50} values for 50 substances listed by Scandinavian poison units. The new assay uses plasma membrane permeant SYTO nucleic assay dyes and relies on dequenching after photobleaching (DAP). Under normal cell culture conditions, self-quenching occurs, and when the cell sample is photobleached by illumination, dye fluorescence is recovered due to photodegradation of molecules involved in quenching. In contrast, when nucleic acid structure is damaged, the distance between dye molecules increases and no quenching occurs.

The DAP assay was performed on HepG2 cells using 25 substances from the MEIC database. Cellular LC_{50} values were determined from dose-response curves with success for all 25 substances. Two LC_{50} values from hardly soluble substances were discarded. A 79% prediction between cellular and human (post-mortem blood concentrations) LC_{50} values was established ($\log LC_{50}$hum. = 0.94 $\log LC_{50}$cell. + 0.65, R2=0.79). Further analysis using blood-brain barrier correction (established by MEIC) increased prediction up to 82%. Because the DAP assay informs on DNA and/or RNA alterations within the cell, the potential for genotoxicity applications was also shown by comparing DAP fluorescence profiles with COMET assay data.

DAP assay experimental procedure is straightforward (simple addition of dye to the culture medium), quick (20 minutes), does not require a washing step and is adapted to HTS instruments. In conclusion, the DAP cell assay predicts acute human toxicity better than rat or mouse assays and equals MEIC assay battery performance, with high information content in one test.

ID ABS: 502

Estimating kinetic parameters *in vitro*

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During drug development, the pharmacokinetic parameters of a compound are determined in animal experiments. To decrease the number of animals needed, *in vitro* and *in silico* approaches are called for that can help predict the kinetics *in vivo*. The aim of this project, part of Predict-IV, is to improve our understanding of the kinetics of compounds *in vitro* and use the data for PBPK modelling for the *in vivo* situation.

The absorption of two compounds, chlorpromazine and diazepam was measured *in vitro* by estimating the permeability across Caco-2 monolayers. The effect of test conditions on membrane permeability was studied, including the effects of protein binding and stirring conditions. In addition, the role of adsorption to the well plate on the test conditions was analyzed. Protein binding of the compounds was also measured by equilibrium dialysis.

Both chlorpromazine and diazepam were well absorbed and were substrates for active efflux transporters. The addition of BSA to the receiver compartment worked as an extra sink condition and increased the amount of compound absorbed. The equilibrium dialysis showed high protein binding for both compounds.

The results from the Caco-2 cell system indicate that chlorpromazine and diazepam have a good absorption which corresponds to *in vivo* findings. Further adjustments to the Caco-2 cell system have to be made to reflect the uptake in the *in vivo* situation, and metabolism of the compounds should be taken into account. Finally, PBPK modelling can be used to convert these *in vitro* data to the *in vivo* situation.

ID ABS: 514

Toxicity profiling using high-throughput cell-based screening *in vitro*

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We have established a state-of-the-art High Throughput Screening (HTS) system, which is widely applicable to cell-based screening for functional target identification, early-stage lead discovery as well as compound cytotoxicity profiling. Our system includes robotic instrumentation with an automated CO_2-incubator, PE-Envision plate reader and ATS-100 acoustic nanodispenser. Additionally, Olympus ScanR system allows High-Content phenotypic assays. A standard principle for cell-based high-throughput functional screening allows testing of multiple chemicals at selected concentrations in 384-well plate format. Compounds are printed on plates, followed by cell plating, incubation and assaying on endpoints like cell proliferation/cytotoxicity or apoptosis, with the capacity of approximately 32,000 cell-biological experiments performed at a time. With our automated system we have already carried out functional screens with a library of 5,000 drug-like small molecule compounds to identify antiproliferative compounds in several breast and prostate cancer cell lines. VTT has also set up a system for protein lysate microarray analyses, where cells are lysed after the compound treatment and printed onto nitrocellulose slides. The lysate microarray is then immunostained for specific protein markers that reflect the cellular status, or phenotype inflicted by the compounds tested. The generation of multiple replicate array slides provides opportunity to typically process 9,000 samples for protein expression by immunohistochemical analysis. Quantitative measurement of the expression of multiple proteins generates a broad, informative toxicity profile of multiple endpoints related to diverse biological fates and cellular processes. The approach enables cost-effective analysis and pattern recognition of mechanisms considered to contribute to the toxicity of chemicals

ID ABS: 525

Development of an *in vitro* angiogenesis model using endothelial cells differentiated from human bone marrow mesenchymal stem cells

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Pathologic excess of angiogenesis as well as deficiencies of angiogenesis are the cause of widespread and severe diseases. Most of our knowledge about the factors, signaling pathways and genes involved in angiogenesis has been obtained from experiments directly carried out on experimental animals. Vascular progenitor cells can be used to study the angiogenesis. Progenitor stem cells from various sources can be differentiated into endothelial cells useful for *in vitro* angiogenesis studies. In this study, human bone marrow mesenchymal stem cells (hBMCs) were differentiated to endothelial cells in the presence of vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1). Then, selected markers were used to characterize the endothelial cells. The differentiated cells were characterized by their molecular markers, such as von Willebrand factor (vWF) and vascular endothelial growth factor receptor 2 (VEGFR2), measured at protein and mRNA levels. The ability of the cells to develop into capillary tubes was also examined in the confluent hBMSCs recovered from MCDB131 media containing 5% FBS, 50 ng/ml VEGF and 20 ng/ml IGF-1. Following induction of differentiation, the cells were transferred to extracellular matrix (ECM) gel solution in wells of 24-well microplates and incubated for 2 h at 37°C for seven days. The area of tubulogenesis and the number of positive cells was assessed on day 12 by counting the number of tubes in each well using a phase contrast microscope. On average >80% of the cells were developed into capillaries.

ID ABS: 527

Effects of chlorpyrifos and deoxynivalenol co-exposure on Caco-2/TC7 cells

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Chlorpyrifos (CPF), an organophosphate insecticide for pest control, and deoxynivalenol (DON), a trichothecene mycotoxin, are common crop contaminants. Our previous studies with Caco-2/TC7 cells demonstrated that CPF, at actual levels of human exposure, interferes with tight junctions altering the barrier integrity and, probably, the absorption of other co-administered chemicals. On the other hand, chronic exposure to DON, can alter the intestinal mucosa integrity and could exacerbate intestinal inflammation. To investigate possible synergic effects on DON toxicity and absorption of CPF, Caco-2/TC7 cells, a clone derived from parental Caco-2 cell line, were treated with different DON concentrations (0.01- 10 μg/ml) with or without CPF (nominal concentrations range from 50 to 625 μM).

Preliminary cytotoxic results (Neutral Red uptake assay) show that only the DON concentration 10 μg/ml is toxic for differentiated (21 days of culture) Caco-2/TC7 cells after 24 hours of exposure.

When the cells were treated, under the same experimental conditions, with both chemicals, a strong decrease of viability was found at 1 μg/ml DON plus the highest CPF concentration tested. Also, cellular morphology was seriously affected by this co-treatment, and the characteristic formations of differentiated epithelia, "domes", were collapsed. No morphological or toxic effects were reported with lower CPF concentrations.

Further investigations on semi-permeable supports are in progress in order to investigate the influence of CPF on DON absorption and uptake in this cellular model.

ID ABS: 529

The true gold standard: a comparison of human immune cells *in vitro* to human *in vivo* immunotoxicity data

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In this paper, we present a strategy of *in vitro* model development that can be adapted to other toxicants and biological endpoints. The specific aim of this study was to develop an *in vitro* model of mercury (Hg) immunotoxicity that could be directly compared to human epidemiological data. To allow a direct comparison, we used primary cultures of human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide, allowing us to study the response to Hg in a mixed population of activated immune cells. In the case of immune responses, as with many other systems, it is important to use environmentally-relevant concentrations in order to avoid non-specific toxic responses which suppress the specific functions of the cells. In our model system, we used $HgCl_2$ concentrations of up to 200 nM, corresponding to a blood Hg concentration of 37 µg/L that is readily observed in fish-eating populations. We used a multi-level study design, where 20 healthy volunteers (10 males and 10 females) were asked to donate blood six separate times, in order to model both inter- and intra-individual variation in response. We measured seven cytokines in the cell culture supernatants, and compared these values to serum cytokine levels from individuals with either high or low mercury exposures. In the *in vitro* model, Hg treatment increased pro-inflammatory cytokine production (IL-1β and TNF-α), and decreased anti-inflammatory cytokine production (IL-1Ra and IL-10). These results are consistent with observations in exposed individuals, where Hg exposure is correlated with increased serum concentrations of the same pro-inflammatory cytokines.

ID ABS: 532

Gene expression in human primary hepatocytes following hypothermic storage

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Human primary hepatocytes are considered to be the closest model to human liver and are thus the gold standard for *in vitro* hepatic models for xenobiotic metabolism and toxicity studies. However, availability of human liver tissue, preservation and distribution of isolated cells to other research centres and pharmaceutical industry limit the more widespread use of this model.

In this study the effect of preservation and hypothermic storage on gene expression profiles was investigated. Human hepatocytes were isolated by a two step perfusion protocol. After attachment human hepatocytes were hypothermically stored at 10°C for 2 and 4 days using a matrix-based system developed by Abcellute Ltd. Following reactivation and recovery, cells were treated for 24 h with 10 μM rifampicin, a known inducer of CYP3A4. A range of cytochrome P450 (CYP) and hepatic transporter genes was analysed using TaqMan real-time PCR.

Preliminary experiments using preserved suspension cells showed that viability was 70.9 ±1.9% and 60.7 ±4.6% after 2 d and 5 d of hypothermic storage at 10°C, respectively.

Gene expression levels in hypothermically preserved cells were compared to levels in hepatocytes 4 hours post-attachment. Rifampicin induction of CYP3A4 was compared to that observed in cells cultured for 24 hours.

Gene	4 hour	2 day preserved	4 day preserved
CYP1A2	100%	117%	94%
CYP2B6	100%	98%	153%
CYP3A4	100%	88%	99%
CYP3A4 (rif induced)	–	73%	87%

The gene expression data indicate that primary human hepatocytes hypothermically preserved for either 2 or 4 days in Abcellute matrix maintain expression and induction profiles of the normally labile CYPs similar to those observed in freshly isolated cells.

ID ABS: 534

Ex vivo colospheres as a potential alternative method for colon cancer xenografts

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Colospheres are 3D multicellular spheres derived from mechanically dissociated colon cancer tissue. We have previously demonstrated that this short-term culture model is exclusively formed by cancer cells and associated with tumour aggressiveness.

To address whether colospheres could be helpful for *ex vivo* drug screening, we used XenoCT320, a colon cancer xenograft established in nude mice from a patient colon tumour, and the colon cancer cell line, CT320X6, derived from XenoCT320. Dissociation of XenoCT320 led to formation of colospheres and CT320X6 culture on agarose to spheroids. Colospheres and spheroids were collected for gene expression studies and drug cytotoxic assays.

Gene expression clustering showed that colospheres were closer to xenograft tissue than spheroids derived from the same xenograft. This has been confirmed in another pair of colospheres/spheroids (i.e. obtained from another xenograft/cell line pair). To compare the sensitivities of paired microtumours to antitumour drugs with *in vivo* response in xenografts, spheroids and colospheres were treated for 4 days with conventional drugs in colon cancer (5-FU, CPT11, or L-OHP). The preliminary data showed: i) despite variable size of colospheres, culture conditions for LDH cytotoxic assays are now validated for reproducibility; ii) all models (*in vivo* xenografts, spheroids and colospheres) displayed resistance to 5-FU. Culture optimization is required for viability assays after drug treatment.

As one xenograft is able to give rise to abundant colospheres, this *ex vivo* model deserves further investigation to determine if it could reduce the number of tumour engrafted mice used in the anticancer drug pipeline.

ID ABS: 547

Cytokine production after acute exposure of precision cut lung slices to nitrogen dioxide and ozone

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Gaseous compounds like nitrogen dioxide and ozone present a major health risk. Inhalation can be associated with exacerbation of asthma, chronic pulmonary disease (COPD) or pneumonia. Precision cut lung slices (PCLS) represent an *ex vivo* technique which delivers the possibility to gain insight into chemical-induced effects in all cell types of the respiratory tract after air/lifted exposure to gaseous mixtures.

PCLS were prepared and exposed air/lifted to different concentrations of ozone and nitrogen dioxide. As negative control PCLS were exposed to clean air. Cytotoxicity was determined by WST-1 and live/dead staining for confocal microscopy. Chemical-induced inflammation was characterized by quantification of cytokine release by ELISA or Luminex technology.

WST-1 or live/dead staining showed that air/lifted cultivation and 1 h exposure to concentrations of 1 to 10 ppm nitrogen dioxide or 3.5 to 8.5 ppm ozone did not induce cytotoxicity. Nitrogen dioxide induced cytotoxicity after exposure to 70 ppm. Expression of IL-1alpha showed for nitrogen dioxide a dose-dependent increase of up to 20% whereas RANTES was decreased. Exposure to ozone resulted in dose-dependent and significant up regulation of the pro-inflammatory cytokine IL-1alpha of up to 80% whereas RANTES showed an increase of up to 800%. Cytokines like MCP-1 or IL-12 showed no changes for any gas compound.

These experiments pointed out that PCLS can be exposed air/lifted to gaseous compounds and show a dose-dependent cytotoxicity and cytokine production. This offers opportunities to investigate *ex vivo* immunotoxicological parameters for gaseous and particulate chemicals in a multifunctional test system.

ID ABS: 553

Reproducibility and interlaboratory transferability of the *in vitro* micronucleus assay in co-culture

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Genotoxicity is usually addressed early in the safety assessment process of chemicals for regulatory purposes. Gene mutation and chromosome damage are the two endpoints that are usually investigated. Although numbers of *in vitro* assays well accepted by regulators are routinely used to check these endpoints, they do have a poor specificity (tendency to yield a high number of positive results whereas *in vivo* genotoxicity results are negative).

To address the need for predictive methods in the context of the European legislation (7th amendment to the European Cosmetic directive, REACH) an *in vitro* micronucleus assay using a human reconstructed skin and target cells grown beneath the skin has been developed. This way of using human reconstructed skin aims at improving the relevance of exposure conditions in *in vitro* genotoxicity assays for dermally applied compounds. The skin is a biologically active barrier driving the exposure to compounds and their possible metabolites. The exposure of the target cells to a given substance can be assessed after topical application as was the case here. Episkin® was used as a metabolically active tissue and a physiologic barrier. The test compound can be metabolized by the skin and/or by the target cells (± S9 if needed).

Chemicals were evaluated using this system by two different laboratories (Institut Pasteur de Lille and the genotoxicity laboratory of l'Oréal).

The results presented here show that the method is reproducible and can be transferred to different laboratories. Furthermore, clastogens as well as aneugens could be detected.

ID ABS: 565

Trans-epithelial electrical resistance (TEER) as a sensitive endpoint for toxin induced sub-lethal injury in human renal tubular epithelial cells

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Epithelial cells function mainly to form a selective barrier between two opposing compartments. Barrier function can be determined in vitro using transepithelial electrical resistance. TEER has been previously demonstrated to be a sensitive endpoint for sub-lethal injury to chemicals and pharmaceuticals. A new human renal proximal tubular cell line has recently become available (RPTEC/TERT1 cells) which displays excellent barrier properties. Thus we tested the use of TEER as a parameter of toxin induced dysfunction in these cells.

RPTEC/TERT1 cells were cultured to confluence on untreated aluminium oxide filters. At steady state TEER, cells were treated with nephrotoxic compounds (cyclosporine A, nifedipine, monuron, KBrO3 and ochratoxin A). Cells were exposed for 72 h and TEER was measured daily for a further 96 h (recovery). In the presence of three of these compounds (nifedipine 30 and 60 μM, monuron 1 mM, and cyclosporin A 25 μM), TEER increased over the exposure time (to 130, 230 and 800% of the starting value, respectively) and returned to control values during the recovery period. Ochratoxin A (10 μM OTA) caused a 40% decrease in TEER followed by a full recovery. KBrO3 at 3 mM caused an irreversible decrease of TEER and at 6 mM a total irreversible collapse.

TEER is a sensitive marker of sub-lethal injury in RPTEC/TERT1 cells. Interestingly, the monolayer responds to certain nephrotoxic compounds by transiently increasing TEER and to others by a TEER decrease. Recovery of the monolayer gives further information to the severity of the toxic insult

ID ABS: 566

Lactate production is a sensitive marker of toxin induced sub-lethal injury in renal proximal tubular cells (RPTEC/TERT1)

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In vitro cell culture models for hazard and risk assessment of chemicals and pharmaceuticals are continuously being developed and improved. However, there is also a need to develop in parallel, more sensitive assays of cellular stress and dysfunction, which go beyond simply measuring cell death. A new human renal proximal tubule cell line, RPTEC/TERT1, exhibits very low levels of glycolysis when differentiated and potentially reverts to glycolysis upon certain types of stress. Here, we evaluate the use of lactate as a marker of toxin induced stress.

Differentiated RPTEC/TERT1 cells were treated with the nephrotoxins monuron, nifedipine, potassium bromate (KBrO3), cyclosporine A (CsA), cadmium chloride ($CdCl_2$) and ochratoxin A (OTA) for 72 h. Supernatant lactate as a marker of glycolysis was measured using a LDH based colorimetric assay. Cell viability was assessed using resazurin reduction and release of the cytosolic enzyme LDH into supernatant medium.

With the exception of nifedipine, all compounds caused a dose and time dependent increase in lactate production. Additionally, this parameter was more sensitive than either LDH release or resazurin reduction. Thus RPTEC/TERT1 cells respond to many different chronic nephrotoxins by increasing glycolysis rates. This parameter is sensitive, simple and cheap to measure and is thus an ideal marker of toxin induced cell stress in renal epithelial cells.

ID ABS: 568

In vitro BBB models suitable for all stages of R&D

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Unlike nearly all other organs of the body where there is a free exchange between blood and interstitial fluid, the cerebral capillaries form a physical and metabolic barrier. The blood-brain barrier (BBB), by regulating the exchange of substances between blood and brain, maintains brain homeostasis and provides a defense against toxic or infective agents circulating in the blood. Unfortunately, this barrier also diminishes the value of many promising drug candidates. The BBB is also implicated in pathologies such as neurodegenerative disorders and stroke as well as infectious processes. Studying the BBB and predicting this permeability is essential. To evaluate pharmacokinetic properties of neurological drugs animal studies can be performed but render the development of drugs a long, high-cost process. Moreover, these methods cannot be adapted at an early stage of R&D. In the early 90's, Cecchelli et al. did pioneering work in establishing a highly predictive BBB model, consisting of the co-culture of bovine brain capillary endothelial cells and rat glial cells. This model is currently used in pharmaceutical industries for mechanistic studies and as a permeability screen. By modifying this well-validated co-culture model, it has been possible to develop a BBB system that is easier to use, faster and suitable for automation and thus fits the needs of High Throughput Screening (HTS). Characteristics of the BBB model, which is ready in only 4 days and also available in frozen ready-to-use format, will be presented. Results which highlight that the *in vitro* BBB model like the 4 day model could improve neurotoxicity assessment will also be shown

ID ABS: 603

Alternative methods for phototoxicity testing using reconstructed human skin models

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Phototoxicity is defined as a skin inflammatory reaction elicited by topical application or systemic administration of chemicals and subsequent exposure to light, particularly UVA radiation. Where substances are intended for use in dermatological products applied to the skin, it is necessary to carry out an assessment of potential phototoxic hazard. The aim of our study was to investigate the ability of reconstructed human skin models (H3D PT) to identify the phototoxic potency of chemicals. We evaluated the phototoxic potential of 4 UV absorbing chemicals (chlorpromazine, promethazine, rose bengal and bergamot oil) and 4 non-UV absorbing chemicals (penicillin G, sodium lauryl sulfate, Eusolex 9020 and Eusolex 6300) using the EpiderMTM, KeraskinTM, and MelaskinTM models. The test is based on a comparison of cytotoxicity of a chemical when tested with or without exposure to a non toxic dose of UVA+ visible light. After chemicals were applied topically to tissue of each model, tissues were exposed to a non-cytotoxic dose of UVA (6 J/cm^2). Cell viability was quantified by MTT assay 22 h after UVA exposure. Our results showed that chlorpromazine, promethazine, rose bengal and bergamot oil were phototoxic, and penicillin G, sodium lauryl sulfate, Eusolex 9020 and Eusolex 6300 were non-phototoxic. These results correspond well with *in vivo* data. Meanwhile, three models showed the same results with the exception that rose bangal was close to non-phototoxic in the MelaskinTM model. These results suggest that the H3D PT is able to discriminate efficiently between phototoxic and non-phototoxic chemicals and is a good alternative method for assessing the phototoxic potential.

ID ABS: 604

Brand-to-brand variation of three dimensional cultured kin models for estimation of membrane permeation and concentration of chemical compounds

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In vitro permeation experiments using excised human or animal skin are widely applied to evaluate the skin permeation profiles of many substances. However, the 3Rs issues regarding animal experiments as well as ethical problems with human tests limit these experiments. Alternative permeation experiments using three-dimensional cultured human skin models have gained attention before this background. Here, six kinds of three-dimensional cultured human skin models were selected and evaluated for their morphological properties and permeation profiles of model compounds, and the obtained results were compared with those found in human and hairless rat skin to examine the utility of the three-dimensional cultured human skin model as an alternative skin for testing. The present experimental results suggest that only LSE-high and EpiDerm can be used as alternatives for testing the skin permeation of drugs and cosmetic ingredients.

ID ABS: 769

Prediction of human pharmacokinetics following dermal administration: integration of a skin absorption module to the Simcyp Population-Based ADME Simulator™ with the aim of avoiding animal studies

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Animal models to assess dermal absorption are common however the physicochemically based methods are now available to predict the fraction absorbed via the skin and its rate. Simcyp Population-Based ADME Simulator™ has been developed to simulate pharmacokinetic clinical trials incorporating inter-individual variability with the purpose of identifying the individuals at extreme tails of population distribution; an attribute which cannot be associated with any animal model. However, until recently the platform did not have any module for assessing and simulating dermal absorption. We now describe a dermal absorption module and demonstrate its functionality in examining the inter- and intra-individual variability.

Simcyp simulator combines information on genetic, physiological, and demographic variability with preclinical *in vitro* data to allow extrapolation to *in vivo* pharmacokinetics. Version 9 now includes skin as a route of drug administration. The skin absorption model incorporates two different characteristics of the skin layers (stratum corneum and viable epidermis) and considers intra- and inter-individual variability in composition and thickness of layers (1). Simulations with the diclofenac, as a commonly used topical drug, are compared with the outcome of human *in vivo* studies. The input parameters for the skin module (such as the partition and permeability constants) were calculated based on the simple molecular descriptors logP, hydrogen-bond donors (HBD) and molecular weight.

Six different clinical studies (2,3,4,5,6,7) with varying demography and different drug formulations (spray and patch), dosing scheme (single and multiple doses), and application places (forearm, thigh, and knee) were simulated. The simulated plasma concentration-time profiles were comparable with the corresponding *in vivo* data.

The outcome of this analysis demonstrates the possibility of replacing animal models of dermal absorption with the *in silico* models implemented within Simcyp skin absorption module. A demonstration session will be available to show the performance of the module.

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ID ABS: 779

Optimization of culturing & measurement conditions for improved stability & sensitivity of cell physiology monitoring systems for toxicology applications

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Long term and on-line monitoring of water quality with cell-based systems requires at least two aspects to be addressed: a) the measurement system including the cellular system has to be stable and to deliver stable data over time and b) the sensitivity of the cell-based system has to be adequate for the proposed application. In this study the Bionas® 2500 analyzing system detects physiological parameters of living cells (HepG2, V79) in a label-free and non-invasive assay. Cells are placed on a sensor chip and supplied with medium in a perfusion system. The multiparametric sensor chip continuously measures a) the oxygen consumption (respiration), b) the extracellular acidification (glycolysis) and c) the cell impedance. Whereas the oxygen consumption and the acidification determine the acute rates of the cellular energy metabolism (bioenergetics) the cell impedance detects alterations in the cellular adhesion/confluence and morphology.

A perfusion system supplies the cells with nutrients and guarantees highly defined cell environmental conditions throughout the whole experiment. The composition of the media used in the system for the measurement has shown to be very important concerning signal stability during the experiment and sensitivity to toxicants. To examine adaptation effects the culture medium was also modified.

Although the composition of the medium was only changed in modest parameters, the effects on stability and sensitivity were significant.

Summarized, simple changes in the culture and measurement medium may affect the outcome of *in vitro* experiments – not only for toxicological applications.

ID ABS: 785

Upcyte hepatocytes as alternative for animal experiments to assess toxicogenomic effects and metabolic profiles of chemical entities

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Worldwide a huge amount of animal experiments is performed in order to evaluate drug candidates with respect to ADME-Tox (Absorption, Distribution, Metabolism, Excretion & Toxicology) or to investigate toxicity of chemical compounds. Generally, it is aimed to obtain as much information as possible on ADME-Tox in cell culture.

Primary human hepatocytes are recognised as the best available *in vitro* model to anticipate the metabolic profile of a drug in men. However, the supply of hepatocytes is limited by the deficit of liver donors and the absence or only modest expansion of these cells in culture.

The aim of this study was the generation of *in-vitro* proliferating primary human hepatocytes with extended lifespan as alternative source for human hepatocytes and animal testing.

Using medicyte's proprietary upcyte technology (upcyte; up-regulated cells), we succeeded in the generation of hepatocytes with the ability to proliferate and showing an extended lifespan. In addition, the upcyte hepatocytes express various liver-specific functions and maintain their specific functional characteristics upon long term cultivation. Analysis of CYP activities, cytoxicity, drug drug interaction and drug transporter expression indicate the high potential for the use of upcytes hepatocyte for new *in vitro* liver cell models for metabolic drug, toxicity and toxicogenomic analysis. This will eventually lead to a *in vitro* hepatocyte system in order to reduce or replace animal experiments in preclinical drug development and toxicity testing of chemical compounds.

PO10: Validation concepts

ID ABS: 26

Regulatory acceptance of an alternative test method at EU level

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Regulatory acceptance is the formal adoption of a validated test method by a regulatory agency/authority. The involvement of regulatory authorities in all stages of the validation process is imperative to accomplish this. The presentation details the principles and criteria for the regulatory acceptance of new or revised toxicological test methods at EU level and the interaction with the process of regulatory acceptance at the Organisation for Economic Cooperation and Development (OECD), notably when appropriate validated methods become available to replace, reduce or refine animal testing. Undue delay in the regulatory context will be addressed. Furthermore, information will be provided on the Tracking System for Alternative test methods (TSAR), which has been developed to provide transparency in the validation process leading up to regulatory acceptance. TSAR provides information on all stages in the process leading up to regulator acceptance, from the initial submission for prevalidation until final adoption by inclusion in the EU legislation (i.e. Testing Method Regulation (TMR), Commission Regulation (EC) No 440/2008 of 30 May 2008) and/or related Guidance Documents). As not all alternative methods (will) need to be included in the TMR, other platforms are being considered for enhancing regulatory acceptance of alterative methods.

ID ABS: 43

Statistical issues in the design and analysis of validation studies

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As statisticians, we have been involved in the planning, conducting, and evaluation of multi-laboratory validation studies for alternatives to animal experiments conducted in Japan. Over the past decade, we have faced certain statistical issues in the design and analysis of validation studies, such as the efficient allocation of materials to multiple laboratories, the evaluation of inter-laboratory reproducibility and transferability, the determination of the cut-off value for assessing target toxicity, the estimation of the sensitivity and specificity of test methods, and data management. In order to obtain reliable scientific evidence for the test method through the validation process, these issues should be appropriately and adequately addressed from statistical and toxicological perspectives. We devised some practical methodologies and procedures for these issues and confirmed their practicability based on real data in validation studies. For example, our results showed that statistical measures such as D-optimality and the intra-class correlation based on a linear model are useful if the endpoint to assess the target toxicity is distributed as a normal distribution. An overview of our approach is presented in this study. The role of statisticians in the validation study is also discussed here.

ID ABS: 126

The international validation study for the ER alpha STTA antagonist assay using HeLa9903

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The new *in vitro* test guideline for the "Stably Transfected Human Estrogen Receptor (ER) Transcriptional Activation Assay (STTA) using the human ERalpha-HeLa-9903 (HeLa9903) Cell Line for Detection of Estrogenic Agonist-Activity of Chemicals" was approved at the 20th OECD Working Group of National Coordinators meeting in April 2008. In accordance with the comments from the Peer Review Panel, the STTA anti-estrogenic (antagonist) assay validation study was initiated and is coordinated by JaCVAM, with membership of the management including representation also of ECVAM, EFSA and US-EPA.

The STTA antagonist assay is a screening method for identifying compounds which inhibit the response of the endogenous ligand, 17beta-estradiol. The protocol of the antagonist assay was designed to ensure the reliability and sensitivity of the assay with testing of "control chemicals" in each assay plate, and testing "reference chemicals" once each day the assay is run.

Five participating laboratories are following three tasks, [Task-1]: Set up of the system and demonstration of the basic skills of the laboratory by testing the reference chemicals in the agonist assay according to the test guideline; [Task-2]: Testing of un-coded reference chemicals based upon the provisional performance standard for antagonist assay; [Task-3]: Coded chemicals will be tested by the laboratories to demonstrate proficiency with the antagonist assay in Task-2 to evaluate the intra- and inter-laboratory reproducibility. All laboratories have successfully completed Task-1, for Task-2 the performance standards for antagonist assay have been revised based on the Task-2 results. Task-3 of the validation study is on-going.

ID ABS: 180

Validation of an *in vitro* skin irritation test protocol (EpiDerm SIT) to replace the *in vivo* rabbit test for hazard identification of chemicals

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

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

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

ID ABS: 400

Test development, validation and implementation: regulatory needs. An exploratory study in the carcinoGENOMICS project (FP6 LSH037712)

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One of the major obstacles in the implementation of 3R methods for regulatory purposes is the acceptance of the newly developed methods by regulatory authorities. The reasons for non-acceptance vary, ranging from non-communication between test developers and regulators, remaining questions about the validation process to poorly defined application domains. In order to identify the requirements for successful 3Rs implementation we have sent a questionnaire to regulators, industry and academia. The outcome of this questionnaire was discussed in an invitational workshop. These activities belonged to workpackage 11 of FP6 IP carcinoGENOMICS, which aims to develop *in vitro* methods for assessing the carcinogenic potential of compounds. Issues being addressed were the relevance of -omics data for risk assessment (e.g. types of biomarkers, data needs), validation criteria (e.g. criteria for acceptance, selection of chemicals), technology transfer (training needs, standardisation) and level of involvement of regulatory agencies (extent, when in the process, kind of involvement). The poster will provide an overview of some of the conclusions of the questionnaire and workshop.

We believe that the outcome on regulatory interaction goes beyond the carcinoGENOMICS project and is relevant for all projects that aim to develop new test methods for regulatory implementation as well as for some of the activities taking place in the European partnership between the Commission and the industry, i.e. EPAA.

The authors would like to thank the members of Working Group 11 of the carcinoGENOMICS project for their input.

ID ABS: 459

Reference laboratories can make validation more efficient

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

ID ABS: 540

Managing uncertainty and chaos: validation study risk management

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Validation studies may fail due to poor performance of the method being evaluated, or due to a myriad of other management, personnel, quality, market, political, or other forces. This study addresses reasons other than test method performance for study failure, and proposes a strategy for reducing validation study project risk. The current approach to toxicity test method validation is relatively new, with criteria and processes being defined by international authorities in the 1990's, and presents new project management challenges. Most organizations assign the management of such large projects to professional project managers (PMs) – a practice largely ignored to date in alternatives test method validation. The inter-laboratory studies are very expensive, so is this considered to be a cost savings? The toxicologists that typically take on the project management role perhaps do not want to relinquish control? Or perhaps this is just an oversight by professionals not accustomed to the need/benefit for a PM. Whatever the reason, the lack of oversight by a professional PM on such a large project as an inter-laboratory validation study adds unnecessary risk to the project. This paper will describe project risk and how it may threaten project success. An historical validation study will be used to illustrate the project risk management process, which includes the following steps: risk management planning; identifying project risks; analyzing the risks; planning risk responses; and monitoring and controlling risk responses. The application of risk management would be valuable considering the high failure rate of alternative test method validation studies.

ID ABS: 582

Testing of coded substances for a multi-phased international validation study of an Estrogen Receptor (ER) Transcriptional Activation (TA) assay

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The LUMI-CELL® ER assay is an ER TA test method developed to detect ER agonists and antagonists. NICEATM, ECVAM, and JaCVAM are conducting an international multi-laboratory validation study to evaluate the reproducibility and accuracy of this assay. This four-phased study will evaluate all 78 of the reference substances recommended by ICCVAM for validation of *in vitro* ER test methods. Phase I results demonstrated acceptable intralaboratory reproducibility and established quality controls for testing of coded reference substances in subsequent phases. In Phase II, repeat testing of coded reference substances covering a range of estrogenic activities was conducted in two stages (four substances in Phase IIa and eight substances in Phase IIb) to optimize agonist and antagonist protocols to be used for the testing of the remaining reference substances in Phases III and IV. A large number of tests failed one or more study acceptance criteria during Phase IIa (52% [46/88]). Therefore, the Study Management Team (SMT) recommended protocol modifications in order to increase plate acceptance without compromising the ability of the assay to detect and quantify agonist or antagonist activity. Phase IIb results indicated interlaboratory differences in the maximum concentration selected for evaluation, based on differences in perceived solubility and/or cytotoxicity. For some substances, this resulted in interlaboratory discordance in calls. The SMT subsequently recommended protocol modifications to better standardize these steps in the assay. These results underscore the importance of a phased study design to allow for necessary protocol refinements. ILS staff supported by NIEHS contract N01-ES-35504.

ID ABS: 586

ICCVAM recommendations for five *in vitro* pyrogen test methods

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Test systems based on the *in vitro* activation of human blood cells have been developed for pyrogenicity testing. Such methods utilize human whole blood, isolated primary monocytes, or a monocyte cell line, and are based on quantifying cytokine release to identify substances containing Gram-negative endotoxin. ICCVAM evaluated the validation status of five of these methods as potential replacements for the rabbit pyrogen test (RPT). In a multilaboratory validation study of 10 parenteral pharmaceuticals spiked with four concentrations of Gram-negative endotoxin, accuracy, false positive, and false negative rates ranged from 81-93%, 3-23%, and 1-27%, respectively. The methods were generally reproducible within and among testing laboratories. ICCVAM subsequently recommended that these *in vitro* test methods be considered to detect Gram-negative endotoxin in human parenteral drugs. Although none should be considered as a complete replacement for the RPT, these methods may be used on a case-by-case basis, subject to product-specific validation by the appropriate regulatory agency. When used in this manner, these methods should further reduce the number of animals needed for pyrogenicity testing. ICCVAM recommends that *in vitro* pyrogen tests be considered prior to testing in animals and that an alternative test method be used when deemed appropriate. ICCVAM forwarded these recommendations to U.S. Federal agencies along with recommendations for research and development needed to further expand their usefulness. Agency responses are due by mid-2009 and will be summarized. The views expressed above do not necessarily represent the official position of any government agency.

Theme 2: Areas of animal use

Breakout Sessions

Session BS11: Basic research

Concept of the 3Rs in basic research

J. Zurlo

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Basic scientific research provides the underpinnings and fundamental understanding of biological processes. In the case of basic biomedical science, most studies are conducted simply to increase our insight into basic biological functions, with longer-term goals of delineating the causes, prevention, and cures for human disease. In the past century or so, many basic research studies used laboratory animals as models for human disease. While much information has been gleaned from the use of animal models, our knowledge and development of sophisticated new technologies have enabled more extensive use of human materials, for example, biomarkers and human cells in culture. In this session, the speakers will provide examples of how each of the 3Rs may be applied to basic research. In some disciplines like neuroscience, it is more difficult to replace animals, but reduction and refinement play important roles. In other areas, it has become essential to work in human cell systems to pinpoint specific pathways unique to humans. The presenters will highlight their own work as well as that of others in the field.

Incorporating the 3Rs into basic mechanistic toxicology research

J. Yager and J. Bressler

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Studies to explore basic cellular processes and mechanisms of disease should be approached from the perspective of the 3Rs. This paradigm will be illustrated using two examples: investigation of the mechanisms of sporadic breast cancer and of factors that contribute to autism. Development of sporadic breast cancer is associated with persistent exposure to increased levels of endogenous estradiol/estrone (E2/E1). *In vitro* studies demonstrate that oxidative metabolism of E2/E1 to DNA damaging reactive quinones may contribute to estrogen carcinogenesis. Modification of this E2/E1 biotransformation process represents a potential target for chemoprevention. Investigations beginning with a rat model progressing to use of a human breast epithelial cell line will be described to illustrate use of the 3Rs paradigm for determining the effectiveness of a chemoprevention approach to reduce breast cancer risk. In autism, most of the behaviors cannot adequately be modeled in rodents because of the involvement of cortical structures unique to humans. Another approach is to model cellular events observed in autistic brains, for example abnormal brain growth, using cultures of human neural cells. Brain size depends upon cell number, dendritic arborization, axonal growth, and myelin biosynthesis. Rodent cell culture and human stem cells are being employed to examine these processes but human cells are more likely to detect chemicals harmful to humans. Indeed, thalidomide is associated with autism in humans but studies in rats indicated that it was safe. Investigators in many areas of basic science should consider conducting mechanistic studies in human cells rather than using animal models.

Three dimensional human tissue structures to study cellular differentiation

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Human multipotent mesenchymal stromal cells or mesenchymal stem cells (MSCs) are found in adult tissues such as bone marrow and are able to differentiate into osteogenic, chondrogenic and adipogenic tissues. There is intense interest in determining how MSCs may be used in future cell-based therapies, including gene therapy and tissue engineering, and as *in vitro* models for fundamental research and drug discovery. The intrinsic self-renewal and differentiation capacity of MSCs allows their use *in vitro* to establish three dimensional (3D) tissue-like structures, such as bone and cartilage, which mimic the *in vivo* environment. We have developed simplified methods for cultivating human MSCs under non-adherent conditions to promote cell-cell interactions to form micro-tissue-like structures. We have also engineered bi-differentiated osteo-chondrogenic spheroids with defined bone and cartilage features and used different biomimetic scaffolds to support osteogenic MSCs. Our 2D and 3D models of MSC growth have been used to determine pathways that regulate osteochondral differentiation using *in vitro* conditions that may reflect more accurately *in vivo* intercellular connectivity. In light of this, we have developed approaches for targeting gene disruption to allow the analysis of the effects of gene knockdown/knockout in tissue-like environments.

Viable alternatives: choosing the proper experimental model for neuroscience studies

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Choosing proper experimental models for neuroscience studies is difficult. Cell cultures, other *in vitro* preparations or non-animal models may be chosen. However, many neuroscience studies need an intact nervous system to control stages of experimentally induced responses.

The choice of neuroscience model systems involves many things. CIOMS Guidelines and U.S. Government Principles provide important tenets when models are selected for studies. When choosing models, consideration should be given to model appropriateness, relevance, minimization of animals when used, data quality, and results validity. Studies designed with proper experimental controls reduce variance. Good design can modify bad initial hypotheses.

Examples will be given that illustrate refinements to best practices that have lead to reductions in the number of animals used as well as reduction in animal pain and distress while under study. Ultimately these refinements, leading to reductions, may lead to replacements as investigators appreciate that valid results can be obtained with fewer animals and less sentient species.

C. F. M. Hendriksen suggests that the 3Rs be implemented with common sense, commitment, and communication. Doing so requires an understanding of experimental basics, blending these into the choice of models, and balancing animal welfare and scientific needs when designing and conducting neuroscience studies.

Restoration and maintenance of the *in vivo*-like hepatocellular homeostatic balance: key for the establishment of long-term liver-based *in vitro* models

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Primary hepatocyte cultures are considered as the golden standard in the field of liver-based *in vitro* modelling. However, long-term cultivation of primary hepatocytes is impaired by the progressive loss of the hepatocyte-specific phenotype. This dedifferentiation process is triggered during the isolation of hepatocytes from the liver and is associated with drastic homeostatic modifications, whereby proliferative activity and apoptosis are induced at the expense of the differentiated phenotype. A number of factors are known to affect the hepatocellular phenotype *in vivo*, including blood components, the extracellular matrix, and intercellular contacts. At a more upstream level, liver-specific gene expression is governed by a subset of liver-enriched tran-

scription factors. These *in vivo* issues will be outlined in a first part and it will be illustrated how these features deteriorate while setting up primary hepatocyte cultures. In a second part, a state of the art of the strategies to counteract hepatocyte dedifferentiation will be provided. These rely on mimicking the *in vivo* microenvironment and include the re-establishment of cell-cell and cell-extracellular matrix connections, and the addition of differentiation-promoting molecules to the culture medium. In recent years, a number of innovative approaches have been introduced that tackle the actual triggers of hepatocyte dedifferentiation, such as the (epi)genetic modification of gene expression in favour of the differentiated phenotype. These methodologies aim at a more direct and persistent restoration of the *in vivo*-like hepatocellular homeostatic balance and thereby provide an essential contribution in the way forward to the development of long-term liver-based *in vitro* models.

ID ABS: 492

Molecular and genomic characterization of estrogenic effects of 3-methylcholanthrene (3-MC)

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The two estrogen receptor isoforms ERa and ERb are ligand-inducible transcription factors that belong to the nuclear receptor (NR) superfamily. These receptors mediate the biological effects of endogenous estrogens like E2, but are also targets for a broad range of natural and synthetic compounds, including dietary compounds, pharmaceuticals and various types of environmental pollutants.

Endocrine disruptive compounds (EDCs) are chemicals that interfere with normal hormonal signaling. Many of these are ligands for the aryl hydrocarbon receptor (AhR) and consequently trigger AhR-mediated signaling. However, several crosstalk mechanisms between the AhR and NR members, e.g. the ERs, have been described.

Here, we compare the effects of the synthetic estrogen diethylstilbestrol (DES), the prototypical AhR agonist 3-methylcholanthrene (3-MC) and the environmental pollutant dioxin (TCDD) on estrogenic signaling. Using whole genome microarray analysis and gene expression profiling, we describe that these compounds regulate separate sets of genes, thus controlling distinct regulatory networks in HepG2 cells which stably express ER. Interestingly, we observe low degree of overlap exists between the genetic network activated following exposure to 3-MC or TCDD, two compounds considered as interchangeable in AhR studies. In addition, we identify novel ERa regulated target genes namely FST, UTRN and PAI-1 genes. The estrogenic effects exerted by 3-MC are exclusively observed in ERa-expressing cells and not in ERb cells, suggesting ER isoform selectivity. Moreover, when analyzed by chromatin immunoprecipitation (ChIP), enrichment of ERa, but not AhR, to ERE regions in IGFBP4 and GREB1 promoters could be observed in 3-MC-treated HepG2 cells.

Session BS12: Chemicals and pesticides

REACH and the need for intelligent testing strategies (ITS)

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The objectives of REACH cannot be achieved under the current risk assessment approach. A change in mind set among all the relevant stakeholders is needed: risk assessment should move away from a labor-intensive and animal-consuming approach to intelligent and pragmatic testing, by combining exposure and hazard data effectively and trying to group chemicals (category approaches and read-across). Intelligent Testing Strategies (ITS) rather than box ticking will be the challenge in the next decade, where more than 55,000 chemicals need to be evaluated. The focus should be on reducing the overall uncertainties of these 55,000 chemicals, while acknowledging the existence of the uncertainty paradox: reducing uncertainty in the assessment of individual chemicals following the classical chemical-by-chemical approach as we have in previous decades will result

in a prolongation of uncertainty for the entire group of >55,000 chemicals as a whole. With the first REACH registration deadline rapidly approaching (2010), a mind set change is urgently needed. We can speed up the regulatory acceptance process, starting with the maximum use of currently available exposure and hazard data, tools and models. Optimal use should also be made of experimental exposure and hazard data generated under REACH. Only such an approach will make it possible to obtain a sufficient level of information within the time frame of REACH. A much more intensive dialogue between stakeholders is necessary

Reduction of fish use in environmental hazard and risk assessment

G. Whale

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Companies have an obligation to ensure that materials used or produced in their operations and treated wastes released to the environment are assessed for their safety to protect the workforce, consumers, neighbours and the environment against adverse effects. In tandem with this, there is an increasing need to reduce laboratory animal experiments where other reliable means of establishing product safety and environmental safety are available. Consequently, many companies have committed to reduce the number of animals used in the evaluation of the safety of their materials and to support research into developing and validating alternate assessment methods. This includes the use of vertebrates in ecotoxicity tests and studies undertaken to assess the environmental safety of products, wastes and effluents.

This presentation provides an overview of how these commitments can be translated into action. As such, it provides an insight into the steps being taken by industry to monitor and reduce animal use for environmental assessments and an overview of inter-industry and international initiatives to support the development of new approaches to reduce the use of "protected stages" of vertebrate animals (predominantly fish) in environmental hazard and risk assessments.

Regulatory requirements for pesticide and biocide testing and scope for alternative approaches

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The current paradigm of toxicity testing used to assess potential risks of pesticide chemicals has been in place for many years. European Directives for pesticides and biocides (91/414/EEC and 98/8/EC) will be replaced by new regulations. According these, substances should only be authorised where it has been demonstrated that they are not expected to have any harmful effect on human or animal health. To avoid unnecessary use of animals, revisions of data requirements need to be consistent with requirements of other regulations.

The new European regulations will comply with the 3R principles, refining, reducing and replacing vertebrate tests where possible. Duplication of tests will be prohibited. The use of tiered approaches to toxicity testing is embraced in the draft data requirements, and should be further developed. Although the new data requirements will specify the use of validated standard test protocols, other methods may be used, if comprehensibly justified by the notifier. A less rigid approach to data requirements and more flexible and targeted selection of studies and study-designs will prohibit redundant testing.

Further, retrospective analysis of the contribution of individual tests of the current standard data requirement package – in particular, the 1-year dog study, the current multi-generation study, and the mouse carcinogenicity study is regarded crucial: analysis of existing extensive toxicity databases will play an important role in refining both, test methods and data requirements.

* presenting author

Initiatives to decrease redundancy in animal testing of pesticides

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The Agricultural Chemical Safety Assessment (ACSA) project was initiated by ILSI-HESI in 2000, involving academia, regulatory agencies and industry, with the purpose to evaluate more efficient ways to provide the necessary safety information for the registration of agrochemicals.

A review of publications on pesticides dealing with the need to investigate toxicity in dogs and the duration of such studies indicates the need for systemic toxicity tests in dogs. However, the value of a 12-month dog study in addition to a 3-month study is very limited, as three key publications independently conclude with the recommendation to limit dog testing with pesticides to 3-month studies.

The value of the second species, mouse cancer bioassay has also been evaluated by ACSA and more recently by toxicologists from regulatory agencies and industry. Both reviews conclude that it does not contribute significant additional information over and above that provided by the carcinogenicity study in the rat.

The two-generation (OECD 416) rat study is the study which uses most animals. It has been proposed by ACSA that this study could be replaced by an extended one-generation study within an intelligent testing strategy: a combination of default (core) components and optional additional evaluations (modules) that are triggered or waived in light of available data or to fulfill information requirements within a given regulatory framework. Thereby, the reduction in animal usage that may be achieved with an extended one-generation study design is substantial, but not attained at the expense of data generation for risk assessment purposes.

Strategies for reproductive and developmental toxicity assessment

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The area of reproductive and developmental toxicity testing provides numerous opportunities for application of 3Rs. In terms of reduction of animal numbers, there are a number of initiatives to make use of screening-level *in vivo* assays (such as OECD 421/2), or to more routinely use one-generation instead of two-generation studies to evaluate reproduction. Proposed refinements to the one-generation assay will also generate more data from a single study, further reducing the number of animals needed to fully characterize toxicity. Beyond simply reducing animal numbers, there are opportunities to take advantage of our increasing understanding of the mechanistic aspects of reproductive toxicity to design more hypothesis-driven approaches to safety assessment that rely more on *in vitro*, *in silico*, and toxicogenomic approaches. Examples of *in silico* approaches are read-across and other SAR-based methods that rely on existing toxicology data sets such as DSSTox that can be searched by chemical structure or substructure to identify analogs to new chemicals that will inform us about the potential toxicity of the new chemicals. Examples of *in vitro* approaches include research that takes advantage of the strong phylogenetic conservation of developmental signaling pathways, which may make it possible to use the embryos of non-mammalian organisms to more accurately predict human teratogenic potential. Toxicogenomic approaches can be used to identify all possible modes of toxicity in a target tissue, permitting the prediction of toxic outcome *in vivo*. These strategies will support movement away from rote testing approaches to a more informed approach to reproductive toxicity testing.

ID ABS: 544

An examination of new chemical regulation policies as a means to revolutionize toxicity testing

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Revision of the main US law governing industrial chemicals, the Toxic Substances Control Act (TSCA), is likely to happen in the near future. At the same time a desire to move away from current testing methods for ethical, scientific, and practical reasons has led to multi-million dollar investments in *in vitro* and computational toxicology methods and programs. Such in-

vestment has been endorsed by multiple scientific bodies, most comprehensively by the US National Academies of Science in its 2007 report, Toxicity Testing in the 21st Century: A Vision and a Strategy. While the field of toxicology transitions from an observational to a predictive science, it is essential that new legislation governing the regulation of chemicals remains open to incorporating integrated approaches to assessment of chemical hazards. We examine the available programmatic options specifically related to TSCA, including proposed legislation such as the Kid Safe Chemicals Act, the EPA's Chemical Assessment and Management Program (ChAMP), and an Intelligent Testing Strategy approach consistent with the NRC vision and the US EPA Strategic Plan for Evaluating the Toxicity of Chemicals. Two main outcomes focus the analysis: first, the numbers of animals used in any of these options are estimated and compared. Second, the potential effectiveness of these three approaches is assessed in numbers of chemicals evaluated per year and after 10 years. Quantitative analyses like these are essential to judiciously select policies that reduce the use of animals in toxicity testing and protect human health and the environment.

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Session BS13: Cosmetics

Challenges and progress in development of *in vitro* assays for eye irritation safety evaluation: a COLIPA update

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An extensive number of *in vitro* assays, many of which find applications in industry, have been developed and proposed as alternatives to the Draize test. Although several have been included in major validation/evaluation studies, none have met all the criteria to replace the Draize test. This has been attributed in part to a lack of understanding of physiological mechanisms of eye irritation. Furthermore, it is generally accepted that combinations of *in vitro* assays will be required to fully replace the Draize test. COLIPA's eye irritation programme is focused on identification of new *in vitro* endpoints more predictive of the *in vivo* human response to chemical injury through understanding mechanisms of eye injury/recovery. A key project on method development/optimisation has focused on Reconstructed Human Tissue assays using human corneal models, enabling these methods to enter a prospective validation study with ECVAM. The research programme focus is the availability of models that address depth of injury/recovery as a mechanistic basis for eye irritation. Ongoing work is focused on continued development/optimisation of multilayer ocular models, including isolated eyes, isolated corneas and bioengineered corneal constructs, and incorporation of evaluation parameters that measure depth of injury. Knowledge gained from these activities will be used to help define combinations of *in vitro* assays that could evaluate eye irritation across the range of irritancy for different chemical classes. In overview, this presentation will discuss progress on *in vitro* assays development to validation and the cosmetics industry's approach to the use of *in vitro* assays for safety assessment of its ingredients/products.

The COLIPA strategy for animal-free genotoxicity testing

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From the 11th March this year, the first of the two deadlines to the 7th Amendment to the Cosmetics Directive came into effect. This implemented a ban on genotoxicity testing of chemical ingredients in cosmetics using animals and has perhaps had less focus from an animal alternatives standpoint, because sensitive *in vitro* genetic toxicology assays already exist. However, it has become clear that 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). Where, in the past, this would have created the need for unnecessary *in vivo* follow up testing, the cosmetics industry is now left with the erroneous rejection of valuable new ingredients.

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: (i) a three year project performed at Covance Laboratories (UK) to optimize current mammalian cell assays in order to improve specificity, (ii) a two and a half year project led by member companies of the cosmetics industry, which aims to establish and validate new methods for genotoxicity testing using reconstructed human 3D skin models, and (iii) research into the metabolic capacity of human skin and 3D models.

Skin sensitization: the COLIPA strategy for developing and evaluating non-animal test methods for risk assessment

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Allergic contact dermatitis is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). Currently, the sensitizing potential and potency of new chemicals is usually characterized using data generated via animal studies, such as the local lymph node assay (LLNA). There are, however, increasing public and political concerns regarding the use of animals for the testing of new chemicals. Consequently, the development of *in vitro*, *in chemico* or *in silico* models for predicting the sensitizing potential and/or potency of new chemicals is receiving widespread interest.

The COLIPA Skin Tolerance Task Force currently collaborates with and/or funds several academic research groups to expand our understanding of the molecular and cellular events occurring during the acquisition of skin sensitization. Knowledge gained from this research is being used to support the development and evaluation of novel alternative approaches for the identification and characterization of skin sensitizing chemicals. At present three non-animal test methods (direct peptide reactivity assay (DPRA), Myeloid U937 Skin Sensitization Test (MUSST) and human cell line activation test (hCLAT)) have been evaluated via COLIPA interlaboratory ring trials for their potential to predict skin sensitization potential and were recently submitted to ECVAM for formal pre-validation. Data from all three test methods will now be used to support the study and development of testing strategy approaches for skin sensitizer potency prediction.

In summary, this presentation represents the current viewpoint of the cosmetics industry on the feasibility of replacing the need for animal test data for informing skin sensitization risk assessment decisions.

The use of *in silico*/QSAR approaches to predict toxicity and fate

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The 7th Amendment to the EU Cosmetics Directive has made developing *in vitro* and *in silico* approaches to assure product safety a key business need to support future innovation. Over the past years there have been considerable efforts to develop *in silico* methods that would comply with regulatory constraints (OECD principles). In this context, transparent and mechanism-based modeling approaches are needed. Biodegradation and bioaccumulation will be exemplified as fate endpoints, whereas skin sensitization and *in vitro* genotoxicity (AMES and chromosomal aberration) will be demonstrated as toxicity endpoints. Environmental degradation pathways and magnitude of stable degradants need to be predicted when biodegradation is simulated. Fish liver metabolism appears to be an important factor when the bioconcentration factor is modeled. Similarly, molecular transformations in skin and rat liver metabolism are critical for adequate modeling of skin sensitization and genotoxicity, respectively. Predictions of these endpoints is extremely important in an early stage of the product development program, where multiple candidate products are evaluated based on their structure and physiochemical properties to support identification of lead product/formulation, at the same time minimizing the risk for environment and human health. The (Q)SAR methods will be discussed in terms of their mechanistic interpretation and applicability domain, which are critical for their regulatory application. The role of chemical categories will be discussed in relation to read-across approach. A case study will be illustrated where the outcome of the traditional (Q)SAR methods will be combined with categorization and subsequent read-across in collecting weight of evidence for decision support. Capabilities of *in silico* methods for selecting chemicals for strategic testing will be demonstrated using phototoxicity.

Experience on product safety assessment within the cosmetic industry

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In March 2009, the 7th Amendment to the European Cosmetic Directive imposed a ban on animal testing for the genotoxicity, acute toxicity, ocular and skin irritancy toxicological endpoints. This major change implied a shift to alternative ways of assessing the safety of cosmetic ingredients on these specific toxicological endpoints and, consequently, led us to refine our current Safety Evaluation paradigm.

A multidisciplinary team at L'Oréal has worked to develop and implement a new safety evaluation paradigm. This effort went through a stepwise process including:

- Evaluation of the performances of the predictive methods available on a significant number of cosmetic ingredients,
- Identification of the applicability domains of these methods in order to fit the chemical diversity that the cosmetic industry is facing,
- To define our own Integrated Testing Strategies, i.e. approaches that integrate different types of data and information (read across, *in silico* models, physical-chemical characterization and *in vitro* methods and assays) into the decision-making process
- Implementation of these Integrated Testing Strategies for routine safety assessment of new ingredients.

The outcome of this strategy and the current status of implementation of the new safety evaluation paradigm will be presented and discussed during the lecture.

Use of alternative methods in the safety evaluation of cosmetic products

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Cosmetic products marketed in the United States are regulated by the Food and Drug Administration (FDA) in accordance with the requirements of the Federal Food, Drug, and Cosmetic Act and other laws including the Fair Packaging and Labeling Act. FDA does not have the statutory authority to approve cosmetic products or their labels before marketing or to require pre-market notification to the Agency of finished cosmetic products and their ingredients. Although pre-market approval of cosmetics is not required, industry safety evaluation is not voluntary. It is the manufacturer's responsibility to assure that their products

are safe and that they comply fully with all applicable laws and regulations enforced by FDA prior to marketing. FDA supports the use of validated alternatives to animal testing as a means of testing for safety. In addition, FDA is actively engaged in the alternatives arena through participation in multiple organizations including the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) and the Institute for *In vitro* Sciences (IIVS). FDA is also an active member of the International Cooperation on Cosmetics Regulation (ICCR), which is a voluntary international group of regulatory authorities from the United States, Japan, the European Union, and Canada. ICCR was formed to establish better cooperation and communication among regulators and industry on topics of mutual interest including alternatives to animal testing. Through these endeavors, FDA supports activities for the international regulatory acceptance of non-animal alternatives for cosmetic products.

International regulatory acceptance for non-animal alternatives in the area of cosmetics regulation

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From a European perspective international acceptance of non-animal alternatives is crucial in the light of the testing and marketing bans under the Cosmetics Directive. The ban on testing of finished cosmetic products has been in force since 11 September 2004, the ban on testing ingredients or combination of ingredients has become effective as of 11 March 2009, irrespective of the availability of alternative non-animal tests. The marketing ban has become effective on 11 March 2009 for all human health effects with the exception of repeated-dose toxicity, reproductive toxicity and toxicokinetics. For these specific health effects, a deadline of 10 years after entry into force of the Directive is laid down, i.e. 11 March 2013, regardless of the availability of alternative non-animal tests.

The process towards regulatory acceptance of alternative methods essentially consists of two stages: the scientific peer review of a validation study ("validation") and its regulatory acceptance by the regulator/legislator. Regulatory acceptance usually follows the recommendation of the validation stage. It is therefore crucial to ensure that, at the validation stage, the assessments internationally do not differ. The Commission therefore promotes intensified cooperation to facilitate the development and the validation of alternative methods and their recognition by our main partners. A framework of scientific cooperation has been established between the US, Japan and Canada, in a multilateral forum called "International Cooperation on Cosmetic Regulation" (ICCR) and has led to the ICCR subgroup called "International Cooperation on Alternative Test Methods" (ICATM).

Looking ahead in Europe efforts will need to concentrate on the outstanding endpoints for the 2009 deadline and on the much more challenging endpoints for the 2013 deadline.

Regulatory requirements for cosmetics in the EU and status of non-animal tests

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Regulatory requirements in EU:

-Cosmetic products must be safe and must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use.

-The responsibility is placed upon the manufacturer, marketeer or person placing the product on the EU market.

-For finished products, a qualified safety assessor must undersign the safety assessment based on the chemical structure, toxicological profile and exposure of the ingredients. For ingredients present on the Annexes of Dir 93/35/EEC, the SCCS undertakes safety assessment when mandated and advices the Commission, responsible for risk management.

-Safety compliance is controlled by means of a post-marketing surveillance system.

-Safety must be guaranteed through validated non-animal alternative tests. Clear testing and marketing deadlines exist.

Status of validated non-animal tests:

Validated Replacement Tests

Skin corrosivity: TER, EpiSkin, EpiDerm

Skin irritation: EpiSkin, modified EpiDermSIT, SkinEthic RHE

Eye irritation: screening for strong/severe irritants (BCOP, IRE, ICE, HET-CAM)

Phototoxicity: 3T3 NRU PT

Dermal absorption *in vitro*: human/pig skin

Mutagenicity/genotoxicity: bacterial reverse mutation; *in vitro* mammalian cell gene mutation; *in vitro* micronucleus; *in vitro* mammalian chromosome aberration

Embryotoxicity: WEC, MM, EST

Validated Reduction/Refinement Tests
Acute oral toxicity: fixed dose; acute toxic class; up-and-down
Skin sensitisation: LLNA
Lacking Validated Replacement Tests
Acute dermal and inhalation toxicity; eye irritation, skin sensitisation; photoallergy; subacute and subchronic toxicity; chronic toxicity; target organ and systemic toxicity; (non-genotoxic) carcinogenicity; biokinetics

Barriers for regulatory acceptance:
Testing and marketing deadlines; validation process: time consuming and costly; lacking of specific applicability domains; trust building process; in use experience and post-marketing knowledge; overselling.
Facilitators:
Collaboration between different industries; early involvement of regulators; better communication and dissemination of 3Rs information; clear indication of limitations of applicability; global harmonisation; education and training in 3Rs.

Review of an alternative to animal testing for safety evaluation of cosmetic ingredients using quasi-drug

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Since 2007, JaCVAM (Japanese Center for the Validation of Alternative Methods) has had the research project H19-Drug-003 Research on establishment of a system of safety evaluation and international cooperation using alternatives to animal testing (Chief researcher: Hajime Kojima, NIHS; the National Institute of Health Sciences) supported by the MHLW (the Ministry of Health, Labour and Welfare). In the mission statement for this project there is the Review of alternatives to animal testing for the safety evaluation of cosmetic ingredients using Quasi-drug. To enforce this mission, JaCVAM has organized an ad hoc exploratory committee. Its members were organized by dermatologists, regulators, representatives of the JCIA (Japanese Cosmetic Industry Association), and researchers of the NIHS. Furthermore, six task forces were organized under the exploratory committee to investigate a detailed system of merits & demerits for *in vitro* tests using safety evaluations of the ingredients by Quasi-drug. These task forces independently reviewed in vitro testing methods for skin irritation, skin sensitization, eye irritation, phototoxicity, genotoxicity, and skin penetration & absorption. This year, research group will make overall discussions on the use of alternative methods for the safety evaluation of cosmetic ingredients using Quasi-drug based on the reports submitted from the task forces. The Japanese main stance is a need for Japan to develop alternative methods. However, people only use cosmetics when they know they are safe. For the reason, evaluation of those products has to be done in a careful manner

Session BS14: Pharmaceuticals

What did we learn up to now from a project such as START-UP?

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START-UP (Scientific and technological issues in 3R alternatives research in the process of drug development and Union politics) is a FP7 EU support action project coordinated by ecopa (European Consensus Platform on 3R-Alternatives), having 16 National Consensus Platforms among its members, each representing the crucial stakeholders (animal welfare, academia, industry, governmental organisations).

The project aims at identifying 3R-bottlenecks in drug development and rate limiting steps on the political, scientific and technological level, with the ultimate goal of seeing whether safety and efficacy of new compounds can be improved by a better implementation of 3R-alternatives.

Three pilot meetings with the pharmaceutical industry, regulators and young scientists took place and provided key information to be loaded into 3 workshops, one on each R.

It became clear that too much focus was on one-to-one Replacement, on regulatory toxicity testing and on chemicals. Refinement and Reduction were often left out of the picture, and so were pharmaceuticals. START-UP opens the way for a new kind of alternative test need identification and solutions, namely ask the experts and have a balanced NGO get the real issues identified. These are concerned with:

- refinement of animal disease models;
- directing transgenics to industrial needs;
- careful evaluation of "promising" alternatives;
- *in vitro* biomarkers of clinical relevance;
- efficacy and safety testing of biopharmaceuticals;
- applicability domain of existing alternatives.

Adherence to the 3R principles in the ICH process

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The international harmonization of testing guidelines and the mutual regulatory acceptance of test data is a very important measure to obviate duplication of animal testing for the same substance due to geographical differences in testing requirements and thus to reduce animal testing worldwide. In this respect, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutcials for Human Use (ICH) had and still has a significant impact on reducing animal use in regulatory pharmaceutical testing. Since 1990, experts from the regulatory authorities and pharmaceutical industry of Japan, Europe and the US have been working to harmonize safety, quality, and efficacy testing procedures for human drug product registration. The ICH Safety guidelines cover most areas of non-clinical/toxicological assessment, including testing for carcinogenicity, genotoxicity, reproductive toxicity, chronic toxicity, immunotoxicity etc.

More recently the European Commission requested the European Medicines Agency (EMEA) to conduct a review of the non-clinical ICH guidelines to see whether they were up-to-date, in particular with regard to new developments to reduce, refine or replace animal studies. This initiative led to a still ongoing process of revision of some of the existing ICH guidelines. In addition, a first meeting took place between the non-clincal safety experts working within ICH and the organizations for "Validation of Alternative Methods" in the three ICH regions, i.e. ECVAM, ICCVAM and JaCVAM, looking for ways of cooperation and sharing of information about existing replacement, reduction and refinement methods and their acceptance by regulatory authorities.

Implementation of the 3Rs in the European pharmaceutical industry

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The role of the pharmaceutical industry is to develop innovative, safe and efficient medicines. While biomedical researchers use animals to understand the different disease processes, highly regulated product safety testing still imposes studies in animals, since the maximal possible safety must be ensured prior to clinical testing.

The pharmaceutical industry faces considerable challenges, of which one is related to the low success rate of new chemical entities. In this context, it was reported (Kola and Landis, 2004) that the major causes of attrition were lack of efficacy (±30%) and lack of safety (±30%). This presentation will focus on the latter and will discuss the possible impact of the implementation of *in vitro* test methods within drug development. It is important to realize that reducing toxicity-related attrition should be in place from the earliest stages of the discovery phase. Examples within the context of genetic and exploratory toxicology, in line with the 3R concept, will be shown.

When implementing alternatives to animal experimentation, different drivers must be considered: besides ethical motivations, predictivity (risk assessment), required resources (throughput), test material availability and regulatory demands become important. In order to secure efficient hazard identification and human risk assessment, the novel test methodologies need to be standardized, formally validated and integrated into test strategies. This requires further collaboration between different industry sectors, academia and governmental (validation) organisations.

Current situation of alternatives to animal testing in Japanese pharmaceutical industry

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In Japan, the social environment of laboratory animals and animal experiments has drastically changed in the past several years. With the amendment of the Law for the Humane Treatment and Management of Animals, the specifications for the use of animals for scientific purposes have been revised, and the 3Rs principle has been given legal standing. The amended law came into force in 2006. Around that time, the Ministry of Health, Labor and Welfare (MHLW), which had jurisdiction over the pharmaceutical industry, established a basic guideline for animal experimentation. In addition, the guideline for the animal experiments was also established by the Science Council of Japan. These guidelines stipulate that each pharmaceutical institute must establish an institutional animal care and use committee (IACUC) and review the animal experiment protocols. Under such circumstances, the consciousness of researchers for the alternatives to animal testing has changed greatly, and many pharmaceutical companies have promoted the introduction of alternative methods in animal studies. In addition, the MHLW established the Japanese Center for the Validation of Alternative Methods (JaCVAM) in Japan's National Institute of Health Science in 2005 and currently supports study and implementation of alternatives in the pharmaceutical industry. However, no pharmaceutical company yet has an organization for alternative studies, while cosmetic companies do. The Japanese pharmaceutical industry needs to carry out more education of the researchers, and it is necessary for them to wrestle with development and implementation of the alternative methods systematically in cooperation with academia and the government.

How we use alternatives in the U.S., and why

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To understand the context of alternative model use in the pharmaceutical industry, one must recognize that the drivers here are 1) the accurate prediction of what will happen *in vivo*, 2) speed, 3) the capability to screen many compounds quickly (throughput), and 4) the ability to deliver an answer using small amounts of compound. Overall, we use the simplest system that is biologically plausible and that makes sense. In drug discovery, a new drug target is transfected into a tractable cell line so that screening of drug substructures can occur rapidly. In drug safety, genotoxic mechanisms (base exchange, frameshift

mutations) are assessed in bacteria and tests of clastogenicity use Chinese Hamster Ovary cells. Similarly, binding to specific channel proteins can be addressed in cell lines (hERG channels, MDR pump binding uses CaCo2's). Mouse embryonic stem cells are used to predict developmental toxicity. Zebrafish are the new darlings of toxicology, because of their speed of development, shared gene expression with mammals, and the small amount of compounds required for a test. They are being evaluated for everything from predictive developmental toxicity to cardiovascular function to integrated CNS function. Finally, the "Toxicity Testing in the 21st Century" book from the US National Academies gives us all a more concentrated and a more comprehensive framework in which to work. The biggest challenge is assuring safety of biologicals while minimizing the use of primates. Thus, while driven more by pragmatic concerns, the use of alternatives in the U.S. pharmaceuticals sector is alive and quite well.

ID ABS 558

Human tissue pharmacology: a true animal alternative

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There have been a number of high profile failures in the drug development arena in recent years. Despite approximately 10 years and $200 million of lead candidate investment, new chemical entities with potentially fatal secondary pharmacodynamics continue to reach patient populations. In a time where the number of animal experiments continues to rise, the need for true translational alternatives has never been greater.

Functional assays in fresh residual human tissue ethically obtained from surgical procedures represent the closest model to human *in vivo* function because species variation is avoided and target patient populations can be assessed. Such tests allow the generation of real human proof of concept data much earlier in the development process. For example, vascular tissue can be used to screen an anti-hypertensive drug, or intestinal tissue can be used to screen drugs for inflammatory bowel disease. In a similar way, certain tissues are useful screening tools for the prediction of off-target effects, for example, isolated airways can be used to detect unwanted bronchoconstriction and cardiac tissue can be used to assess the risk of arrhythmias.

Using real examples from cardiovascular, inflammation and permeability experiments we describe how data obtained from human tissue (both healthy and diseased) can reduce the amount of animal experiments and ultimately reduce the risk and cost associated with drug development process.

Session BS15: Food improving agents

A detailed description of QSAR and TTC and their application to a variety of toxicology endpoints for food additives

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Over the past 10 years, the FDA's Center for Food Safety and Applied Nutrition, Office of Food Additive Safety (OFAS), in collaboration with the FDA's Center for Drug Evaluation and Research Informatics and Computational Safety Analysis Staff, has, through various cooperative research and development agreements, acquired access to several quantitative structure activity relationship (QSAR) programs and chemoinformatics systems. This software is being used in the evaluation of new food contact materials, food additives, dietary ingredients, impurities and breakdown products. An important aspect to creating better QSAR models is the identification and incorporation of high quality toxicity data into QSAR training sets. To this

end, OFAS has begun to capture its historical data in a structurally searchable format and to incorporate this information into existing QSAR models and a new food additives knowledge base. The food additives knowledge base is an electronic representation of structural and biological rules derived from both OFAS's historical data as well as public sources of toxicity information. The knowledge base consists of modules of structural alerts and chemical class-driven QSAR models. The structural alerts/chemical classes along with threshold of toxicological concern (TTC) values are stratified across multiple toxicity endpoints allowing for the pre- and post-market evaluation of food additives under a TTC paradigm. Knowledge derived from this evaluation can be used to eliminate unnecessary toxicity testing or to identify new safety concerns as new exposure and toxicity data are incorporated into the model. The knowledge base modules are designed to be delivered within a configurable workflow management tool.

In vivo approaches to simultaneously assess both genotoxicity and carcinogenicity of food additives

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For the safety evaluation of flavoring agents, both 90-day repeated dose toxicity and genotoxicity studies are required in Japan, although such agents have been evaluated on TTC basis in JECFA separately from the other food additives. Recently, even the JECFA procedure could not be applied to some flavoring agents, such as furan- or alkoxy-substituted chemicals, because of the unsolved carcinogenic potentials. In general, it is sometimes difficult to discriminate genotoxic and non-genotoxic carcinogens, because each assay is carried out separately. Both assays are basically independent of each other, which raises a simple query as to how much the detected genotoxic potential can contribute to carcinogenicity. To solve such issues, we have studied the mechanisms of action of carcinogens in transgenic rodents carrying reporter genes, which are expected to provide powerful tools for the evaluation of both genotoxicity and carcinogenicity at the same organ level. For example, several comparative studies of genotoxic carcinogens, such as environmental pollutants, nitrosamines and heterocyclic amines in these transgenic animals have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity. These results clearly indicate that understanding of the detailed mechanism of carcinogenic action could be crucial for more precise risk assessment. Bioassay systems, such as the 90-day repeated dose toxicity study using transgenic rodents carrying reporter genes, would be extremely useful for the purpose of simultaneously assessing both *in vivo* genotoxicity and carcinogenicity, which could contribute to 3Rs.

An industry perspective on alternative methods to establish level of safety concern of food chemicals in absence of sufficient toxicological data

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Over recent years, there has been mounting concern about food as a source of exposure to potentially toxic chemicals. It has been estimated that there are over five millions man-made chemicals known, of which 70,000 are in use today. Furthermore, there are about 100,000 naturally occurring chemicals of known structure. Since for the vast majority of these chemicals, toxicological information is absent or limited, the assessment of their health significance is therefore difficult. In such situations, the availability of reliable tools allowing establishing levels of safety concern appear of particular importance to ensure adequate consumer protection without undue over-conservatism.

Solutions to this general issue are not straightforward. In this context, *in silico* predictive models have obvious advantages in terms of time, cost and also animal protection. Ideally, such models should predict safe levels of exposure. Promising models have been developed to predict animal chronic toxicity. For chemicals expected to act through a threshold mechanism, the size of the margin between the actual exposure and the predicted chronic toxicity (margin of exposure, MoE) could be used for decision making. The MoE should account for potential interspecies and intraspecies differences as well as uncertainties related to the performance of the models used. Since genotoxic carcinogens are considered to act through a non-threshold mechanism, the interpretation of MoEs would currently be much more questionable. Therefore, the efficient application of an *in silico* strategy in food safety requires the possibility to differentiate

DNA-reactive genotoxic carcinogens from other chemicals.

A battery of *in silico* toxicology tools may also be envisaged to establish the level of safety concern associated with putative new food additive candidates and key constituents of toxicologically uncharacterized plant extracts. Such an approach would likely be valuable for early decision making in new product development (to screen out candidates) and to decide on the need for toxicological studies.

Session BS16: Genetically modified organisms

Safety assessment of GMOs and derived food and feed: the role of animal feeding trials and alternative methods

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The various elements of the safety and nutritional assessment procedure for genetically modified (GM) plant derived food and feed will be discussed, in particular the potential and limitations of animal feeding trials for the safety and nutritional testing of whole GM food and feed. General principles for the risk assessment of GM plants and derived food and feed are presented, as described in the Guidance Document of the EFSA Scientific Panel on genetically modified organisms.

Toxicological *in vivo*, *in silico*, and *in vitro* test methods will be discussed, which may be applied for the safety and nutritional assessment of single, specific compounds present in food and feed derived from GM plants. Testing of the safety and nutritional value of the whole GM plant or derived food and feed should be considered where the molecular, compositional, phenotypic, agronomic and other analyses have demonstrated differences between the GM plant derived food and feed and their conventional counterpart, apart from the inserted trait(s), or if there are any indications or remaining uncertainties for the potential occurrence of unintended effects. In such a case, the testing program should include at least a 90-day rodent feeding study.

The sensitivity and specificity of such a test model will be discussed. There is a need for a more uniform approach to the design and analysis of animal feeding trials, and in particular for appropriate statistical analysis of data. Furthermore, alternative strategies in order to reduce, replace or complement animal testing of whole GM food/feed will be analyzed.

Safety testing of GM crops for human and animal consumption

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Food safety assessment strategies for GMOs used by developers are based on international guidelines (Codex, WHO, EFSA, etc.). These assessments include evaluating the safety of introduced proteins, as well as intended and potential unintended changes in the whole food. For whole foods with intentional composition changes, such as introducing heart healthy long chain omega fatty acids into vegetable oils, the safety assessment can focus on the changes in fatty acid content. International guidelines recommend agronomic and compositional assessments as powerful tools to detect potential unintended changes in biotech crops. A 90 day rat toxicology study with the whole food has also been utilized case-by-case to provide additional confirmation of food safety. Even though these studies are conducted under GLPs and accepted by international regulatory agencies, they are sometimes repeated by in-country laboratories which raises questions about the inappropriate use

of research animals. Moreover, EFSA has concluded that such studies generally provide little useful information when analytical methods have already confirmed food safety. Furthermore, biological systems are generally less sensitive than analytical methods to detect changes in endogenous nutrients or toxicants. Genomics, proteomics and metabolomics profiling technologies have been proposed as alternatives to animal studies because they can make many more comparisons between conventional and GM crops. However, they do not help to interpret the potential health consequences of minor changes in food composition. Furthermore, limitations of profiling technologies include a lack of quantitative rigor, difficulties in identifying analytes, and a lack of baseline information on natural variability on food composition.

Latest developments in allergenicity testing of GMOs

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The pre-market assessment of GMOs includes the assessment of the allergenicity of both the newly expressed proteins and the whole GM food. They should be assessed for both the capacity to trigger an allergic reaction in individuals already sensitized to cross reactive proteins and to *de novo* sensitize predisposed individuals.

The development of bioinformatics tools and of allergen databases has increased the accuracy of identification of structural similarity between a novel protein and known allergens. In the case of indication of such similarity and also when there is a possible impact of the genetic modification on the qualitative and quantitative pattern of expression of endogenous allergens in the GMO, additional analyses should be performed, including IgE binding studies. Normally, sera from allergic humans should be used, which may be difficult. Sera from animals experimentally sensitized in well defined conditions, using different procedures, could provide a substitute for a preliminary screening.

The potential of novel proteins or whole GM food to *de novo* sensitize predisposed individuals cannot be assessed on humans and no single animal model can provide conclusive information. However, if there are indications from the structure of the protein or the origin of the food that a risk of sensitization exists, a combination of *in vivo* tests in various strains of animals and conditions of exposure may provide useful information on the likelihood of such potential. Animal models may also allow maximizing the risk or reproducing particular conditions of exposure in some at risk groups of the population.

Omics approaches for the safety assessment of GMOs

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Risk assessment frameworks, such as those used for GM crops, have detailed comparative analysis with appropriate non-GM counterparts as their cornerstone. Opinions have been voiced that current analytical approaches are too specific and need to be complemented by more unbiased, larger scale analysis of gene and protein expression using transcriptomics and proteomics, respectively. In parallel, the use of metabolomics has been advocated as an approach to expand significantly the range of metabolites that can be measured to assess more stringently the potential for any unintended effects cause by the genetic engineering process. Transcriptomics, proteomics and metabolomics have been termed collectively omics technologies. Omics outputs are extremely data rich and are already providing useful information on the major factors influencing variation in gene, protein and metabolite expression. These include crop management practices, genotype, interactions between genotype and growing environment and the nature of the breeding approach used. This information on sources and extents of natural variation provides an important benchmark for risk assessors when examining the safety of GM crops compared with their non GM counterparts. Omics technologies are also being used with *in vitro* and *ex vivo* model mammalian systems to provide insights into potential toxicity issues, for example, and could offer a route to reducing the need for animal testing per se. The paper will examine the potential for deploying use of omics approaches in the saftey assessment of GMOs.

Session BS17: Nanomaterial toxicity testing

Cell culture models of the epithelial airway barrier to study the toxic potential of nanoparticles: comparison to *in vivo* models

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Although many structural and functional barriers exist in the lung, a number of epidemiological studies have indicated that inhalation of particulate matter can cause increased pulmonary morbidity and mortality. It is the smallest fraction of particulate matter, known as combustion-derived nanoparticles (NPs) (<0.1μm in diameter), which are particularly harmful. The potential adverse effects of exposure to NPs is of great concern due to the constantly increasing production of engineered NPs as well as the accidental release of NPs into the environment in relation to the nanotechnology industry.

To assess the potential adverse effects of NPs, many cell culture models have recently been developed in a standardized and well-characterised environment. As the lung is known to be the first target organ following NP exposure, a number of lung cell culture models have been created, which may help to elucidate the mechanisms of how particles that are inhaled and deposited on the lung surface can interact with the cells and induce cellular responses. In relation to this, a 3-dimensional model of the epithelial airway barrier including several cell types (epithelial cells, monocyte-derived macrophages and dendritic cells) barrier will be presented.

Although *in vitro* models exhibit a number of limitations, they can be used for the screening of large numbers of newly developed NPs within a short time at a relatively low-cost compared to *in vivo* analysis. Considering all the limitations when working with cell culture models, they might be a great tool to perform basic research including toxicity tests.

Genotoxicity of nanoparticles – comparison of *in vivo* to *in vitro* models

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Genotoxicity assays have been introduced to allow for improved cancer risk assessment strategies. Nanopartices (NP) form a rather specific group of compounds, whereby mechanisms of DNA damage induction have been shown to depend on their physicochemical properties, such as size, surface reactivity and solubility. For the evaluation of genotoxicity of NP both *in vitro* and *in vivo* models are available. *In vivo* genotoxicity studies have shown that NP can cause DNA damage and mutagenicity in the lung epithelium of rats. However, significant genotoxic effects have been observed only at exposure conditions in which also significant pulmonary inflammation was induced. These findings relate to the concept of secondary genotoxicity in particle risk assessment, whereby genotoxicity is considered to result from inflammatory cell-derived reactive oxygen species (ROS). In contrast, primary genotoxicity is defined to be caused by particles in the absence of inflammation. Secondary genotoxicity is considered to involve a threshold, determined by the exposure concentration that will trigger inflammation and overwhelm antioxidant and DNA damage repair capacities in the lung. This concept will be discussed along currently available *in vitro* models to screen for the genotoxicity as well as inflammatory potency of NP.

Use of *in vitro* and inhalation models for assessment of nanoparticle effects on lung cells

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Nanomaterials are respirable in humans and may have the potential, based upon their geometry, composition, size and durability, to cause adverse effects in lungs, especially if they are inhaled at high concentrations. Physiologically relevant inhalation studies to predict the toxicity and pathogenicity of nanomaterials are prohibitive in terms of time and expense. For these reasons, a panel of *in vitro* assays using established lines of human or rodent lung epithelial and mesothelial cells, target cells of lung cancers and mesotheliomas, can be evaluated to determine the cytotoxicity (altered metabolism, decreased growth, lytic or apoptotic cell death), proliferation, genotoxicity, and altered gene expression or methylation. These assays should include pathogenic minerals (asbestos fibers) as positive controls and nonpathogenic particles (fine titanium dioxide or glass beads) as negative controls. Recent work emphasizes the importance of microarray profiles in human mesothelial cells *in vitro* that can discriminate between asbestos fibers and noncarcinogenic particles (fine TiO_2, platy talc, glass beads) (Shukla et al., *Am. J. Respir. Cell Mol. Biol.*, Dec 18[Epub ahead of print], 2008). This data base can be compared to gene profiling in lungs of rodents inhaling respirable fibers, particles and nanomaterials.

ID ABS: 445

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

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Development of nanomaterials with new functionalities facilitates a wide range of innovative applications. Along with the design of nanomaterials, the safety of these materials and its applications must be addressed, applying a valid and pragmatic testing strategy. In order to improve testing efficiency with a minimal need for animal testing, we focus our research on five areas:
(1) Comprehensive physico-chemical and biophysical characterization of the nanomaterial and the test items.
(2) *In vitro* screening providing guidance for further testing and aiding product development. A genomics approach was used, enabling assessment of mechanisms of toxicity and identification of cellular pathways affected by particles. In addition, methods to study the toxicity of several metal-based nanomaterials in precision-cut lung slices were developed, taking into account artifacts due to interactions of the material with the test system.
(3) Genotoxicity testing, including Comet assays and chromosomal aberration tests both *in vivo* (by different application routes) and *in vitro* (with different preparations of the test suspensions).
(4) Short-term *in vivo* testing. Due to the lack of sufficiently validated *in vitro* testing methods, the potential hazard of nanomaterials needs to be complemented with targeted *in vivo* testing. We developed a 5-day inhalation study design optimizing aerosol generation and characterization as well as selecting relevant and predictive biological parameters. Twelve nanomaterials were tested and results were compared to longer-term toxicity assays and to results from *in vitro* studies.
(5) Human exposure assessment for risk assessment, including the development of sampling strategies and a relational database for exposure modeling.

ID ABS: 69

Implications of action plans and research strategies towards replacing animal testing in nanotechnology

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Animal experiments are increasingly being performed in nanotechnological research. In nanotoxicology, they are used to evaluate the hazard of nanomaterials and nanoproducts and in nanomedicine to develop nanotechnological medical products and techniques. However nanotechnological research also leads to new *in vitro* methodologies with great potential to improve biomedical research without animals.

Nanotechnology is considered to be a key technology of the 21st century. The European Union, EU member states and other European Countries have set up action plans, platforms and research programmes supporting nanotechnology. These initiatives also address human health and environmental issues. The OECD has established a Working Party on the Safety of Manufactured Nanomaterials.

The presentation provides an overview on animal experiments and alternative methods in nanotoxicology and nanomedicine and on the animal welfare relevant scopes and contents of different action plans, platforms and research programmes. Own surveys reveal incentives and initiatives to replace animal experiments in the area of nanotechnological safety testing, but not in nanomedical fundamental research. Also in nanotoxicology, research programmes, as a rule, do not aim to develop entirely animal-free testing strategies. It will be discussed how different action plans, platforms and research programmes should complement each other to ensure efficient cooperation aiming at replacing animal experiments both in nanotoxicology and nanomedicine while at the same time ensuring human health and environmental safety. Additional political and scientific topics will be addressed which should be pursued to achieve a paradigm change in nanotechnology and biomedical research as such turning away from animal testing altogether.

Using *in vitro* models to predict the *in vivo* pathogenicity of fibres and high aspect ratio nanoparticles

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Nanotechnology has generated a wide variety of different nanomaterials including high aspect ratio (fibre-like) nanoparticles such as nanotubes, nanowires and nanofibrils. Concern has been raised about the potential for such particles to behave like pathogenic fibres. Fibres such as asbestos are pathogenic due to their length and biopersistence (low dissolution), both of which are characteristics shared by carbon nanotubes. Fibres of longer than 15-20 μm are considered to be of greater hazard due to the inability of macrophages to effectively clear such particles via phagocytosis due to their length being greater than the size of the macrophage cell. Engulfment of the fibres is accompanied by the release of damaging reactive oxygen species, which is prolonged during this process of frustrated phagocytsis. This study demonstrates that using *in vitro* cell models, fibres and carbon nanotubes can be predicted in terms of their pathogenic potential in the mouse intraperitoneal injection model. The *in vitro* model used is the macrophage, which is found to generate ROS and pro-inflammatory cytokine production (TNFalpha) on exposure to long amosite asbestos and long carbon nanotubes, but not on exposure to short amosite asbestos or short/entangled carbon nanotubes.

Session BS18: Vaccines and biologicals

3R trends and opportunities in vaccine quality control: a general introduction

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Would an elimination of animal use in quality control of vaccines ever be feasible? I might be too optimistic to say "soon", but we are on the right track.

For a long time, quality control of vaccines was characterised by extensive numbers of animals and high severity levels of the experimental procedures used. However, times are changing. This is due to developments both at the level of vaccine production and at the level of quality control. Production processes are continuously standardised and optimised, allowing production of better defined vaccines. Furthermore, a range of non-animal models is now available that can be used both for in-process control and for lot release testing. In case replacement of animal models is not (yet) within reach, industry and regulatory agencies are increasingly aware of the opportunities to reduce and refine animal use. This presentation will provide an overview of existing trends in 3R developments. Until recently, emphasis has been given to replacement of individual animal tests. However, a generic approach is now becoming fashionable. This trend, the consistency approach, challenges the idea of a vaccine lot being a unique product. The key point in the consistency approach is demonstration of consistency in consecutive vaccine lots produced in relation to a clinical lot that has shown to be safe and effective, both in the surrogate (animal) model and in the target species, such as man. Preferred models to demonstrate consistency are physico-chemical and immuno-chemical methods, such as fluorescense spectroscopy and biosensor analysis.

Potency testing of rabies vaccine for human and veterinary use, possibilities for alternatives

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Each batch of rabies vaccine for veterinary use is tested for potency. *Ph.Eur.* (Europe), 9CFR (USA) and OIE prescribe a vaccination-challenge test in mice. The test uses high numbers of mice, involves severe distress and pain and is poorly reproducible. Attempts were undertaken to reduce, refine and replace the test. *Ph.Eur.* introduced human endpoints to reduce animal suffering. Currently, acceptance criteria are under revision. It should be possible to increase the number of valid tests without loosing information, thus reducing test repeats and animal usage. *Ph.Eur.* has taken the initiative, starting a project to replace challenge tests by serological testing after vaccination. Full replacement of the mouse test by an *in vitro* method is still not possible, because of adjuvants in the final product, but *in vitro* methods represent a suitable method for in process testing.

For human use vaccines the same test in mice is used, but with even higher numbers of animals per dilution and, for some guidelines, with repeated testing. WHO (2005) recommends further development and use of *in vitro* assays, currently used as in process controls, for testing the final vaccine, since antigen concentration studies at this stage of the production has not generally been reported. Authorities keep expecting potency assays that assure the efficacy in humans despite extensive validation during licensing processes. Good Manufacture Practices and Quality Control during production should be seen as a guarantee of the consistency of production. Acceptance of this may facilitate the replacement of the *in vivo* test by an *in vitro* assay.

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In vitro tests for the assessment of clostridial vaccine antigens

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Toxin-producing clostridial species are responsible for a variety of severe animal diseases. As a result, vaccine manufacturers produce a wide range of clostridial toxins which, once chemically inactivated to form toxoid antigens, are used in the production of many vaccines. There is a legal requirement that each batch of these materials is subjected to toxicity testing and antigen quality measurement. All of these tests use mice and it is estimated that in Europe alone tens of thousands of mice are used annually for these purposes. Certain cell lines are known to be sensitive to specific clostridial toxins. Based on this knowledge, it has been proposed that it should be possible to replace these mouse tests with cell line assays. Our initial research concentrated on replacement tests for the toxin and toxoided antigen of *Clostridium septicum*. The VERO cell line was found to be suitable and it has been used to develop *in vitro* replacement assays for the mouse toxicity and antigen quality tests. The cell line assays have been shown to have a greater than 98% correlation with the *in vivo* assays. Overall the *in vitro* assays have proved to be more sensitive and accurate than the original mouse tests. They provide results far more rapidly, are cheaper to perform and allow more accurate blending of vaccines. Similar cell line assays are being developed for other clostridial toxins and toxoid antigens which should result in a subsequent substantial reduction in mouse usage.

ZEBET expert meeting on alternative methods to replace the LD_{50} potency test in botulinum neurotoxin product testing: scientific and legal challenges

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Botulinum neurotoxin (BoNT) is used for treatment of medical disorders such as cervical dystonia, blepharospasm and migraine. Prior to release, production lots of BoNT require characterization in an LD_{50} potency test in mice according to the monograph Botulinum Toxin Type A for Injection, issued in the European *Pharmacopoeia* (EP) 6.0. Due to the paralysing toxic syndrome of BoNT, the treatment of animals is associated with severe suffering. On the other hand, the mentioned monograph in EP 6.0 already indicates three alternative methods, i.e. (i) an *in vitro* endopeptidase assay, (ii) an *ex vivo* assay using the mouse phrenic nerve diaphragm, and (iii) a mouse bioassay using paralysis as endpoint. These methods could be employed to assay BoNT-products after successful validation with respect to the LD_{50} assay (reference method).

As part of the German Federal Institute for Risk Assessment (BfR) in Berlin, the Center for Alternative Methods to Animal Experiments (ZEBET) has organised an international expert meeting in April 2009 dedicated to elaborate on the issue of alternative methods to replace the LD_{50} Potency Test for BoNT Testing. Participants of this meeting were representatives of German ministries, national and international competent authorities, validation organizations and manufacturers of BoNT products, as well as academia and animal welfare organisations. The aim of this expert meeting was to clarify the regulatory requirements regarding BoNT product testing on a national and international level, to select the most promising alternative test methods and to pinpoint further steps necessary to succeed with their validation and regulatory acceptance.

ID ABS: 358

Transgenic biopharming: ethical, scientific and safety considerations

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A number of human biopharmaceuticals are already produced by expression in farm animals and rodents. Amongst these are human haemoglobin and protein C produced in pig blood and milk, tissue plasminogen activator from goat milk and factor VIII and IX produced in sheep's milk.

The quality control standards expected for biopharmed products are considerably higher than those expected for products expressed in and isolated from *in vitro* culture systems. Nevertheless, since biopharming is considered to be up to 10 times more cost effective than cell culture production methods, it is entirely possible that biopharming will continue to be seen as a viable option for cost effective production of complex proteins.

Here, we examine the new technologies available for improving biopharmaceutical production using *in vitro* methods, such as high density and secretory culture systems, and transgenic plants, such as safflower and maize. A comparison of the clinical safety and pharmacological activity of a number of biopharmaceuticals produced using different technologies has been made to support the case for *in vitro* and transgenic plant expression as alternatives to biopharming. Specific reference will be made to biosimilars development and to existing products that have been expressed in both transgenic animals and alternative expression systems, including human insulin, growth hormone and recombinant antibodies.

ID ABS: 372

The development of new adjuvants with minimal side-effects

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Adjuvants are used to enhance cellular and antibody-mediated immune responses. Whereas relatively mild adjuvants have been developed for animal and human vaccination purposes, stronger adjuvants are widely used to induce human-like diseases in experimental animal models. These stronger adjuvants often cause side-effects such as the granulomatous skin lesions that can develop upon immunization with complete Freund's adjuvant (CFA). CFA consists of a mix of mineral oil, surfactant and heat-killed *Mycobacteria* (hkMy). We aim to develop an adjuvant with similar potency as CFA but with minimal side-effects.

In a bottom-up approach we have mapped which components in hkMy contribute to the adjuvant effect. To achieve this, we have developed bioassays of Toll-like receptor (TLR)-transfected cell lines to screen for adjuvanticity. TLR form a family of 10 recently identified receptors that can potently activate the immune system. Using progressive fractionation, we have identified several new molecules in hkMy that cause TLR activation, which might be used to construct a cleaner adjuvant.

In a top-down approach we have characterized a selection of genetically modified strains of *Mycobacteria* for their potential to cause side-effects using an *in vitro* granuloma model. Modified strains that cause fewer granulomas as compared to unmodified strains will subsequently be tested for adjuvant activity in our TLR bioassays. If adjuvant activity is comparable, such modified strains are good candidates for new experimental adjuvants with fewer side-effects.

Replacing CFA by an adjuvant with fewer side-effects will decrease immunization-associated discomfort and represents a major refinement for studies performed in non-human primates, rabbits and rodents.

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Session BS19: Education and training

Institutional animal user training in Canada

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The Canadian Council on Animal Care (CCAC) is the peer-based organization overseeing the ethical care and use of animals in research, teaching and testing throughout Canada since 1968. Institutional Animal Care Committees (ACCs) pioneered by the CCAC are the keystone of the Canadian oversight system. ACCs act as local quality control structures responsible for informed decision-making based on science and societal values, while the CCAC provides quality assurance at the national level.

Adequate training of all personnel is an essential component of any institutional animal care and use program to ensure that animals are used in the most humane and ethical manner. The CCAC guideline on institutional animal user training was published in 1999 to present theoretical and practical training requirements for animal users including investigators, post-doctoral fellows, research staff and graduate students. An accompanying Recommended Syllabus indicates the core topics to be covered. Twelve web-based modules on the core topics of the Recommended Syllabus were posted on the CCAC website with other resources prior to the mandatory implementation of the CCAC guidelines on institutional animal user training through the CCAC Assessment Program, beginning in 2003.

While the CCAC certifies institutional training programs as part of its certification of complete institutional animal care and use programs, the examination of individuals' competencies is the responsibility of the institution and its ACC.

Education and training for researchers in laboratory animal science in Australia

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Under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), which is the key national policy concerning such activities, primary responsibility for the welfare of the animals is vested in the scientist. The Code details principles that guide a decision as to if and how animals are used. Institutional Animal Ethics Committees must be satisfied that a case has been made that any use of animals is justified taking into consideration the scientific merit of a proposal and evidence that the principles of Replacement, Reduction and Refinement have been critically applied in the planning and proposed conduct of the study.

The Code requires institutions to ensure that scientists are aware of their responsibilities and so to provide education programs, including on-going training, seminars and workshops. For students undertaking research training, the Code requires they receive instruction in their ethical and legal responsibilities, as well as the appropriate methods for animal care and use.

The nexus between achieving scientific and animal welfare outcomes is a core element of the Code. Evidence-based guidelines such as those recently published on ways to promote wellbeing promote a focus on the 3Rs and support education programs.

Institutions have developed programs customised to their particular needs. This paper will describe several such programs and how the use of alternatives is promoted both in the context of educational activities and as a key component to planning projects and the responsible conduct of such research.

Asian trend of education and training in laboratory animal medicine for refinement

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Refinement is one of the main themes of laboratory animal medicine in terms of the 3Rs. In particular, the alleviation of pain and distress is the main concern of the general public for refinement of animal experimentation. However, the refinement may be the most complex issue in alternatives to animal experimentation, and therefore the research in refinement should be strengthened. The results of research in this area should be distributed to the investigators and technicians. The advanced countries in laboratory animal science have stringent regulations requiring that the alleviation of pain and distress by anesthesia and analgesia is clearly documented. Veterinary care may be a key word for laboratory animal welfare in these countries. Unfortunately, some Asian countries do not have advanced veterinary education systems for animal welfare, and there is a paucity of knowledge and technology of anesthesia and analgesia among veterinarians. This reflects less advanced laboratory animal welfare as a part of veterinary care for laboratory animals. Refinement of the treatment of laboratory animals with the knowledge of anesthesia and analgesia is now influenced by several laboratory animal welfare advocates in Asian countries, such as the International Association of Colleges of Laboratory Animal Medicine (IACLAM), Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC International) and Asian Federation of Laboratory Animal Science Associations (AFLAS). The recent activities of these organizations are introduced. The laboratory animal welfare advocates in Asian countries seek the support of the more advanced countries in the education and training of laboratory animal veterinarians in anesthesia and analgesia.

FELASA category C course

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FELASA defines Category C Personnel as persons responsible for directing animal experiments. It recommends they be regarded as competent when they possess a Bachelor's or Master's degree and subsequently complete training equivalent to a basic 80-hour course as addressing topics including husbandry, animal health and experiment procedures. The training is designed to give scientists the knowledge and skills necessary for responsible and ethical use of animals in experiments. The course should address alternatives to the use of animals, as well as how to design experiments which use the minimum number of animals and ensure the highest ethical standards. The syllabus recommends demonstration and practice of basic experimental procedures, but adoption of replacement training methods is encouraged. FELASA does not prescribe how practical training should be undertaken and both formal laboratory sessions held during the course, or tutorial training by an experienced and accredited scientist during authorised investigations are considered satisfactory. The FELASA Accreditation Board adheres to the published recommendations for category C training (Laboratory Animals 1995; 29: 121-131) but has established some expectations to assist in accreditation. In relation to the use of animals these include: 1) most common species need to be covered in the program; 2) there should be a minimum of 10 hours practical training on at least two species of animals; 3) the competence of students must be assured.

Education and training for researchers in laboratory animal science in the United States

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Laboratory Animal Use in the United States is primarily governed by two regulations, the Animal Welfare Regulations – AWRs (enforced by the US Department of Agriculture – USDA) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals – PHS Policy (overseen by the Office of Laboratory Animal Welfare – OLAW – of the PHS). In addition, most major users of laboratory animals are involved in the voluntary accreditation program of the Accreditation and Assessment of Laboratory Animal Care, International – aaalac, Intl. The aaalac and the PHS require com-

pliance with the Guide for the Care and Use of the Laboratory Animals – the Guide.

The AWRs and the Guide both address the training of individuals involved in animal research. The AWRs require that "Personnel conducting procedures on the species being maintained or studied will be appropriately qualified and trained in those procedures". Section 2.32 of the AWRs specifically addresses personnel qualifications. It is the institution's responsibility to ensure training, and this responsibility is "fulfilled in part through the provision of training and instruction...". The AWRs go further and describe the general areas which must be included. Training is one of the areas evaluated as part of unannounced USDA inspections, which occur at least annually. The Guide also has a section on personnel qualifications and training, however, details of researcher training are not provided, except to indicate that they must comply with regulations. When conducting site visits, aaalac site visitors will often review training records and, through observation of activities and questioning research personnel, make an assessment of the adequacy of training at the institution.

The Institute of Laboratory Animal Resources (ILAR) National Academy of Science (NAS) published a manual on training that provides additional guidance on development of training programs (Education and Training in the Care and Use of Laboratory Animals: A Guide for Developing Institutional Programs). The basis for such programs includes a list of subjects which should be included in core material and additional modules, which are generally provided on an "as needed" basis. In addition, a full issue of the ILAR Journal was devoted to Training and Adult Learning Strategies for the Care and Use of Laboratory Animals.

Therefore, in the US, researcher training is not formalized at a national level as it is in some other regions. Using more of a performance based approach, researcher training is individualized at both the institutional level and the individual researcher level, based upon what species and procedures are involved. While this may seem to be a weakness by those who use other systems, the success of training (competency) is regularly assessed externally by regulators and site visitors and internally through biannual thorough review of institutional programs by the Institutional Animal Care and Use Committees. Reports of these reviews must be sent to the Institutional Official, who has legal responsibility to assure compliance and be available for review by external regulators and site visitors.

This presentation will elaborate on the approach to researcher training in the US and discuss the strengths and weaknesses of this system.

Session BS20: Animal use policies

Animal experiments – criteria for ethical evaluation and ethical limits

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Since 1986, animal experiments in Germany may only then be approved by the authorities if they are indispensible for a legitimate purpose ("unerlässlich") and the inflicted suffering is not classified as disproportionate to the benefits thereof ("ethisch vertretbar", Article 8, paragraph 3 German Animal Protection Act, TierSchG). In order to verify these conditions each competent authority shall convene one or more committees consisting of scientists experienced with the assessment of animal experiments and of selected members of proposed lists of animal welfare organisations (Article 15 TierSchG). These Animal Experimentation Committees have been gaining experience in the ethical review of applications for animal experiments since 1986. Because rejected applications of animal experiments are often resolved judicially, an ethical testing procedure has been developed, which - based on the Principle of Proportionality - not only fulfils bioethical but also legal requirements. In accordance with the application of animal experiments subject to authorisation, the four steps of quality assurance of the Principle of Proportionality will be introduced during the presentation and illustrated with examples.

Special protection for primates – the need for faster progress

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It is widely recognised that the senses and communication abilities of non-human primates are similar to those of humans, as is their capacity to experience pain and negative and positive emotions. Their use in scientific research raises serious ethical and welfare concerns, which has resulted in the requirement for additional justification for primate use in some countries.

In the last ten years, there have been a number of authoritative reports urging tighter controls on primate use, higher standards of husbandry and care, and an end to the capture and use of wild animals. However, there appears to be great resistance to changing the status quo. Any attempt to restrict primate use, such as in the 2008 European Commission proposal revising Directive 86/609, is met with serious opposition.

At the 2005 World Congress, a resolution urged industry and regulators to accept ending primate use as a desirable goal and to work together to achieve this. Given the special nature of primates this seems a perfectly reasonable proposition. Four years on, we have seen little change. There has been no decrease in their use; regulations around the world still allow species which have highly developed brains, rich and complex social lives, and extensive natural home ranges to spend their lives in laboratory caging in which they can only take a few steps in any direction; they are still captured from the wild, and transported around the world. This is unacceptable and there is a desperate need for more progress and greater commitment.

Applying the information gained from animal harm categorization systems – case study of Canada's Categories of Invasiveness

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The Canadian Categories of Invasiveness (CI) animal harm categorization system serves three purposes: 1) it alerts investigators and animal care committees (ACCs) to the degree of pain and distress that experimental procedures cause animals; 2) it informs the public about the numbers of animals that potentially experience each category of harm; and 3) it provides data which informs national policies on animal use in science. The CI system provides important input to the harm-benefit analysis of animal use, because it assists ACCs to assess the harms, and provides some national consistency in ethical review. Individual ACC members may use classification in a high category of invasiveness as a cue to more closely scrutinize the justification and benefits of a protocol. CI data is published annually online and is accessed by the media and humane organizations, however the extent of wider public access is not known. To identify trends in animal use and inform national policy, CI statistics are reviewed annually. The information has been used to evaluate the effect of guidelines to establish humane endpoints, and to identify problems in the harms categorization of genetically-engineered animals (GEAs). Emerging challenges to the current CI system include: categorizing harms to animals in breeding colonies, especially animals bred specifically to have a disease condition; categorizing GEAs at different stages of new animal line generation; and ensuring publically reported information more accurately reflects the pain and distress experienced by animals. Future policy goals include using CI data to drive Replacement efforts in Canada.

Why should mammalian fetuses be included in regulations protecting animals during experiments if they do not become conscious until after birth?

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The definition of "animal" in welfare regulations often includes life stages where suffering may occur through the conscious perception of noxious stimuli (e.g. feeling pain), and excludes those stages when, at the time the regulations were framed, the young were presumed to lack the capacity for conscious perception. For instance, mammalian fetuses and avian or reptilian young may be protected only after the first half of pregnancy or development has elapsed, and marsupial pouch young from immediately after birth. Recent re-evaluations of the literature and our related research on mammalian young suggest that states of unconsciousness, which preclude suffering, persist to developmental stages beyond those where the young are protected under such regulations. For instance, unconscious states persist until about 140-150 days of in-pouch age in the Tammar wallaby joey, which is neurologically extremely immature at birth, until 4-14 days after birth in the neurologically moderately immature newborns of cats, dogs, mice, rats and rabbits, and until minutes or hours after birth in the neurologically mature newborns of farm animals. These observations challenge the basis of regulations framed in these terms and raise several questions. Should such regulations now be changed to better reflect our current understanding of developmental neurobiology? If they were to be changed, what would be the main areas of concern? Is the precautionary principle of ensuring that mammalian young are protected well before there is any likelihood that they have reached a developmental stage when suffering could occur sufficient to maintain the status quo?

ID ABS: 9

Reviewing the reviews: an analysis of the process of ensuring regulatory compliance in the use of animals in science in New Zealand

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In New Zealand, the Animal Welfare Act 1999 requires that organisations that use animals in research, testing and teaching be audited at least every five years for compliance with both the Act and the organisations' individually approved codes of ethical conduct. The reviews look at both the use of animals by the organisations and the working of their animal ethics committees. This paper looks at the results of this ongoing review process since the introduction of the Act at the beginning of 2000, during which time most of the organisations have been reviewed at least twice. It includes an analysis of the non-compliance issues that have arisen.

Extra Breakout Sessions

Session EB4: Status report on ICATM

What is the International Cooperation on Alternative Test Methods (ICATM) and what is its role?

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In the fall of 2007, the International Cooperation on Cosmetics Regulation (ICCR), tasked ECVAM, NICEATM-ICCVAM, JaCVAM, and Health Canada with developing a framework to promote harmonization of scientific recommendations on alternative toxicity testing methods. After several meetings, a framework was developed and ICCR approved the framework in the fall of 2008.

In order to implement the framework, the International Cooperation on Alternative Test Methods (ICATM) participating national validation organizations (ECVAM, NICEATM-ICCVAM, JaCVAM, and Health Canada) signed a Memorandum of Cooperation (MoC) on April 27, 2009. The MoC promotes enhanced international cooperation and coordination on the scientific validation of non- and reduced-animal toxicity testing methods. Three critical areas of test method evaluation are covered validation studies, independent scientific peer review meetings and reports, and development of test method recommendations for regulatory consideration.

By communicating and working together throughout the process, it is anticipated that we will avoid duplication of work while at the same time identify and embrace scientifically sound and robust test methods that will maintain or enhance protection of human and animal health and the environment. This should foster their application in industry and for regulatory purposes and hence reduce, refine, and replace the use of animals in testing where scientifically feasible.

Note: This abstract reflects the views of the authors and has not been reviewed or approved by any agencies. Since this abstract was written as part of the official duties of the authors, it can be freely copied.

ICATM's importance for ECVAM and the 3Rs in the EU

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The 7th amendment to the EU Cosmetics Regulation bans the marketing of any animal-tested substance in cosmetics as of 2013, independent of where the testing took place. REACH, the EU's Chemicals Regulation allows for animal testing only as a last resort. Scientific progress leads to alternative tests that are equal or better than animal testing in terms of their capacity to precisely predict the potential impact of a substance on the human body or the environment. The US EPA has decided to move towards modern testing technologies.

All these developments point in the same direction: for many reasons alternative testing will become the norm rather than the exception once they are accepted as offering the

same or better capacity for health and environmental safety assessment.

Given that chemicals and products made from them are internationally traded, a large interest exists to ensure similar regulatory requirements worldwide. ICATM, bringing together the validation centres of Japan, the US, and the EU, will help to ensure the necessary harmonisation, at least of the scientific/technical validation of new testing methods. This in turn should support their international acceptance at OECD level as well as regulatory acceptance at national level. It can be expected that, as a consequence, these validated alternative tests will be more widely and more quickly applied in industry and for regulatory purposes, thus strongly supporting the 3Rs – not only in the EU but worldwide.

Current and future contributions of NICEATM and ICCVAM to the international cooperation on alternative test methods

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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) work collaboratively to promote validation and regulatory acceptance of new, revised, and alternative test methods that are based on sound science and that will provide continued or improved protection of people, animals, and the environment while reducing, refining, and replacing the use of animals where scientifically feasible. The United States, Canada, the European Union, and Japan signed a Memorandum of Cooperation on International Cooperation on Alternative Test Methods (ICATM) in April 2009. The participating national validation organizations will work to expand and strengthen cooperation, collaboration, and communications on the scientific validation and evaluation of new alternative testing methods proposed for regulatory health and safety assessments. NICEATM and ICCVAM, as the designated participating national validation organizations for the U.S., will collaborate with JaCVAM, ECVAM, and Health Canada's Environmental Health Science and Research Bureau. NICEATM and ICCVAM have implemented procedures to ensure consistent collaborations with the other validation organizations during the design and conduct of validation studies, the evaluation and independent scientific peer review of proposed test methods, and the development of harmonized test method recommendations for regulatory consideration. The enhanced international cooperation in these three areas is expected to result in more efficient test method validation and review, and more rapid national and international acceptance of scientifically valid test methods.

Note: The views expressed above do not necessarily represent official positions of any federal agency.

JaCVAM's role in the 3Rs and ICATM

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In November 2005, the Japanese Center for the Validation of Alternative Methods (JaCVAM) was established as part of the Division of Pharmacology at the National Center for Biological Safety and Research, affiliated with the National Institute of Health Sciences (NIHS) in Japan. The key objectives of JaCVAM are: 1) to ensure that new or revised test methods are validated, peer-reviewed, and officially accepted by regulatory agencies and 2) to expand international cooperation on alternatives to animal testing. JaCVAM's goals are to facilitate the validation of alternative methods using domestically developed test methods, conduct peer reviews of these and internationally certified test methods, and promote the practice of the 3Rs in the area of animal testing to accomplish our mission.

Therefore, it is important that JaCVAM connect with the International Cooperation on Alternative Test Methods (ICATM). JaCVAM will work hard to maintain a framework for enhanced international cooperation, collaboration and communication in three related but independent critical stages: 1) test method validation studies, 2) independent peer review of the validation status of test methods, and 3) development of formal test method recommendations. Furthermore, JaCVAM will enhance relations with ICATM and Japanese researchers in all fields.

Health Canada's role in ICATM

D. H. Blakey

Environmental Health Science and Research Bureau, Health Canada, Canada

The Environmental Health Science and Research Bureau is a member of ICATM along with ICCVAM, ECVAM, and JaCVAM. Unlike the other members, Canada has no formally designated agency to coordinate or conduct validation studies for alternative methods. Instead, Canada's participation in ICATM will be coordinated through the Bureau.

The Bureau is interested in cooperating on the validation of alternative methods and facilitating acceptance of alternative methods that offer equivalent or better protection of human health while reducing animal use.

Health Canada will contribute to ICATM activities by providing expertise to plan and review validation studies conducted by member agencies and by participating in discussions regarding ICATM recommendation on the suitability of validated alternative methods as suitable for regulatory use.

Session EB5: Status report on EPAA

EPAA: paving the way towards new perspectives on safety

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One of the aims of EPAA is to provide a fresh perspective on, and further impetus for, the development and implementation of alternative approaches for safety assessment. With regards to this aim, a workshop with some unique features was sponsored and organized by EPAA in April 2008 in Brussels. The workshop, populated not only with experienced toxicologists but also with eminent scientists from other disciplines who would not normally engage with toxicology issues, was part of a strategy to identify new approaches to hazard identification and risk assessment without the need for repeated systemic exposure of animals to test materials. The challenging goal is to be able to predict, without recourse to animal models, the likelihood that adverse health effects of any kind might result from long term exposure of people to a new product. A variety of novel strategies were explored and areas of research identified that may be of particular relevance. Among the areas that were discussed and are considered ripe for exploitation – or further exploitation – in this context are: computational chemistry, mathematical modelling, stem cell biology and harnessing the power of various "omics" technologies in tandem with systems biology.

Although informing the future research agenda in this way is important, there are two other, arguably less tangible, benefits that derive from initiatives of this kind. The first is to harness more effectively the very substantial achievements that have been witnessed in biology and chemistry during the last 10 years. The second issue is the need to further legitimise alternatives research within the general scientific community, emphasizing the impact of pursuing modern biology to deliver animal welfare benefits while improving our ability to ensure the safety of new products. Among the great scientific challenges is provision of truly innovative approaches to testing that will allow the safe development of new products without recourse to animal experiments.

The European partnership for Alternative Approaches to Animal Testing: acute toxicity testing: analysis across sectors

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) is an unprecedented collaboration between the European Commission and major companies from seven industry sectors. The partners are committed to pooling knowledge, research and resources to accelerate the development, validation and acceptance of alternative approaches and share best practice to further the use of 3Rs methods in regulatory safety testing.

Acute toxicity tests are conducted in most sectors and therefore are an ideal study type for cross-sectoral analysis and sharing of best practice in order to identify opportunities for application of the 3Rs. Acute toxicity studies have been some of the most criticized of all regulatory toxicity tests, as they may be associated with substantial adverse effects in animals and often provide limited data.

A review of the scientific drivers for acute toxicity testing within the pharmaceutical industry led to a successful challenge to the requirement for acute toxicity testing in that sector (Robinson et al., 2008. *Regul. Toxicol. Pharmacol. 50,* 345-352). Based on this experience, the EPAA is analysing regulatory and scientific drivers for acute toxicity testing in different sectors where the overall data requirements and the use of data may vary. Where this type of study cannot be waived, the collaboration may lead to improved study designs or testing strategies with direct 3Rs benefit. Activities and results to date will be presented

In vitro metabolism test systems as essential parts of integrated test strategies for long-term toxicities reinforced by EPAA's joint efforts

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To meet the remaining requirements of the Cosmetics Directive, information about toxicokinetics and metabolism are pivotal for the safety assessment of cosmetic ingredients and vital for designing the necessary *in vitro* toxicity tests. A collaborative effort between academia, industry and the European Commission, including the active involvement of three established validation bodies (ECVAM, ICCVAM and JaCVAM) has been undertaken to provide a reliable and relevant *in vitro* test system that is metabolically competent.

This effort aims to develop one of the elements needed by integrated testing strategies (ITS) to enable the highly complex processes by which our body handles xenobiotics to be modelled. In this case it is a metabolically competent cell system that models the process of xenobiotic biotransformation by hepatocytes. This will hopefully contribute to the assessment of complex long(er)-term human health effects and reduce animal numbers.

The second element for any successful strategy will be the integration of information provided by building blocks such as *in vitro* test systems. In the end, any integrated test strategy will need to model the complex interplay of physicochemical, toxicodynamic (the number of targets that can interact with the toxicant), toxicokinetics (the number of molecules that can reach the target) and toxicological processes in order to enable a reliable prediction of adverse human health effects of xenobiotics.

The paper presented focuses on the *in vitro* metabolically competent cell system under validation, which could potentially become a central part of an ITS, and informs on the progress made so far.

Session EB6: Status report on ReProTect

The ReProTect project

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Testing in animals is the only tool available today for hazard and risk assessment of chemicals that affect the reproductive cycle. The number of animals used for reproductive toxicity testing is expected to further increase due to the European legislation REACH and the 7th amendment to the Cosmetics Directive. A reduction in animal number can only be achieved if testing strategies are developed that integrate *in vitro* alternatives. On this background, an integrated project, named ReProTect, was started in 2004, sponsored by the 6th Framework Program of the European Union. The aim of ReProTect (www.reprotect.eu) is to develop/optimize batteries of *in vitro* assays to detect adverse effects on the reproductive cycle and to unravel, if possible, their underlying mechanisms of action. The project consortium is composed of 33 partners from 12 European countries, coming from academia, industry, small medium enterprises and government institutions. The total budget of the project is 13.2 m€. ReProTect consists of four research areas, which cover aspects related to fertility, implantation, and prenatal development, and a fourth research area, cross-cutting technologies, explores the use of innovative methodologies in toxicity testing. In ReProTect more then 20 alternative tests have been developed or optimized, and their reproducibility and transferability between labs have been studied. In total, more than 100 peer-reviewed chemicals have been investigated. An independent statistical analysis is ongoing to assess the applicability of the newly developed tests.

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Session EB7: Status report on OSIRIS

In vitro toxicological alerts in ecotoxicological hazard assessment

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To date, *in vitro* assays have not been employed in regulatory testing of ecotoxicological properties of chemicals. With the implementation of new chemical regulations such as REACH in Europe, the possible role of *in vitro* approaches in integrated testing strategies for ecotoxicological hazard assessment is reconsidered. Current focus is on the use of non-animal methods to substitute for *in vivo* ecotoxicity tests with vertebrates, for instance, the fish embryo assay as an alternative to acute lethality assays with fish, or metabolically competent subcellular and cellular preparations as alternatives to *in vivo* bioconcentration testing with fish. Another possible role of *in vitro* approaches that is emphasized by the OSIRIS project is to reduce uncer-

tainties in ecotoxicological hazard assessment. An example of this case is endocrine disruption. Environmental risks due to endocrine activities of chemicals were completely missed by the conventional regulatory tests relying exclusively on apical endpoints. Here, *in vitro* assays, through their ability to screen for specific toxic reactivities and modes of toxic action, can provide "alerts" that help to prioritize and guide subsequent testing. Such "toxicological alerts" obtained from *in vitro* tools may also be of value in acute-chronic as well as in across-species extrapolations, which are both key elements in ecotoxicological hazard assessment.

Developing strategies for integrating *in vitro, in vivo* and *in silico* information in mutagenicity/carcinogenicity

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In vivo mutagenicity studies, closely followed by carcinogenicity, pose a high demand for test-related recourses: therefore, the development and use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals might have a huge saving potential for these endpoints. The structure-activity relationships paradigm provides a wide range of tools.

Some are coarse-grain approaches such as structural alerts (SA): these have a crucial role in risk assessment for: a) description of sets of chemicals; b) preliminary hazard characterization; c) formation of categories; d) generation of subsets of congeneric chemicals to be analyzed subsequently with quantitative (QSAR) methods; e) priority setting.

On the other side, there are fine-tuned QSARs for congeneric classes of chemicals. Good quality, local QSARs for mutagenicity and carcinogenicity, when challenged for their predictivity in respect to real external test sets were 70 to 100% correct in their external predictions.

A crucial issue is that of the uncertainty of the modeling approaches. More properly, their uncertainty should be compared with that of the competing experimental tests. For example, the ability of SAs to predict rodent carcinogenicity is of the same order as the Ames test (around 65% accuracy). Equally illuminating is the fact that the external predictivity of good local QSARs (70 to 100% accuracy) is of the same order as the reported inter-laboratory variability of the Ames test (85%).

Thus, uncertainties are found in both modelling and experimental systems. The crucial issue is that of exploiting and combining – at their best – both methods.

Bayesian evaluation of non-animal information to support decision making – skin sensitization test case

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Following increasing use of *in vitro/in chemico/in silico* tests in management of chemicals, there is a need to develop data integration frameworks allowing the interpretation of results from test batteries and making an inference about an *in vivo* endpoint. The framework should be transparent and structured, and allow for consistent and rational reasoning. We developed a formal weight-of-evidence framework for a multiple test battery that meets these requirements using Bayesian probabilistic inference. It resolves conflicting evidence, reasons consistently given different data sets and/incomplete data sets. To assess skin sensitization we developed a Bayesian Network with the target variable LLNA assay and input variables grouped into 3 lines of evidence: bioavailability, peptide reactivity and dendritic cell activation. Inputs to the bioavailability line of evidence are all generated *in silico* and include: Log Kow and calculated variables related to penetration from a dynamic skin model: dose absorbed systemically, free chemical concentration in the skin, maximum concentration in the epidermis. Inputs to the peptide reactivity line of evidence include data from in chimico tests such as lysine, cysteine, luciferase reactivity. Finally, the dendritic cells line of evidence is based on U937 CD86 expression and IL-8 production. The input variables are ranked relative to their importance in explaining a chemical's potency in the LLNA and help define an optimal testing strategy. Reduction in the certainty of the battery outcome due to conditional dependence between tests is demonstrated and taken into account while assessing information gain from multiple assays.

Lunch Sessions

Session SL3: 2009 developments in the field of alternative methods

No abstracts arrived

Session SL4: Recent progress and future directions in the validation and regulatory acceptance of alternative test methods that reduce, refine, and replace animal use

Recent progress and future directions at NICEATM-ICCVAM: validation and acceptance of alternative methods that reduce, refine, and replace animal use

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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) work collaboratively to promote the validation and regulatory acceptance of new, revised, and alternative test methods that are based on sound science and that will provide continued or improved protection of people, animals, and the environment while reducing, refining, and replacing the use of animals where scientifically feasible. ICCVAM was organized in 1997 and US law established it as a permanent interagency committee in 2000. ICCVAM consists of representatives from 15 regulatory and research agencies that generate, use, or require toxicological testing data to safeguard the health of people, animals, and the environment. NICEATM administers ICCVAM and provides scientific and technical support, including the conduct of high priority validation studies. Since its establishment, ICCVAM has contributed to the evaluation of 27 alternative test methods that have now been accepted or endorsed by national and international authorities. NICEATM and ICCVAM developed a Five-Year Plan in 2008 in conjunction with member agencies to advance alternative methods. The plan identifies priority areas for research, development, translation, and validation activities necessary to support the regulatory acceptance of alternative methods. Implementation of the five-year plan and expanded international collaborations are expected to result in significant progress on alternative methods that will support improved safety assessments while reducing, refining, and replacing animal use.

Note: The views expressed above do not necessarily represent the official positions of any federal agency.

Recent progress and future directions at JaCVAM

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The Japanese Center for the Validation of Alternative Methods (JaCVAM) is currently coordinating validation studies and peer reviews for several test methods. Most of the test methods will be used to assess the safety of cosmetic products. In 2007-2008, we supported international and national validation studies on *in vitro* skin irritation testing and skin sensitization testing, which included the following assays: non-radioisotope LLNA (LLNA: BrdU-ELISA), comet assay (*in vitro* and *in vivo*), reporter gene assay for endocrine disrupter screening, and Bhras cell transformation assay. Similarly, up to peer review, the *in vitro* skin irritation testing included the following assays: EPISKIN, *in vitro* eye irritation testing, BCOP (Bovine Corneal Opacity and Permeability Test) and ICE (Isolated Chicken Eye Test Method), phototoxicity testing using a battery of yeast membranes and red blood cells, and *in vitro* pyrogen screening. Furthermore, the non-commission members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statements:

1) Vitrolife-Skin, a 3-dimensional cultured skin model, can be used to distinguish between corrosive and non-corrosive chemicals within the context of OECD testing guideline No. 431 on *In vitro* Skin Corrosion: Human Skin Model Test.
2) LLNA (Local Lymph Node Assay)-DA can be used to distinguish between sensitizing and non-sensitizing chemicals within the context of OECD testing guideline No. 429.

Recent progress and future directions at ECVAM

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ECVAM, the European Centre for the Validation of Alternative Methods, is based in the Institute of Health and Consumer Protection (IHCP) of the European Commission's Joint Research Centre (JRC). ECVAM is supported by its scientific advisory committee ESAC that peer reviews validations studies of methods that can replace, reduce, or refine animal experiments. ESAC is currently being renewed by means of an open call for the expression of interest that invites individual experts to apply before 1 October for becoming a member of the committee.

So far the focus of ECVAM's work is on toxicology testing methods as EU legislation requires using alternative methods whenever possible – and for cosmetics it makes them mandatory. For topical toxicology, a range of alternative *in vitro*-based test methods have been validated by ECVAM or are forthcoming. However, repeated dose toxicity and other complex endpoints pose major challenges.

While ECVAM continues validating alternative methods by comparing them to *in vivo* reference data, it will therefore also embark on developing validation methods for the complex methods needed for complex endpoints. Often these methods will consist of integrated testing strategies, combining *in silico* with *in vitro* and possibly even *in vivo* approaches. The 'omics', like metabolomics for analysing the metabolites produced in cells challenged by xenobiotics, or genomics, to look at their genetic impact, will also play a role and have to be made use of. This presentation will conclude by briefly outlining ECVAM's plans to approach this highly complex challenge.

Poster Section

PO11: Basic research

ID ABS: 14

Connexin32 hemichannels facilitate the apoptotic-to-necrotic transition during Fas-mediated hepatocyte cell death

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Background/Aims: Although it is widely accepted that the establishment of gap junctional intercellular communication (GJIC) is a prerequisite for maintaining liver homeostasis, its role in the occurrence of apoptotic hepatocyte cell death still remains elusive. Most hepatic gap junctions are formed by two hemichannels of neighboring hepatocytes, which are composed of connexin32 (Cx32). The present study was set up to investigate the fate of Cx32 and its channels in Fas-mediated hepatocellular apoptosis.

Methods: Primary hepatocytes were exposed to Fas ligand, and Cx32 expression was studied by means of immunoblotting and qRT-PCR analysis. GJIC was monitored by fluorescence recovery after photobleaching analysis. Cx32 hemichannels were approached through Cx32 siRNA-mediated gene silencing, cell surface biotinylation, application of a Cx32 mimetic peptide and measurement of ATP release.

Results: GJIC rapidly declined upon progression of the cell death response, which was associated with a decay of the gap junctional Cx32 protein pool. Simultaneously, levels of newly synthesized Cx32 protein increased and gathered in a hemichannel configuration. This particularly became evident towards the final stages of the cell death process and was not reflected at the transcriptional level. Both the silencing of Cx32 expression and the inhibition of Cx32 hemichannel activity prior to cell death induction resulted in a delayed termination of the cell death response.

Conclusions: (i) Cx32 hemichannels facilitate the apoptotic-to-necrotic transition, which typically occurs in the final stage of Fas-mediated hepatocyte cell death. (ii) Primary hepatocyte cultures are suitable *in vitro* tools to elucidate the mechanisms that underlie hepatocyte cell death.

ID ABS: 24

Influence of the three-dimensional culture model on epithelial-mesenchymal transition in oral squamous cell carcinoma

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Several proteins related to cancer growth, invasion and metastasis, such as vimentin, are being widely associated with carcinogenesis and tumor progression. Vimentin expression is commonly observed in mesenchymal cells, however, it is also found in epithelial carcinomas such as cervix, breast and bladder tumors. Its presence in epithelial tumor cells contributes to

epithelial-mesenchymal transition and is associated with tumorigenesis and cancer progression. The aim of this study was to analyze vimentin expression in head and neck squamous cell carcinoma (HNSCC) cell lines in different conditions. Three HNSCC cell lines and the HaCat cell line (immortalized keratinocytes) were submitted to a 3D assay in Matrigel®. As control the same cell lines was used without any treatment. Cytoplasmatic staining of vimentin was observed in some of the carcinoma cells studied except for HaCat, which was negative. Western Blotting demonstrated a statistically significant decrease in vimentin expression when cells were cultured in Matrigel®. The results suggest that in squamous cell carcinoma vimentin can be expressed in some cells and its expression is directly linked to the microenvironment of the tumor. Therefore, the relationship between cells and extracellular matrix plays an important role in tumorigenesis and metastasis.

ID ABS: 48

Evaluation of the anti-arthritic potential of *Colchicum luteum* baker

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Rheumatoid arthritis (RA) is a debilitating disease that severely affects the quality of life. Its treatment is further complicated by non-compliance to drug regimens due to various side effects. This leads to patients seeking treatment alternatives in traditional systems of medicine. In traditional medicine *Colchicum luteum* (CL) is one of the important plants of most anti-rheumatic formulations, but its efficacy has not been experimentally validated. Therefore, the present study was carried out to evaluate the therapeutic potential of CL hydro-alcoholic extract (CLHE) in an experimental model of RA. Experiments were carried out after clearance from the Institutional Animal Ethics Committee. Animals were anaesthetized and baseline recordings of joint size were made on Day 0. Arthritis was induced by a subplantar administration of 0.1 ml Freund's Complete Adjuvant (FCA: Difco) into the left hind paw. CLHE (17 mg, 34 mg, 68 mg/kg) and standard drug indomethacin (3 mg/kg) were administered in 1% gum acacia suspension from Day 0-20. Increase in joint size was measured on Days 3, 7, 14 and 21. Thereafter terminal blood collection was carried out and TNF-α levels were estimated. In this study, CLHE produced a dose dependent and significant ($p<0.01$) reduction in joint swelling. Inhibition of joint swelling in the 68 mg/kg CLHE treated groups on Days 14 and 21 (66.01% and 68.37%) was comparable to the indomethacin treated group (65.53% and 72.55%). TNF- α levels were significantly ($p<0.01$) reduced in all treatment groups as compared to control. CLHE has the potential to be used as an adjuvant in the treatment of RA.

ID ABS: 57

Alternative infection models

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The study of microorganisms is an important and prolific field of basic research. The stimulus for some of these investigations comes from the fact that bacteria and fungus are responsible for numerous human diseases, some of them lethal. While antimicrobial drugs are widely available, for various reasons, they are not always capable of clearing the infection. In this context, understanding host-pathogen interactions and the mechanisms that lead to disease may open new opportunities to fight these microorganisms. Many infection studies involve rodents as the experimental animal and several alternative models are available. Because various molecular pathways are conserved during evolution, it has been shown that it is feasible to use fish, insects, protozoans and plants to study specific aspects of human infections. We aim to highlight the usefulness of lower organisms, plants and 3D cell systems as *in vivo* and *in vitro* infection models.

ID ABS: 112

Quantitative assessment of genotoxicity induced by simulated solar UV on reconstructed skin: DNA damage, p53 status and apoptosis

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Normal human keratinocytes in culture constitute a relevant and validated model for photogenotoxicity studies. However, complementary information on 3D tissues in conditions closer to human skin is necessary. In this regard, industrial reconstructed skin models such as the Episkin model are very convenient. Numerous studies on photobiology have been reported in such systems, but generally, data were obtained using immunohistochemistry analysis, a technique which is rather qualitative than quantitative. The aim of this study was to provide quantitative methodologies in order to precisely assess the genotoxic impact of sunlight. Here, a full thickness reconstructed skin model (including dermis with fibroblasts embedded in collagen) was exposed to simulated solar UV radiation similar to zenithal sunlight in terms of spectral power distribution. Biological endpoints related to genotoxicity were then analyzed quantitatively. A dose dependent induction of DNA breaks and pyrimidine dimers was characterized using an adapted protocol of the single cell gel electrophoresis (comet assay). P53 accumulation measured with an ELISA 5 hours post UV exposure was shown to reach a plateau by exposure times over 15 minutes. By then, the apoptosis rate related to Caspase-3 activity, measured 18 hours post exposure, started to increase. Finally, quantitative RT-PCR on genes controlled by p53 showed that p21 and GADD45 were induced, whereas a clear induction of MDM2 required relatively high UV doses. This study shows that reconstructed skin is a useful tool as part of *in vitro* strategies for the assessment of photoprotection and photogenotoxicity.

ID ABS: 127

Prediction of the toxicity score by *in vitro* test: an application of Bayesian linear regression

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When a new alternative test is established, a dichotomized assessment of the toxicity of a chemical is frequently performed. However, in an animal test, a toxicity-scoring method based on continuous measurement would facilitate the development of a method for directly predicting the score by using the measures of a corresponding alternative test, which would be useful for assessing the chemical. Here, we propose a statistical method for predicting the toxicity score of an animal test by using the measurements obtained in the corresponding alternative test.

The proposed method is based on the assumption that the alternative test has several concurrent experimental positive-control groups, and we can also refer to extra data about the alternative test from a validation study or historical control. Thus, we developed a prediction method based on Bayesian linear regression.

We applied the proposed method to predict the maximum average score of the Draize eye irritation test by using the data from a series of multilaboratory studies on a cytotoxicity test, which was performed in the 1990's.

The proposed method requires background data of the relationship between the scores of the targeted animal test and those of its alternative test. Once such a relationship is validated, this method will be useful.

ID ABS: 138

In vivo approaches to assess both genotoxicity and carcinogenicity

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Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. However, it is currently difficult to discriminate genotoxic and non-genotoxic carcinogens because both assays are basically independent of each other, which raises a simple query as to how much the

detected genotoxic potential can consequently contribute to carcinogenicity. To clarify this critical issue, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected to prove to be powerful tools for the simultaneous evaluation of both genotoxicity and carcinogenicity on the same organ level. A number of studies of genotoxic carcinogens using these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action. On the other hand, a known non-genotoxic carcinogen, dicyclanil, increased *in vivo* genotoxicity as well as oxidative DNA damage in female mice, consistent with the sex specificity of its carcinogenicity, albeit without clear evidence of direct DNA reactivity. In contrast, a genotoxic chlorinated water by-product, MX, failed to exert *in vivo* genotoxicity and carcinogenicity in mice. We also confirmed that such reporter gene carrying rodents are not more susceptible or resistant to carcinogenicity as compared with intact counterparts. These results thus indicate that understanding of the detailed mechanism of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose.

ID ABS: 144

Efficacy of *Lawsonia inermis Linn.* in experimental arthritis

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Lawsonia inermis Linn. (LI) is a medicinal plant that finds mention in various anti-inflammatory, antipyretic, analgesic, and anti-microbial preparations in traditional medicine. In Unani medicine, *Lawsonia inermis* is an important constituent of anti-arthritic preparations. There are a number of reports on antiinflammatory and antipyretic actions of this plant, but information regarding its anti-arthritic activity is lacking. Therefore, the present study was carried out to evaluate the therapeutic potential of LI hydro-alcoholic extract (LIHE) in experimental arthritis. Experiments were carried out after obtaining clearance from the Institutional Animal Ethics Committee. Animals were anaesthetized and base line recordings of joint size were made on Day 0. Arthritis was induced by subplantar administration of 0.1 ml Freund's Complete Adjuvant (FCA:Difco) into the left hind paw. LIHE (4.5 mg, 9 mg, 18 mg/kg) and indomethacin (3 mg/kg) were administered orally as 1% gum acacia suspension from Day 0-20. Increase in joint size was measured on Days 3, 7, 14 & 21. Thereafter terminal blood collection was done and serum TNF-α levels were estimated. In this study, LIHE produced a dose dependent and significant ($p<0.01$) reduction in joint swelling. Inhibition of joint swelling in the 18 mg/kg LIHE treated group on Days 14 and 21 (65.53% and 72.55%) was comparable to the indomethacin treated group (65.53% and 72.55%). Paradoxically, TNF-α levels were elevated in all LIHE treated groups and the increase was significant in the 4.5 mg/kg LIHE treated group as compared to control. The results suggest the potential for use of LIHE as an adjuvant in the treatment of Rheumatoid arthritis.

ID ABS: 153

Time to challenge fundamental research

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The 7th world congress on alternatives and animal use in the life sciences is an opportune occasion to examine general trends and to try to plot future developments in the light of these trends. Industry appears to be leading the way through its decreased reliance on animal testing and the concurrent development of new testing strategies. This has been particularly evident in the cosmetics industry, following the entry into force in March 2009 of the EU Cosmetics Directive. In contrast to this encouraging trend, animal use in basic research would appear to be increasing. Much of this increase can be attributed to the production of genetically modified rodents. Another area of basic research that is cause for concern is the use of non human primates in neurological research. Ironically, although the non human primate model is considered the most vital for the study of human disease, it is probably also the most amenable to legal challenge in the courtroom.

ID ABS: 175

Genotoxicity evaluation of *Dipteryx alata Vogel* hydroalcoholic extract through micronucleus assay using CHO cells

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Dipteryx alata Vogel, popularly known as "baru", is an herbal medicine that grows in the poor soil of the Brazilian savanna. Its bark is used for anti-inflammatory treatments by the "Quilombolas". The present study determines the genotoxicity of *Dipteryx alata Vogel* hydroalcoholic extract (30:70), using Chinese Hamster Ovary (CHO) cells. The test was conducted in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin and incubated at 37ºC, 97% humidity, 5% CO_2 in cell culture flasks. The cells were exposed to different extract concentrations (0.005 mg/ml; 0.016 mg/ml and 0.05 mg/ml), positive controls and cytochalasin B during targeted mitosis in the presence and absence of an appropriate metabolic activation system (Mix S9 solution). The regression formula used to estimate the cytotoxicity as described in OECD 487 – Draft Proposal for a New Guideline – in 2007 was applied to determine cell proliferation as the cytokinesis-block proliferation index (CBPI). The results for the absence of S9 were CBPI 1.70 ±0.035 and MF 4.00 ±0.58, and in the presence of S9, CBPI 1.78 ±0.015 and MF 7.13 ±0.94, both for the highest concentration of the extract. Although, the micronucleus frequency (MF), in the presence of S9 was above 5%, these negative results indicate that, under the test conditions used, the test substance does not induce chromosome breaks in cultured CHO cells.

ID ABS: 278

Antiproliferative effects of celecoxib through Akt signaling pathway

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Celecoxib, a cyclooxygenase-2 (COX-2) selective inhibitor, non-steroidal anti-inflammatory drug, has been shown to be an important cell proliferation inhibitor in squamous cell carcinoma. It represents a potential adjuvant in the treatment of this lesion. Nevertheless, it remains unclear which signaling pathways are influenced. Proteins involved in cell proliferation and apoptosis control have been pinpointed as probable targets of celecoxib. Additionally, its antitumor action has been suggested to be COX-2 independent. This study aims at analysing the effects of celecoxib on the Akt signaling pathway (PTEN, Akt, NFκB, CyclinD1) in two HNSCC (head and neck squamous cell carcinoma) cell lines by protein expression analysis. Cells were treated with celecoxib (10 μM) for 48 h. Cell proteins were submitted to Western blotting analysis. A significant reduction in PTEN, CyclinD1 and pAkt expression was observed in the presence of celecoxib ($p<0.05$). A slight decrease was noted for NFκB expression ($p>0.05$). This work suggests that celecoxib is an effective inhibitor of cellular proliferation in HNSC.

ID ABS: 291

Mechanisms of aryl hydrocarbon receptor induction by pifithrin

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The aryl hydrocarbon receptor (AhR) can be activated by a variety of xenobiotics and mediates different cellular detoxification pathways. One protein whose expression is controlled by the activated AhR is cytochrome P4501A (CYP1A), as evidenced at the enzymatic level by measuring ethoxyresorufin-O-deethylase (EROD) activity. There exist also a number of studies that relate AhR activation with induction or inhibition of tumoral phenomena. It has been traditionally assumed that AhR activators share some structural features: they are polycyclic, polyaromatic, planar molecules. However, some compounds without planar

feature are also able to activate the AhR. In the present work, we studied the ability of pifithrin, an inhibitor of signaling by the tumor suppressor protein p53, to activate AhR. This will give new information about the structural features necessary for activation of AhR that can be helpful for the understanding of AhR mediated processes. Pifithrin was able to activate EROD activity in a fish cell line. *Ab initio* computational calculations showed that pifithrin meets the requirements to act as an AhR activator. The possible interaction of the activated AhR with specific DNA responsive elements was studied in a rat hepatoma cell line expressing luciferase as a reporter gene under the indirect control of AhR. The binding of pifithrin to the AhR was observed using marked antibodies directed against the AhR-activated complex. The concrete mechanism of AhR activation was studied by means of co-incubation with AhR and cytochrome P450 activators and inhibitors.

ID ABS: 293

Focus on alternatives: a consortium for an advanced and ethical research

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Focus on Alternatives (FoA) is a consortium of British non-profit organisations that fund the development of methods which replace the use of laboratory animals in research, education and testing. FoA has been proactive on several topics, the most recent of which include:

• Human tissue campaign – Survey results

A survey was conducted to understand the limitations and challenges scientists face when using human cells and tissues. The survey results will prove useful in overcoming existing barriers regarding the donation and availability of human tissues for research.

• Foetal calf serum-free table

An updated list of commercially available foetal calf serum (FCS)-free media for a wide range of defined cell lines is now available from FoA. This is an invaluable tool for scientists aiming at defining culture media to improve the control and consistency of culture conditions, and decrease the risk of contamination.

• Human Volunteers – Replacing Animal Experiments in Pain Research

A workshop was organised with leading experts, from both industry and academia, to assess the validity of the animal model in pain research. The conclusions were published in a report in the journal Neuroimage, exploring how studies with volunteers, in combination with *in vitro* methods, can address human pain conditions whilst replacing the use of animals.

• Replacing Primates in Medical Research

Members of FoA produced a report available through our website, which highlights the use of primates together with current and future potential replacement techniques, in five areas of medical research – malaria, stroke, hepatitis C, AIDS and cognition research.

ID ABS: 295

Cytotoxicity evaluation of *Plathymenia reticulata Benth* hydroalcoholic extract using CHO cells

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Plathymenia reticulata Benth is a typical plant from Brazilian savanna areas, popularly known as "vinhático", which belongs to the *Leguminosae* family. Its stems are used in anti-inflammatory treatments. According to Brazilian standards, in order to register a medicinal plant, it is required that a technical bulletin containing scientific data assures its safe use. The present work evaluated the cytotoxicity of a hydroalcoholic extract obtained from samples collected in Tocantins, Brazil, using Chinese hamster ovary cells (CHO). The tests were performed in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin, and incubated at 37ºC, 97% humidity and 5% CO_2 in cell culture flasks. The cytotoxicity was evaluated by exposing CHO cells to different concentrations of the extract. The viable cells were quantified using MTS/PMS. The regression formula used to estimate the starting dose was based on gram units, so it was applicable to mixtures and unknown substances, as described in the peer review panel report from ICCVAM and NICEATM in 2006. The IC_{10} corresponded to 0.331 mg/ml and the IC_{50} to 0.598 mg/ml. These values established the starting doses for the acute oral systemic toxicity test of the hydroalcoholic extract (1719.26 mg kg^{-1}), reducing the required number of animals for further scientific investigations. Although reports suggest the use of 3T3 cell culture and the NRU method to detect cell viability, data generated using different cells brings new perspectives to the *in vitro* methodology validation.

ID ABS: 389

Human amniotic membrane as a suitable cover in jaw injuries – a substrate for bone regeneration

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Amniotic membrane (AM), the inner most layer of fetal membrane, has been introduced as an appropriate membrane for burn injuries and skin ulcer,as its beneficial influences like epithelialization and inhibition of inflammation, and its growth factors like PDGF, BMP4 and TGFβ could accelerate the healing process. In the present study its effects on bone regeneration and wound healing were investigated. 10 male and female adult dogs weighing 20 kg underwent a transplantation operation in mandible and maxillary, and AM was applied as a membrane to promote healing. Each animal had both control and experimental injury sites. Tissue samples were obtained after 2, 8 and 12 weeks for histological assay. There were no significant differences between male and female or right and left side of jaws, while the amniotic membrane reduced the development of a fibrin leukocyte layer and inflammation. It also promoted healing and, interestingly, bone regeneration ($p<0.05$).The present results suggest that the human AM could be a suitable cover for various injuries, specifically bone defects, and even the accellular AM could have the property to accelerate healing and bone regeneration, which probably could be attributed to the permessive induction provided by amniotic membranes. AM contains collagen, laminin and fibronectin that could be suitable substrates for the permessive induction in the process of bone regeneration and induce the progenitors or maybe the stem cells to differentiate into osteoblasts.

PO12: Chemicals & pesticides

ID ABS: 34

Reducing animal use under the implementation of REACH – science, policy and legislation working together

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The European regulation relating to the safe use of chemicals REACH (the Registration, Evaluation, Authorisation and Restriction of Chemicals) entered into force on 1st June 2007 after six years of negotiation. A total of 143,000 chemicals have been pre-registered with the Agency responsible for the implementation of REACH (ECHA), although ultimately only 30,000 of these may require REACH registration. The numbers of animals estimated to be required under REACH for these chemicals at the last assessment by the European Commission (JRC 2006) was 8-13 million.

There has been increased research into alternative methods for the toxicological endpoints required under REACH, but now that REACH has started there are immediate deadlines for registration starting with 2010. It is therefore imperative to disseminate the practical, scientific based steps that can be made to reduce animal use prior to these deadlines. This study updates current knowledge by evaluating the further reduction in animals that can be achieved in the immediate to short term by the use of "common sense" animal reduction strategies within the Annexes (existing data not withstanding). For example, use of a combined study for reproductive screening, repeat dose and acute dose endpoints could save up to 5 million animals under Annex VIII, use of 90 day studies instead of both 28 day and 90 day studies could save a quarter of a million under Annex IX. Further savings in animals can be achieved if lessons are learned from the strategies employed under the US HPV programme.

ID ABS: 55

Examining the "value added" of multi-route acute systemic toxicity studies

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Current regulatory frameworks for pesticides and industrial chemicals call for acute systemic toxicity testing by up to three different exposure routes. For example, in the pesticides sector, acute oral, dermal and inhalation studies are required for all active ingredients as well as for all finished formulations/end-use-products. A similar trend is evident in the industrial chemicals sector, with the EU's REACH regulation having initially called for acute systemic toxicity data via a single route, and only for chemicals marketed in volumes of of 10 or more metric tonnes per annum. However, amendments tabled by several EU member states during political negotiations led to acute lethality data being required for all substances covered under REACH (i.e., 30,000 chemicals marketed in volumes 1 or more tonne per year), plus a further requirement for lethality data via a second exposure route for chemicals marketed annually in volumes of 10 or more tonnes. To ascertain the "value-added" by multi-route testing for acute systemic toxicity, a retrospective data analysis was undertaken under the auspices of the European Partnership on Alternative Approaches to Animal Testing (EPAA), which examined the concordance among regulatory classifications for acute oral and dermal toxicity for 1,569 chemicals and 186 pesticides. The findings from this review will be presented, together with recommendations for amendment of relevant sectoral data requirements and criteria for data waivers.

ID ABS: 121

OECD approves updated inhalation toxicity guidelines and guidance document

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In April 2009, the OECD WNT Committee approved four inhalation guidelines and an inhalation guidance document. This was the culmination of a project that began in 2002 and involved the participation of inhalation toxicologists from industry and regulators in eight countries and representatives from FRAME and ICAPO. The primary goals were to 1) provide updated inhalation guidelines that provide robust data while using fewer animals, and 2) minimize the need for multiple studies (and animals) to satisfy diverse regulatory needs of individual countries. Acute inhalation toxicity guideline TG 403 was revised to meet modern testing standards and the specific needs defined by Emergency Response Guidelines. TG 403 gives a study director a choice of two testing protocols – a traditional protocol that uses fewer animals, and a Cxt protocol that yields a value for n in Cnxt. Both protocols allow considerable flexibility in study design. TG 436, an alternative guideline developed by Germany (BfR), uses the acute toxic class testing method. It uses fixed concentrations and requires a minimum of animals, and is primarily designed for classification and labeling. TG412 and TG 413 are 28 day and 90 day inhalation toxicity guidelines, respectively, that were revised to meet modern testing standards. Finally, GD 39 is an inhalation toxicity guidance document that provides additional information for study directors and regulators beyond that contained in the TGs. Other projects still in progress are 1) an inhalation toxicity pathology guidance document by an international team of pathologists, and 2) an acute reference concentration guidance document.

ID ABS: 556

The U.S. Environmental Protection Agency's high production volume challenge program: lessons learned

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The U.S. Environmental Protection Agency's (EPA) High Production Volume Challenge Program, the largest U.S. animal testing program of its time, required industry to make available baseline health and environmental effects data on nearly 2,800 chemicals. As the program ends, we present a quantitative examination of all test plans submitted for compliance with program guidelines on the use of animals. Contrary to claims by the EPA and Environmental Defense that few animal tests would be conducted, new animal tests were proposed in 211 (48%) of the 439 test plans submitted. Many chemical sponsors failed to conduct comprehensive analyses of available data, instead proposing duplicative tests. Opportunities to group chemicals into categories, which reduces testing by allowing "read-across" of data from one category member to others and provides a contextual basis to evaluate toxicity, were often overlooked. However, even when sponsors took this approach, the EPA rejected the proposed categories 59% of the time. Further, although the EPA's guidelines specifically encouraged the use of *in vitro* methods for genetic toxicity testing, *in vivo* tests were still proposed in 32 test plans – and EPA objected to only 11 of these (34%). While we find that guidelines on the use of animals in the voluntary program were followed inconsistently by sponsors – and enforced even less consistently by the EPA – we present successful strategies to reduce testing developed by PETA and the Physicians Committee for Responsible Medicine in collaboration with companies, and discuss their application to future testing programs.

ID ABS: 573

Use of read-across of existing hazard data to fulfill HPV chemical program requirements without the need for new animal testing

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The Soap and Detergent Association (SDA) is a leading manager of chemical consortia fulfilling commitments to the voluntary global International Council of Chemical Associations (ICCA) and U.S. Environmental Protection Agency (EPA) high production volume (HPV) chemical programs. SDA's commitment to compile and make publicly available a baseline set of health and environmental effects data covers 291 chemicals sponsored by 62 companies within ten chemical consortia. The chemical categories represented by these consortia include: aliphatic acids, aliphatic alcohols, alkoxides, alkyl sulfates, amine oxides, glycerides, hydrotropes, LAS/ABS, methyl esters, and triclocarban. Due to the structural similarity of the chemicals within a category their physiochemical and toxicological properties are likely to be similar. This has allowed the utilization of the read-across technique where the data available for some substances satisfy the data need for member chemicals without data. SDA has found read-across to be especially useful in assigning data for ecotoxicity and human health endpoints to many chemicals among its 143 completed chemical commitments to date. Consequently, the use of read-across has eliminated the need for new animal testing while allowing SDA consortia to meet those data requirements. The use of thousands of test animals has been avoided. SDA anticipates additional reductions in animal testing by utilizing read-across in ongoing efforts to complete the data sets for the balance of its sponsored chemicals.

PO13: Cosmetics

ID ABS: 72

Development of human reconstructed epidermis in Brazil and analysis of ceramide production

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Human epidermis models have been identified as useful tools for the testing of phototoxicity, corrosivity and irritancy, and test protocols have been developed for such applications and animal replacement tests. However, these products or services are available only in Europe and EUA markets, where the biggest cosmetics companies of the world are localized. In Brazil, Natura, one of the most important cosmetic companies, has supported research for the development of a human reconstructed epidermis and also a methodology to quantify ceramides in *ex vivo* skin and in human epidermis models. Ceramides (CER) in human stratum corneum play physicochemical roles in determining the skin's barrier and water-holding functions. The aim of the present study was develop a human epidermis model by seeding dissociated keratinocytes in an insert and growing such recombined cultures for 1 wk, exposed to air, at the surface of the culture medium and also to evaluate lipid composition measured by HPTLC. Lipid analyses revealed the presence of CER I, III, VI(1) and VI(2), gliceryl/lactosylceramide (Gli), phospholipids (PL), cholesterol (CHO) and fatty acids (TG). Compared with native epidermis, the content of CER III, IV, VI and fatty acids was lower in the human reconstruction model. Only CHO, TG and CI were higher in the *in vitro* model, indicating the lower presence of complex ceramides. In conclusion this skin model provides a promising means for studying ceramide production, although the observed results need to be improved and morphological analyses provides.

ID ABS: 95

Improving genotoxicity testing: comet assay with 3D skin models

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Since March 2009 animal testing is prohibited for the safety assessment of cosmetic ingredients due to the 7th Amendment of the Cosmetic Directive. The currently available *in vitro* tests are of low predictive value due to a high rate of false positive results. One strategy to address the demand of improved *in vitro* genotoxicity tests is to introduce more relevant test systems into safety assessment (Pease et al., WC7; Carmichael et al., WC7). Since the skin is the first site of contact with maximum exposure for many chemicals, skin models were introduced into genotoxicity testing within the framework of a COLIPA sponsored joint research project involving four laboratories and three different skin models: An epidermal model: EpiDerm™ (MatTek)

and two full-thickness-models: Phenion®-Full-Thickness-Skin-Model (Henkel), and RealSkin (SkinEthic™).

The following technical bases were implemented:

a) Specific protocols for the single cell isolation of keratinocytes
b) A harmonized Comet assay procedure for each skin tissue
c) An SOP for Comet assay slide analysis
d) Statistical criteria to evaluate and define experiments

Based on these standards the effects of genotoxins (MMS, 4NQO) were investigated after topical application to the skin model. A clear dose-dependent increase of the intensity of the comet tail was observed with each skin model. The results obtained with EpiDerm™ could be confirmed in a second laboratory, proving the good transferability of the test system. The harmonized protocol is suited for multiple donors and the inter- and intra-experimental variabilities were within acceptable ranges.

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

ID ABS: 157

An *in vitro* model for assessing the effectiveness of antioxidants in cosmetics

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Oxidative stress in skin has been linked to the development of wrinkles, aging, and other more serious effects. *In vitro* cell-based systems that can rapidly evaluate whether new chemicals/formulations have antioxidant properties are important in the development of new products for the cosmetic industry. The aim of this study was to induce oxidative stress in normal human epidermal keratinocytes (NHEK) and then demonstrate protection in the presence of Trolox (Vitamin E) and test products. Oxidative stress was induced with menadione, which requires metabolism to reactive quinone intermediates, and cumene hydroperoxide, which does not require metabolism to produce oxidative stress. Formation of reactive oxygen species was monitored with CM-H2DCFDA and glutathione (GSH) status. Cell viability was measured with propidium iodide. NHEK cells were seeded into 96-well plates (10,000 cells/100 µL) and cultured with keratinocyte growth medium/10% BSA. Exposures to menadione (20 µM) and cumene hydroperoxide (100 µM) were for 3, 6, or 24 hr. Menadione increased DCFDA signal by approximately 2-3 fold after 6 hr and reduced GSH to less than 50% of controls after 6 and 24 hr. These effects were greatly reduced in the presence of Trolox (50 µM). Cumene hydroperoxide (20 and 100 µM) also produced significant oxidative stress, increasing DCFDA by 8-10 fold vs. controls and reducing GSH levels after 6 hr exposure. These oxidative stress effects were significantly reduced in the presence of Trolox. When the NHEK model was used to evaluate antioxidant properties of new products, it was possible to demonstrate clear antioxidant properties.

ID ABS: 244

Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays II: importance of accurate toxicity measurement

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Current *in vitro* genetic toxicology assays have a high rate of reported positive results when compared with negative rodent carcinogenicity data. A recent analysis of published data highlighted the inaccuracy of current *in vitro* assays, the rate of misleading positive results with a combination of assays was found to be at least 80% (Kirkland et al., 2005, Mutat. *Res. 584(1-2),* 1-256).

As a result of the 7th Amendment to the Cosmetics Directive, which came into force in March 2009, positive results for

cosmetic ingredients can no longer be followed-up by *in vivo* testing. Therefore, *in vitro* assays must be more predictive in order to avoid attrition of promising chemicals. One focus of a European funded project is to determine the optimal measurement of cytotoxicity used alongside measurements of genotoxicity. Comparisons have been made between different measures of toxicity after exposure to previously identified chemicals, giving rise to misleading positive results in the *in vitro* micronucleus assay. Emphasis has been placed on chemicals that have a steep toxicity profile and a maximum testing concentration limited by toxicity. Comparisons were performed between cell counts, mitotic index and replication index measurements. Intracellular toxicity endpoints were also investigated, including apoptosis.

Our results demonstrate that certain measures have the potential to seriously overestimate toxicity, the implication of which is a higher maximum testing concentration, which may contribute to the generation of misleading positive results with *in vitro* genotoxicity assays.

This work is funded by the European Cosmetic Industry Association COLIPA, ECVAM and NC3Rs.

ID ABS: 245

Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays I: choice of cell type

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Current *in vitro* genetic toxicology assays have a high rate of reported positive results when compared with negative rodent carcinogenicity data. Moreover, the rate of misleading positive results with a combination of assays was found to be at least 80% (Kirkland et al., 2005). Poor predictivity was expected to be worst in p53-deficient cell lines of rodent origin, particularly in the long established and widely used Chinese hamster cell lines. Since *in vivo* models have been banned by the EU Cosmetics Directive since March 2009, *in vitro* models need to be more predictive for the risk assessment of cosmetic ingredients.

As part of a larger framework for improvement of *in vitro* genetic toxicology assays the performance of currently used cell lines is being investigated and compared with p53-competent cells (Kirkland et al., 2007). Comparisons have been made between Chinese hamster Lung, Chinese hamster Ovary, V79, TK6, HepG2 and human peripheral blood lymphocytes. These comparisons were made using the *in vitro* micronucleus assay to evaluate clastogenic potential and highlight any differences in sensitivity between cell lines, with a selection of compounds that are accepted as producing misleading positive results in *in vitro* clastogenicity assays (Kirkland et al., 2008).

Micronucleus assay data from these comparisons shows similar patterns of responses between different cell lines; however sensitivity differs markedly, particularly when comparing levels of toxicity and when compared with p53-competent cells.

This work is funded by the European Cosmetic Industry Association COLIPA, ECVAM and NC3Rs.

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ID ABS: 246

COLIPA validation of the reconstructed human skin micronucleus assay (RSMN): a novel micronucleus assay in a 3D human skin model

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Current mammalian cell *in vitro* genotoxicity assays induce a high level of false positive results, leading to a large number of costly and time consuming follow-up *in vivo* genotoxicity studies. As of March 2009, the 7th Amendment to the EU Cosmetics Directive prohibits the use of *in vivo* genotoxicity tests in safety assessments for cosmetics, greatly impacting the assessment of genotoxicity of new ingredients. To address this, the European Cosmetic Toiletry and Perfumery Association (COLIPA) initiated an international project to establish and evaluate more predictive *in vitro* genotoxicity assays using 3D human tissues. One focus has been on the 3D human skin micronucleus assay (RSMN) in EpiDerm™. Since skin is the first site of contact with maximum exposure to many different products including cosmetics, the RSMN assay offers the potential for a more realistic application/metabolism of test compounds for evaluating genotoxicity (Curren et al., 2006; Mun et al., 2009; Hu et al., 2009). The COLIPA RSMN project is a multi-lab initiative involving Procter & Gamble (US), L'Oréal (France), Henkel (Germany), and the Institute for *In vitro* Sciences (IIVS, US). Intra-laboratory and inter laboratory reproducibility have been investigated with model genotoxins mitomycin C and vinblastine sulfate as well as a variety of chemicals that require metabolic activation. In addition studies with coded chemicals are in progress. This model is a promising new *in vitro* method for detecting micronuclei induction in human skin.

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

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ID ABS: 277

Human keratinocytes as *in vitro* model to predict skin metabolism of aromatic amine hair dyes *in vivo*

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As skin contact to aromatic amines can occur during oxidative hair colouring, characterisation of dermal metabolism is important for their safety assessment. We have compared their metabolism in the human keratinocyte cell line HaCaT with that observed *ex vivo* in human skin and *in vivo* after topical and oral application to rats. For 1,4-diamines, the major metabolic pathway was N-acetylation of the amino groups, which was independent of the route of application. The metabolism of aminophenols was via N-acetylation, O-sulfation and, to a lesser degree, O-glucuronidation. After topical application, the relative contribution of the different pathways was altered such that N-acetylation was the predominant pathway. For all aromatic amines incubated with HaCaT cells, N-acetylated products were the only metabolites detected. N-acetylation was found to be the key metabolic pathway of m- and p-aminophenols in *ex vivo* whole human skin discs, and additional experiments are underway for o-aminophenols and 1,4-diamines. Since N-acetyltransferase 1 (NAT1) is the responsible enzyme, kinetics was further compared to the standard NAT1 substrate p-aminobenzoic acid (PABA) in the HaCaT model, revealing similar values for Km and Vmax for m-aminophenols.

In conclusion, NAT1 dependent dermal N-acetylation is considered to represent a "first-pass" metabolism effect in the skin for most diamines and aminophenols prior to entering the systemic circulation. Since the HaCaT cell model represents a suitable *in vitro* assay for addressing the qualitative contribution of the dermal metabolism of topically applied aromatic amines, it can contribute to a reduction in animal testing.

ID ABS: 342

New risk assessment approaches for cancer: assuring safety without animal testing

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In the safety testing of personal care product ingredients, cancer is an extremely important safety endpoint, but current strategies rely heavily on results from non-animal tests being confirmed by "definitive" animal studies. We believe that a new non-animal strategy can be developed. Novel insights are being generated that will be capable of informing a risk-based approach and we are investigating several new technologies to increase our understanding of the complex interactions that occur in biological systems. For example, technology from the field of biophysics is proving to be valuable in understanding the transformation of cells in culture, in response to chemical carcinogen exposure. Successes with several other new technologies (e.g. transcriptomics, metabolomics) are also beginning to show that discrimination between chemicals known to cause cancer and those that do not, can be achieved *in vitro*, based on mechanistic understandings. The challenge ahead will be to integrate these data to allow risk assessment to be performed for new chemicals in consumer products under the conditions of use. The application of systems biology approaches to anchor the *in vitro* measurements to relevant biomarkers and pathology pathways will be key in this regard, along with a better understanding of exposure and low-dose considerations, in order to establish the thresholds at which these events occur.

ID ABS: 381

Comparison of skin sensitization potentials of decursin with different purities by evaluating the expression of surface markers on THP-1 cells

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Evaluation of the skin sensitization potential is an important part of the safety assessment of new ingredients in cosmetics and drugs to be applied topically. Human hematopoietic cell lines respond to a variety of chemical allergens by up-regulating expression of the various co-stimulatory molecules. Especially, the expression of CD54 and CD86 on the human monocytic leukemia cell line THP-1 is used as an indication for skin sensitization potential after intensive investigation as an *in vitro* alternative approach for the identification of contact sensitizers. Plant-derived bioactive materials have long been used as a good source of active cosmetic ingredients. However, many plant extracts have been excluded from the list of usable cosmetic ingredients after *in vivo* clinical tests due to skin sensitization potential, although pure active agents were not skin sensitizers. In this study, we evaluated skin sensitization potentials of decursin (CAS No. 5928-25-6), a major pyranocoumarin isolated from the roots of *Angelica gigas* by measuring the expression of CD54 and CD86 on THP-1 cells after 24 h exposure to various concentrations of decursin with 70% and 98% purities. The decursin with 70% purity showed a mild skin sensitization potential but the decusin with 98% purity did not. These data suggest that the impurities in crude decursin are the major origin of the sensitization potential, and expression of CD54 and/or CD86 in THP-1 cells may discriminate the slight difference in sensitizing potential of materials.

ID ABS: 447

Eye and skin irritation in 3D human tissue constructs using MTT and ATP endpoints

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The irritation potential of formulations and ingredients for industrial screening and product development is often conducted using *in vitro* 3D ocular and epidermal tissue constructs. To predict irritation potential, tissue viability is determined by MTT reduction in live cells. Two issues may contribute to inaccurate viability assessment: subtoxic exposures that induce higher metabolic rates (i.e., hormesis) and chemicals that directly reduce MTT (e.g., NaOH, α-tocopherol (α-t), ascorbic acid). For such chemicals, freeze-killed tissues are used to estimate chemical-mediated reduction of MTT. Alternative methods of measuring tissue viability, such as adenosine triphosphate (ATP) measurement may be useful. We compared these two methods by testing a model mild skin care formulation spiked with and without α-t and with various concentrations of Triton® to induce a range of toxic effects. For formulations with α-t, freeze-killed tissues were tested in parallel. The MTT assay results showed the same irritancy predictions for the 4 formulations containing α-t as without α-t (e.g., formula with highest Triton conc.: ET_{50} eye = 172 and 157 min, ET_{50} skin = 778 and 772 min, with and w/o α-t). The ATP assay provided the same rank, although the relative viability values were overall lower (e.g., formula with highest Triton conc.: ET_{50} eye = 14.5 and 12.9 min, ET_{50} skin = 202 and 231 min, with and w/o α-t). In summary, the MTT assay of formulas capable of MTT reduction should include freeze-killed tissues, and the ATP assay can confirm the relative rank order of the irritancy predictions.

ID ABS: 450

The safety evaluation of cosmetic ingredients at the EU level and the use of 3R alternatives: an update

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The testing and marketing ban in the EU cosmetic legislation calls for urgent substitution of *in vivo* toxicological studies by replacement alternatives.

As the European Scientific Committee on Consumer Safety (formerly called SCC(NF)P) routinely assesses cosmetic ingredients for which suspicion of potential toxicity exists, a database compiling the contents of the freely available SCC(NF)P opinions was programmed and updated meticulously. The aim was to extract objective and statistically relevant information on the incorporation of 3R alternatives in the submitted cosmetic ingredients' dossiers.

The presented results cover 250 SCC(NF)P opinions issued between 2000 and 2009 and reveal a steady incorporation of 3R alternative methods into the industry submissions. Besides the well-established *in vitro* dermal absorption and *in vitro* battery of mutagenicity/genotoxicity tests, the majority of encountered alternatives concern refinement and reduction tests for acute oral toxicity and skin sensitisation. Only few replacement assays are present, and these are mostly concerned with skin and eye irritation. Some reoccurring comments in the SCC(NF)P opinions reveal problems with the replacement methodologies mentioned, such as false positives for mutagenicity.

For the endpoints of repeated dose and reproductive toxicity, full replacement alternative strategies do not exist, and therefore animal studies are still performed.

It remains crucial to focus not only on development and validation of alternatives, but also on post-validation, including a clear definition of the applicability domain of the alternative method. The latter is indispensable for the highly needed regulatory acceptance of alternative methods, not only for cosmetics but also for other sectors.

ID ABS: 466

In vitro eye and skin irritation assessment of consumer products containing volatile organic compounds (VOCs) in 3D tissue constructs: modified dose

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The Alberto-Culver Company uses a topical application time-to-toxicity protocol in *in vitro* 3D human ocular and epidermal tissue constructs to assess the irritancy of a wide range of product formulations and ingredients. The irritation of some products with VOCs (e.g. hair sprays, mousses, etc.) has been over-predicted by these methods relative to clinical results. Since there is evidence that small chain organic solvents may induce excessive toxicity, we compared the irritation potential of a series of hair sprays with varying VOC concentrations using the standard 100 μl "infinite" dose and a modified 30 μl dose (the minimum volume assuring full coverage of the tissue surface). The rationale was that a lesser dose volume would result in similar or lesser irritation prediction and would more accurately model typical incidental exposure of eye and skin to alcohol-containing hair care products. Eight hair sprays containing 13%-93% VOC along with 3 reference materials (ethanol at 80%, 55%, and 6%), were evaluated. In the EpiOcular™ assay, these materials resulted in ET_{50}'s ranging from <1 minute to 60 minutes when dosed with 100 μl, and 1.1 minutes to 3.3 hours when dosed with 30 μl; in the EpiDerm™ assay, these materials resulted in ET_{50}'s between 2 to 20 hours when dosed with 100 μl, and >24 hours when dosed with 30 μl, indicating a significant dependence of cytotoxicity on dose volume. The effectiveness of the system has been assessed by comparing the *in vitro* results with clinical data and consumer experience information.

PO14: Pharmaceuticals

ID ABS: 4

Multiple drug interaction in chick embryos

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It is not surprising that the administration of one drug may modify the action of another drug when given simultaneously. However there are not very many reports of multiple drug interactions. Almost all of the studies involve just two drugs. In the present study, we investigated the multiple drug interaction between amitriptyline, fluconazole and disopyramide in chick embryos. Fertilized eggs of White Leghorns were incubated, and amitriptyline, fluconazole and disopyramide were injected into the fertilized eggs. Electrocardiograms were recorded 60 minutes after the injection, and heart rate was determined from RR intervals. 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. In this study, the heart rate was significantly decreased by combination of amitriptyline, fluconazole and disopyramide. In addition, arrhythmia was produced by combination of amitriptyline, fluconazole and disopyramide. These findings indicate that the multiple drug interaction of amitriptyline, fluconazole and disopyramide has a marked influence on the heart rate of chick embryos. Although the exact mechanism underlying the multiple drug interaction of the pharmacological effects of the drugs remains to be clarified, the multiple drug interaction seems to enhance the toxicity of the drug in chick embryos.

In conclusion, our *in ovo* recording system for ECG of chick embryos may be useful for investigating the multiple drug interaction of amitriptyline, fluconazole and disopyramide.

ID ABS: 148

Cardiotoxicity of anticancer drug in chick embryos

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Arsenic trioxide (ATO) is an anticancer drug and is currently one of the main treatments for relapsed or refractory acute promyelocytic leukemia. However, adverse effects have been observed after ATO treatment, including cardiac toxicities. It is important that we consider the carefully monitored use of ATO. With the recent concern for animal rights, experimental studies using mammals have been limited in number and methods. Thus, based on social acceptance, experimental studies using chick embryos have drawn attention. Chick embryos have been widely used in pharmacological and toxicological experiments for evaluating drug action. In this study, we investigated the cardiotoxicity of ATO using a chick embryo model. The chick embryonic heart has been used often in pharmacological and toxicological experiments. Electrocardiograms (ECG) were recorded 60 minutes after the injection, and the heart rate (HR) was determined from RR intervals. ATO (0.25 mg-1.0 mg/egg) was injected into fertilized eggs. In the low dosing, HR was not different compared with control. However, HR was significantly decreased by high dosing. HR significantly decreased in a dose- and time-dependent manner ($p<0.05$). In addition, arrhythmia was produced by high dosing. These findings indicate that ATO has a marked influence on the heart rate dose- and time-dependently in chick embryos. We have demonstrated in this study that an electrocardiogram system using chick embryo may be applied as an animal test alternative. In conclusion, our *in ovo* recording system for ECG of chick embryos may be useful for investigating the cardiotoxicity of anticancer drugs.

ID ABS: 189

HepaRG® cell line: a new model for the detection of reactive chemicals and for the possible prediction of chemical-induced cholestasis and steatosis

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Human HepaRG® cells represent the first human hepatoma cell line able to differentiate *in vitro* into hepatocyte-like cells and display several hepatocyte-like functions at levels comparable to those measured in primary human hepatocyte cultures.

Several lines of evidence have demonstrated that HepaRG® cells exhibit (I) a hepatocyte-like morphology; (II) a greater metabolic competence for phase I and II enzyme activities; (III) a concomitant expression of hepatic influx and efflux transporters; (IV) a good inducibility of drug metabolizing enzymes (Aninat et al., 2006; Le Vee et al., 2007; Kanebratt and Andersson, 2008).

Recent results suggest that the HepaRG® cell line could be used as a promising *in vitro* and long-term hepatocyte model for:

- investigation of enzyme induction in drug discovery
- evaluation of hepatic drug transport function
- characterization of hepatotoxic effects of cholestatic and steatotic molecules

References

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ID ABS: 262

Effect of silymarin on the apoptotic index of the adrenal cortex of male hamsters

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Silymarin is a polyphenolic flavonoid that has a cytoprotective effect and is used as a phytopharmaceutical drug in some supportive therapy. The adrenal cortex is essential for life and its function can be affected by many chemical agents and drugs. This study was conducted to quantify the apoptotic index in the adrenal cortex of silymarin treated hamsters.

In this study 10 adult male hamsters were randomly allocated to two groups, i.e. control and experimental group, which was injected i.p. with 100 mg/kg of silymarin for seven consecutive days. After eight days, the animals were euthanized and the adrenal glands were quickly removed, weighed and fixed in buffered formalin. The samples were processed by routine and standard paraffin embedding and serially sectioned in 3ì thickness. Detection of apoptotic cells was performed by nonradioactive *in situ* end labeling using the TUNEL immunocytochemical technique, and the apoptotic index in different adrenocortical zones was assessed by unbiased stereological methods.

Results showed that the highest and lowest apoptotic index (AI) was observed in *zona reticularis* and *zona glomerulosa* of both groups, respectively. However AI decreased in all adrenocortical zones of silymarin treated hamsters compared to controls, but was significant only in *zona reticularis* ($p<0.05$).

It can be concluded that silymarin seems to be a suitable protective drug for adrenal glands and it reduce normal apoptosis in the adrenocortical structure.

ID ABS: 371

Papain *in vitro* cytotoxicity determination by simultaneous bioassays using murine fibroblasts

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Enzymes are essential to any biochemical industrial process. Papain is a cysteine protease, obtained from papaya latex, which holds among its textile, food, dentistry, pharmaceutical, cosmetic and medical applications, cutaneous absorption enhancement and debridement properties, which are particularly useful for scar and wound treatment, highlighting its relevant potential in such fields and also conferring this biomolecule an ability to be characterized as a model enzyme. Simultaneous *in vitro* cytotoxicity bioassays using vital or non-vital colorants were applied in order to quantify, qualitatively and quantitatively, its bioactivity on murine fibroblast cells (CCL 92). Such tests were performed using papain in several different concentrations (from 0.005 to 0.4 % (w/v)) for 24 or 48 hours of contact at 37°C, 97% humidity and 5% CO_2 in cell culture flasks. The viable cells were measured by the MTS/PMS method, where the active component is a tetrazolium compound and the vital cells reduce it to a colored formazan product that is quantified at 490 nm. After this procedure, the adherent cells were colored using Rhodamine B as a non-vital dye. The qualitative analysis revealed an inhibitory concentration (IC_{50}) of 0.001004 mmol/l after 24 h and 0.001017 mmol/l after 48 h exposition and a lethal dose (LD_{50}) of 0.2092 mmol/kg (24 h) and 0.2104 mmol/kg (48 h), respectively. The qualitative assay indicated a trypsin-like action at the 0.02-0.03% (w/v) concentration range. Despite the difference between the experiments, they seemed to be complementary, revealing the need for a better understanding, standardization and validation of *in vitro* assays for enzymatic compounds.

ID ABS: 376

Experimental animal alternative model for diabetes in chick embryos

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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. Streptozotocin (STZ) has been used as a model of insulin-dependent diabetes in animals. However, it was not known whether STZ induces any changes in the pancreas of chick embryos. In the present study, we determined the levels of blood glucose in chick embryos treated with different doses of glucose aiming to establish a model of diabetes in chick embryo by treatment with STZ. Furthermore, we evaluated the effects of human insulin using this diabetes model. Fertilized eggs of White Leghorns were investigated. The different doses of glucose were injected into the eggs. STZ was injected into each egg. Regular human insulin was injected into the air sac of STZ-treated eggs. The levels of blood glucose and serum insulin were determined. Levels of blood glucose and serum insulin in chick embryos increased with developing stages. Levels of blood glucose increased dose dependently. Blood glucose levels of chick embryos treated with STZ were significantly higher than those in the controls. Conversely, the serum insulin levels were lower than that in the controls. In addition, the enhanced levels of blood glucose in STZ-treated embryos were reduced by injection of human insulin. In the present study, we propose an experimental animal model for diabetes in chick embryos treated with STZ and describe the effects of human insulin using this model.

ID ABS: 592

Good practice guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals

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The National Centre for the Replacement, Refinement and Reduction of Animals in Research and the Laboratory Animal Science Association share the goal of promoting the principles of the 3Rs. They have formed an expert working group of toxicologists from the UK's major pharmaceutical companies and contract research organisations to facilitate the sharing of best practice in the design/conduct of regulatory toxicology studies.

Since the objective of toxicology studies in animals is to identify potential toxic effects in humans, some of the animals used will suffer adverse effects. Careful design of studies can reduce the impact on the animal without compromising scientific goals or human safety. Within this context the group have produced a guidance document on dose level selection for regulatory toxicology studies for pharmaceuticals (see www.nc3rs.org.uk) which is intended to promote the application of the 3Rs.

By applying the principles in the document it is possible to reduce and refine the use of animals by:

Preventing repetition or unexpected termination of a study by:
- Minimising variation between sequential studies
- Applying prior knowledge and findings from database searches
- Interactively managing studies

Reducing additional animal studies by:
- Identifying the maximum tolerated dose in early studies
- Identifying target organ toxicity early in test item development
- Not using practical limitations to determine dose level selection

Minimising the number of animals at risk of experiencing adverse effects by:
- Employing a staggered approach to dosing
- Predicting adverse effects from knowledge of the target
- Use of humane endpoints

PO15: Food improving agents

ID ABS: 41

Physiological studies of rats fed large doses of Jerusalem artichoke concentrate

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Jerusalem artichoke is a well known source of inulin, which acts as a prebiotic, a stimulator of calcium absorption and a sweetener in the diabetic diet. Tubers of the Jerusalem artichoke variety "Dag Neutral" were obtained from the Horticultural Study Centre "Pure" Ltd (Latvia). Jerusalem artichoke concentrate (JAC) is produced by traditional technologies with preservation of the biologically active substances and microelements.

The study aimed to determine the influence of JAC on body weight and lipid metabolism in laboratory Wistar rats in comparison with another preparation of inulin called raftilin from the company "Orafti". The animals were fed large doses of JAC (1.5 g/kg) and raftilin (0.8 g/kg) daily for 90 days. During the experiment, the rodents were held in individual feeding cages and had unlimited access to water and standardized food.

Body weight and the levels of cholesterol (total, HDL, LDL) and triglycerides were determined every 30 days. The group being fed JAC displayed lower weight gain compared with the raftilin and reference groups ($P<0.05$). At the end of the experiment a decrease in cholesterol and triglyceride levels was found in blood serum of rats that had been fed JAC. The levels of total cholesterol (16%) and triglycerides (15%) in the blood serum lower in the JAC compared with the raftilin and control groups. No negative influence of large doses of JAC on the general health status of the rats was observed. JAC produced by traditional technologies has a positive impact on the levels of lipoproteins and may be safety used to produce functional food.

ID ABS: 101

In vitro system for testing the safety and the effectiveness of functional food ingredients using zebrafish embryos

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There is an increasing interest of the food industry to employ functional molecules in foodstuff. Each new ingredient intended for human consumption must be tested for its safety as well as for its actual effectiveness. Methods for assessing toxicity and effectiveness in this field are expensive, time consuming and involve the sacrifice of a high number of laboratory animals. The present work describes an alternative method for such purposes, based on the use of zebrafish embryos. The method combines traditional morphological observations with the analysis of the differential expression of a number of selected biomarker genes for detecting toxicity and a determinate type of effectiveness. Fish embryos are not considered animals from a legal point of view, and this implies that tests carried out on them can be regarded as *in vitro*. However, unlike other types of *in vitro* tests, experiments carried out with embryos provide much wider information, as the effects detected represent the response of an entire organism.

So far we have used this method for assessing the possible toxicity of nanosilver and for confirming the immuno-stimulant effect of some compounds such as β-glucan. We identified specific biomarker genes able to detect toxicity of nanoparticles and biomarker genes specific for detecting an immunostimulant effect. The method proved to be rapid, reliable and cost effective.

ID ABS: 281

Green tea flavonoids inhibit migration of human neural progenitor cells *in vitro*

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Flavonoids have been proposed to protect against a multitude of diseases like cancer or cardiovascular disease. Therefore, these compounds are increasingly used as dietary supplements in functional food. The daily intake of flavonoids can exceed 1 g, and plasma levels of 0.3-7.5 μM were measured in humans. Their potential for toxicity, however, is an understudied field of research.

Flavonoids interact with a large number of cell signaling pathways contributing to their beneficial effects, e.g. their antitumorigenic properties. However, many of the signaling pathways which are stimulated during tumor growth are also necessary for developmental processes like cellular survival and migration. Therefore, the aim of this study is to assess the effects of flavonoids on developmental processes *in vitro*.

Treatment of normal human neural progenitor cells with different green tea flavonoids (epigallocatechingallate, epicatechingallate, epigallocatechin and epicatechin; 1 to 10 μM) disrupted cell migration without causing cytotoxicity. Furthermore, adhesion assays pointed out that disturbed migration is caused by interference with normal cellular adhesion. We showed that this is due to an interaction of catechins containing a gallate group with the extracellular matrix. We ruled out that integrins are involved in this effect and are currently pursuing the underlying mechanisms.

PO16: Genetically modified organisms

ID ABS: 177

Sharing and archiving of genetically altered mice: opportunites for reduction and refinement

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The number of genetically altered (GA) mice used in scientific procedures has risen significantly over the last 15 years and continues to do so. This raises animal welfare and ethical concerns, as well as scientific and logistical issues with respect to the generation, breeding, maintenance and use of these animals. Implementation of the 3Rs in this field presents particular challenges.

With this in mind, the RSPCA in the UK convened a working group, in association with the Medical Research Council (MRC), Biotechnology and Biological Sciences Research Council (BBSRC), and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) to discuss how archiving and sharing of information and material relating to GA lines could provide opportunities for Reduction and Refinement.

In 2009, the Group published a report which provides an overview of current "best practice" in this area and sets out simple measures by which every researcher who creates or uses genetically-altered mice can reduce and refine their animal use. The key points and recommendations from the report will be presented, including information on what to archive, when to do so and how it can be done. How to share GA lines is also addressed.

For more information and a copy of the report contact: nosborne@rspca.org.uk

This poster is presented by Paul Littlefair and Nikki Osborne, on behalf of the RSPCA Resource Sharing Working Group

ID ABS: 178

20 years of hypertension research using genetically modified animals: no clinical approaches in sight

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The incidence of essential or primary hypertension is increasing, but although the disease displays clear symptoms, its aetiology appears very complex, and thus no causal treatment is available yet. In the 1990's, genetically modified animals (GMO) were considered to be the key to solving this problem of high complexity. However, until now, although a few approaches have shown that old, well-known drugs have a positive effect (decrease of blood pressure) on such animal models of hypertension, no approach has appeared in the literature of this area of research which might indicate a direct connection between GMO and a therapeutic strategy to treat or prevent this type of hypertension in humans. Instead, criticism of the GMO approach has accumulated in the last years, arguing that it is misleading as this disease does not have a monogenic cause and so complementary regulatory mechanisms could prevent the true identification of the function of the modified genes. Furthermore, the technology is best developed in mice, whose physiology of blood pressure is different from that of humans. Because of species specificity, it is not easy to extrapolate the results from animal models of hypertension to human hypertension. Also, in the years 2000 to 2004 a reorientation of the technology and the aims of this kind of research took place. Therefore, although these approaches are without exception deemed "very promising" in the literature, it cannot be expected that research on GMO will make any contribution to a new therapeutic strategy in the near future.

ID ABS: 212

Histopathological evaluation of liver and kidney in rats fed with genetically modified Bt corn for three generations

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Novel foods and ingredients which do not have a history of safe use are required to be evaluated for safety. That is the reason for an increased number of feeding studies with genetically modified (GM) foods. However, the evidence is still far from proving whether the long-term consumption of GM foods poses a possible danger for human or animal health. This study was designed to evaluate the effects of transgenic Bt corn on the rats that were fed through three generations with either GM corn or its conventional counterpart. To investigate the probable toxic effects, we obtain the liver and kidney tissues of F3 rats for histopathological examinations. Besides serum analysis was performed for liver and kidney function tests.

Relative organ weights of rats in Bt and reference groups were decreased. Focal mononuclear cell infiltration, congestion, granular degeneration, nuclear border changes in liver and hemorrhage, enlargements in parietal layer of Bowman's capsule and minimal tubular degeneration in kidney were observed. The average diameter of glomeruli and glomerular volume decreased while cortex thickness did not change in the kidney of rats in Bt and reference groups. According to biochemical analysis there were some changes in creatinin, total protein and globulin levels.

ID ABS: 476

Report of the ZEBET/BfR expert workshop: implementation and enforcement of the 3Rs principle regarding transgenic animals used for scientific purposes

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In spite of great efforts to lower the number of vertebrates used for scientific purposes in Germany, records indicate rising numbers over the past years, thereby exceeding the 2.6 million mark for the first time in 2007. To great extents, this development is due to the increasing use of genetically modified (i.e. transgenic) animals. In May 2009, the German Federal Institute for Risk Assessment hosted an international workshop to conjointly evaluate specific measures and approaches to effectively confine the use of transgenic animals for scientific purposes in the future. During the first day of the public symposium experts from both scientific institutions and affected stakeholders discussed the current status and value of transgenic animals used for scientific purposes. In following themed breakout groups, short lectures were given by selected experts on the kinds of methods they utilize, thereby focusing on the respective potential to replace, reduce or refine experiments that exploit transgenic animals. Existing gaps of knowledge and demand for future research directions toward alternative methods have been identified and specified in a round table discussion. Participants developed recommendations in concordance with the 3Rs principle, addressing both regulatory authorities and scientists applying transgenic animals. A workshop report will be published shortly.

PO17: Nanotechnology applications

ID ABS: 356

Precision cut lung slices to assess the toxicity of nanomaterials: limitations of the lactate dehydrogenase (LDH) cytotoxicity assay

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The increased use of nanomaterials demands toxicological test systems which comply with the unique physiochemical characteristics of nanomaterials compared to the bulk material. Respiratory exposure is of very high concern for nanomaterials, and precision cut lung slices (PCLS) may offer a test system to assess the lung toxicity of nanomaterials without *in vivo* inhalation studies. We used PCLS to study the cytotoxicity of titanium dioxide and several cobalt ferrite nanomaterials with and without serum protein stabilization to prevent agglomeration of the nanoparticles.

Using mitochondrial activity as an indicator of cytotoxicity (WST assay), increasing concentrations of particles (with and without serum protein stabilization) caused increasing levels of cytotoxicity in PCLS. However, there was no change in the lactate dehydrogenase (LDH) levels caused by serum protein-stabilized particles. Moreover non-stabilized particles caused a decrease of background LDH levels in the PCLS culture supernatant, which we have shown to be due to adsorption of LDH onto the surface of the non-stabilized particles, whereas serum protein stabilization blocked the absorption of LDH.

We have thus demonstrated that the cytotoxicity of nanomaterials in PCLS does not correlate with disrupted membrane integrity followed by LDH release. And furthermore, we established that intracellular enzymes, such as the marker enzyme LDH, are able to bind onto surfaces of nanoparticles and thereby adulterate the detection of toxic effects. Thus the method(s) to assess nanomaterial-mediated effects have to be carefully chosen based on the cellular effect and possible nano-specific artifacts.

This study was supported by EC FP6 funding – CellNanoTox research (NMP-CT-2006-032731).

ID ABS: 420

No evidence of uptake of oxidized multi-walled carbon nanotubes by Caco-2 cells, by TEM analysis

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Carbon nanotubes have many current and anticipated applications due to their high strength to mass ratio, their electrical and thermal conductivity, and the ease with which their surfaces can be functionalized with biological molecules. Various studies have demonstrated that multiwalled carbon nanotubes (MWCNTs) can cause oxidative stress, cell death, and genetic damage. Although there are little data on environmental and occupational releases and exposures to MWCNTs, it is anticipated that exposures will occur as production and applications increase. However, it is not clear whether MWCNTs will be taken up following dermal, inhalation, and ingestion exposure. Due to difficulties in detecting carbon nanotubes, *in vitro* studies of isolated systems coupled with sensitive imaging techniques are useful for understanding how MWCNTs interact with biological tissue. In this study, intestinal transport of oxidized MWCNTs was assessed using the Caco-2 cell culture model, which is often used for studying intestinal transport of pharmaceuticals. The oxidized MWCNTs were well-characterized in terms of size, surface area, and surface oxygen content. Cells were exposed to MWCNTs for 15 minutes to 24 hours and analyzed by transmission electron microscopy (TEM). The images revealed that the oxidized MWNCTs did not aggregate in the media and were not taken up by the Caco-2 cells. The oxidized MWCNTs were present outside the cell and adjacent to the microvilli, yet did not interact with the cell membrane and were not visible within the cell. These results suggest that oxidized MWCNTs are not readily taken up by absorptive intestinal cells.

ID ABS: 465

In vitro approaches to characterizing interactions between engineered silica nanomaterials and DNA

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The rapid transition from research to application in nanotechnology presents a challenge to existing toxicological testing paradigms. Nanomaterials are engineered materials that can have a range of shapes, sizes, and surface modifications, creating a large number of materials requiring assessment. There is concern that standard *in vivo* testing methods may not detect their hazards, which may differ from those of their constituent materials (such as heavy metals). Nanomaterials are deliberately designed to possess specific physical or biological actions, such as binding to biological molecules for diagnostic or therapeutic purposes. These properties are difficult to detect in the *in vivo* systems often used in toxicity testing. In this study, we explore interactions of engineered silica nanoparticles (SiNPs) with DNA as an example of applying alternative methods to understanding both benefits and risks. We used SiNPs manufactured at the Institute of NanoBioTechnology (INBT) at the Johns Hopkins University. These are amine-functionalized spherical silica particles, 25 or 250 nm in diameter. These SiNPs are made with embedded fluorescent dyes that permit unambiguous detection of cellular uptake and binding to intracellular macromolecules. With these properties, we can determine the interactions between SiNPS and DNA qualitatively and quantitatively using techniques that include electrophoresis, densitometry, fluorimetry, and fluorescent microscopy. This system can also be applied to studying a range of NPs in order to determine the impacts of varying physical, chemical and surface properties.

Research supported by grants from INBT, Johns Hopkins University.

ID ABS: 528

In vitro methods for nanotoxicity testing

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Nanomaterials present unique challenges to researchers seeking to assess toxicity, targeting, and chemical activity. Because animal testing has proven cumbersome due to problems with measurement, dosing and tracking, and is often unpredictive, many researchers in the field of nanotechnology aspire to use high-tech *in vitro* methods for scientific as well as for ethical reasons. In a recent landmark report, Toxicity Testing in the Twenty-first Century: A Vision and a Strategy the National Academy of Sciences states that, "Toxicity testing is approaching a scientific pivot point… It is poised to take advantage of the revolutions in biology and biotechnology. Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods…". The field of nanotechnology is in a position to make that vision a reality. This review describes the benefits of using *in vitro* human cell-based organotypic models and highlights some of the most promising methods for nanotoxicity studies. Included in the discussion will be human cell-based models of exposure routes (dermal fibroblasts, lung epithelial cells, and colon cells); movement across barriers (blood brain barrier, lung epithelia); target effects (astrocytes, glial cell, macrophages, T-cells, liver cells, kidney cells); and diseased tissues (liver carcinoma cells, B2-microglobulin cells, corneal cells, and lung cancer cells). In addition, lab-on-chip devices with the ability to test toxicity and targeting will be described.

PO18: Vaccines and biologicals

ID ABS: 3

An *in vitro* biochemical assay system alternative to the *in vivo* histamine sensitisation test for pertussis vaccines

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Pertussis vaccines are important for the prevention of the disease pertussis (whooping cough). The histamine sensitisation test (HIST) is currently the official toxicity test for acellular pertussis containing combination vaccines. HIST is a lethal challenge procedure which requires a large number of animals due to large variations in test performance. There is an urgent need to develop alternatives to the HIST. Pertussis toxin (PTx) has the typical A-B type structure of many bacterial toxins, having an enzymatic A-monomer, the S-1 subunit and a binding B-oligomer of subunits S-2 through to S-5. An *in vitro* enzymatic-HPLC coupled assay to measure the A-monomer and a carbohydrate binding assay to measure the B-oligomer activities were developed. Further validation of the developed assay system with the *in vivo* HIST was carried out. Using multiple regression analysis, a mathematical equation linking with the *in vitro* multi-functions of carbohydrate binding and enzymatic activities has been identified for predicting the *in vivo* pertussis toxin activity. There is a clear correlation between the *in vivo* and *in vitro* results. However, the regression coefficients and constant factors were found to be product specific, which indicates and emphasises the importance of validation for each product.

ID ABS: 6

Progress in the development of *in vitro* alternatives to the *in vivo* histamine sensitisation test for pertussis vaccines

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Pertussis vaccines are commonly used in Europe and worldwide for the prevention of the disease pertussis (whooping cough). The histamine sensitisation test (HIST) is currently the official batch release test for acellular pertussis containing combination vaccines. HIST is a lethal challenge procedure which requires a large number of animals due to large variations in test performance. This often leads to repeated tests caused by invalid assays, a frequent occurrence with this procedure. Thus, there is an urgent need to develop alternatives to the HIST. Pertussis toxin (PTx) has the typical A-B type structure of many other bacterial toxins, having an enzymatic A-monomer, the S-1 subunit and a binding B-oligomer of subunits S-2 through to S-5. An *in vitro* enzymatic-HPLC coupled assay to measure the A-monomer and a carbohydrate binding assay to measure the B-oligomer activities were developed. Further validation of the developed assay system with the *in vivo* HIST was carried out. The results indicate that regression coefficients and constant factors are product specific. By using a mathematical equation linking with the multi-functions of carbohydrate binding and enzymatic activities, there is a clear correlation between the *in vivo* and *in vitro* results. In addition, in order to understand the mechanisms of the interaction between host cell and PTx/ or plus other vaccine antigens in *in vivo* HIST system, human cell-based assays were developed to investigate the biological and physiological effects of PTx on cells in particular in combination with other vaccine components/final formulation.

This project is supported by APC (Home Office, UK) and NC3Rs (UK)

ID ABS: 23

Development of an alternative *in vitro* method to test vaccines against pertussis for the presence of active pertussis toxin

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Pertussis, or whooping cough, is caused by the bacterium *Bordetella pertussis*. The disease is highly contagious and mainly affects young children. Currently used acellular vaccines contain inactivated pertussis toxin (PT). Regulatory bodies such as the European Pharmacopoeia require that all vaccines are thoroughly tested for residual active pertussis toxin, as this could cause severe side effects. The standard test for PT is performed *in vivo*, based on the fact that PT increases the sensitivity of mice to histamine. Since this conventional test involves lethality for part of the animals involved there is a clear need for replacement, all the more since standardisation of the test proves difficult.

Cultured Chinese hamster ovary cells (CHO) exhibit a highly interesting property: they cluster upon exposure to active PT in a dose-dependent manner. Although the underlying molecular mechanism remains to be elucidated, this phenomenon might well provide the basis for a novel animal-free test for the presence active PT in vaccine batches. The present study was performed to explore the potential of this CHO-based PT test by optimizing the conditions for CHO cell culture and PT exposure. In this project, the presence of aluminium phosphate (the PT vaccine adjuvant) proved a complicating factor since this salt turned out to interfere with CHO cell growth. Software was developed to objectively assess the extent of CHO clustering in a high-throughput manner. The lower limit of detection (10 ng/ml of active PT), as obtained by automated assessment, was comparable to the results that were achieved by visual inspection.

ID ABS: 42

Routine and experimental application of the monocyte activation test (MAT) in the Paul-Ehrlich-Institut (PEI)

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Several products are tested for pyrogens (fever inducing substances) by the Rabbit Pyrogen Test (RPT). For more than ten years the PEI has been validating and optimising alternative pyrogen tests based on the release of fever inducing cytokines by human monocytes (Monocyte Activation Test). More than 150 batches of various products have been tested in parallel. Until recently we estimated (calculated) the intravenous pyrogenic dose (limit) of the utmost important pyrogen lipopolysaccharide (LPS) for humans at 40-50 pg LPS/ml blood. An *in vivo* calibration (volunteers vs. MAT using blood of the volunteers) was performed, which confirmed the threshold pyrogenic level of 50 pg LPS/ml. For intravenous application the Pharmacopoeia-threshold is 500 pg LPS/kg body weight. Based on the knowledge of the LPS limit for human beings (and the resulting cytokine release) the limit test can be easily applied for products with a defined endotoxin limit and/or known dosage.

The MAT Monograph (2.6.30) was adopted in March 2009 by the European Pharmacopeia Commission.

The MAT outperforms the "Gold Standard" RPT in several ways. The MAT is more predictive than the RPT, our data indicate that there are species-specific pyrogens like purified lipoteichoic acid. In addition to routine testing the MAT offers the first possibility to test advanced therapeutic medicinal products (ATMP), which could not be tested by RPT or LAL (Test for LPS) until now due to interferences.

Altogether the MAT is not solely a replacement for an animal experiment. It is widening the possibilities for providing pharmaceutical safety.

ID ABS: 99

Replacement assays for the testing of *Clostridium septicum* vaccine antigens

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Clostridial species, such as *Clostridium septicum*, which produce powerful cytotoxins, are responsible for a variety of severe animal diseases. As a result, vaccine manufacturers produce clostridial toxins which, once chemically inactivated to form toxoid antigens, are used in many veterinary vaccines. Each batch of these materials has to be subjected to toxicity testing and antigen quality measurement. These tests use mice, and it is estimated that worldwide tens of thousands are used annually for these purposes. Certain cell lines are known to be sensitive to specific clostridial toxins. It should therefore be possible to replace these mouse tests with cell line assays. Our research has initially concentrated on replacement tests for the toxin and toxoid antigen of *Cl. septicum*. The VERO cell line was found to be suitably sensitive, and it has been used to develop *in vitro* replacement assays for the mouse toxicity and antigen quality tests. The cell line assays have been shown to have a 99% or better agreement with the mouse tests. Similar *in vitro* assays are being developed for other clostridial toxins and toxoids. Based on this work it should be possible to develop cell line replacement assays for many of the common clostridial vaccine antigens with a resulting substantial reduction in mouse usage.

ID ABS: 102

Pertussis toxin-associated toxicity in combination vaccines: an *in vitro* model of leukocyte-endothelial interactions at the blood-brain barrier

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Residual pertussis toxin (PTx) activity in pertussis combination vaccines is thought to play a major role in adverse vaccine reactogenicity, including encephalopathy and other neurological disorders. The *in vivo* histamine sensitisation test (HIST) is the current mandatory toxicity test, but it is difficult to standardise and a priority for replacement. Although *in vitro* biochemical methods measuring the enzymatic and binding activities of PTx have been developed and validated, they do not address the physio-pathological mechanisms of PTx and its interaction with other vaccine components at the blood-brain-barrier level. With this aim, we study the effects of PTx in combination with other vaccine components on leukocyte-endothelial cell-to-cell interactions. Our results showed that incubation of human brain microvascular endothelial cells (HBMEC) with PTx enhanced the permeability of the endothelial monolayer in a dose-dependent manner and at high concentrations increased the expression of surface adhesion molecules (ICAM-1 and VCAM-1). Interestingly, PTx induced only a significant release of MCP-1 by HBMEC amongst a panel of selected cytokines and chemokines and significantly reduced IL-8 levels induced by other vaccine antigens and pro-inflammatory molecules. However, in a co-culture system involving HBMEC and human mononuclear cells, PTx induced a significant release of IL-8, MCP-1, IL-6, TNF-α and MIP-1β. A more complex synergic release of these molecules was also observed when other vaccine components were added. We hope these cell-to-cell interaction studies will enable us to establish a relevant *in vitro* cell model to complement the biochemical toxicity assays in view of replacing the current *in vivo* HIST.

ID ABS: 176

Obstacles and opportunities for the implementation of the Three Rs in Canadian vaccine quality control testing

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The biological origins of vaccines confer risks unique among pharmaceuticals. Careful processing of vaccine components is important to avoid side-effects in susceptible individuals. For this reason, vaccines are highly regulated and testing for safety and efficacy is required before they can be released onto the market. This testing typically requires large numbers of animals and often involves substantial pain and distress. As Canada's organization for setting and maintaining standards for the care and use of animals in science, the Canadian Council on Animal Care (CCAC) requires that animals be used only where no replacement alternative exists, that animal use is reduced and that pain and distress are minimized (refined) as far as possible. These Three Rs principles are also recognized as an essential basis for better science. We have used a case-study approach to identify opportunities and obstacles to the implementation of the Three Rs in vaccine testing in Canada. Preliminary data will be presented, drawn from semi-structured interviews with regulators, scientists and industry representatives involved in the production and testing of vaccines. The study data probes awareness/knowledge of Three Rs methods, the role of *in vitro* data in testing, the mandate of organizations with regards to test development, and international factors which may affect Canadian vaccine policies.

ID ABS: 229

Modified binding assay for the improved detection of residual pertussis toxin in vaccine preparations

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The histamine sensitization (HIST) test in mice is the standard accepted safety test for residual pertussis toxin (PTx) activity in acellular pertussis vaccines. This assay requires a large number of animals, is non-quantitative, difficult to conduct, and has a high false-positive outcome. Recently, an in vitro assay was described in the literature which could differentiate between the active PTx and inactive pertussis toxoid (PTd) based on the selective binding of PTx to fetuin and its detection with a polyclonal anti-PTx antibody. Contrary to expected results, we found significant binding of PTd to fetuin, thereby reducing the assay specificity. This non-specific binding was eliminated at pH 10.2, and the limit of quantitation (LOQ) was 11.2 ng PTx/ml binding buffer when analyzed using 5 parameter logistic regression. To further improve sensitivity and specificity, we screened a panel of monoclonal antibodies (mAb) to PTx and identified one which did not cross-react with PTd. Using this mAb reduced the assay LOQ to 0.5 ng PTx/ml binding buffer, and 2.4 ng PTx/ml vaccine matrix. Six commercially available pertussis-containing vaccines were tested and shown to have PTx levels below LOQ. Desorption with citrate did not increase PTx above LOQ. Our data show that both PTx and PTd bind to fetuin and that specificity in this assay must be conferred by the primary antibody. The mAb used was previously shown to neutralize PTx toxicity *in vivo*, thus lending support to the biological relevance of this assay in the replacement of the HIST test.

ID ABS: 235

Can the worm be turned? – Schistosomiasis vaccine research in men, mice and monkeys

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Schistosomiasis is a macroparasitic disease that affects approximately 200 million people at any one time. Vaccine development has been limited by species differences and problems with extrapolating relevant information from studies in a variety of animal models to humans. To date, vaccines have only been able to confer partial protection against Schistosomiasis infection. In the 30 year history of Schistosomiasis research there has been a convincing physiological argument for using rhesus macaques and olive baboons as infection models, based on features of these species that facilitate a study of concomitant immunity and which allow a more realistic schistosome worm burden to be achieved. These are not easily replicated in rodent models due to size and immunological differences. However, a great deal of the discovery and development of potential therapeutic candidates has also been a result of studies in mice, humans and by using *in vitro* techniques. None of this research has thus far resulted in the availability of a long-term solution to infection, but a number of potential antigens have been identified using these approaches. Here the pros and cons of each model are reviewed with a view to devising a testing strategy that will encompass a multifaceted approach to enable the use of primates to be kept to a minimum and to expedite the progress of vaccines through to clinical trials.

ID ABS: 238

The cAMP assay: a functional *in vitro* alternative to the *in vivo* histamine sensitization test

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An important component of all acellular pertussis (aP) vaccines is detoxified pertussis toxin. For safety reasons, the performance of the *in vivo* histamine sensitization (HS) test is mandatory to guarantee that the quantity of residual pertussis toxin (PT) does not exceed a specified level. Due to its high variability and because the test could be accompanied by severe animal suffering, we decided to develop an alternative *in vitro* assay. This cell culture assay, which mechanistically reflects the HS test, uses a vas-

cular smooth muscle cell-line (A10 cells). The assay is based on the phenomenon that PT, but not detoxified PT, down-regulates the inhibition of adenylate cyclase. This results in an increase of intracellular cAMP levels after stimulation with isoprenaline. Our experiments demonstrated that PT induces cAMP levels in a dose dependent matter. This dose dependent increase is undisturbed by the combination vaccine DTaP-IPV. In addition, cAMP levels were not influenced by other components of aP vaccines including the adjuvant aluminium phosphate. The experiments further demonstrated that the sensitivity of this assay equals the sensitivity of the HS test. Although several causes of variation were identified and the test was further optimized, the variation remains an aspect that requires further study. Nevertheless, our data show that this assay is a promising, sensitive and specific alternative for the HS test.

ID ABS: 251

The consistency approach in vaccine development and quality control

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Well defined biologicals like recombinant proteins can be released based on a series of relatively simple *in vitro* assays like purity, molecular weight, glycosylation pattern, tryptic digest map, cell based assays, binding to target ligand, etc. Product release is based on these tests, in-process controls and process parameters demonstrating consistent production. Most vaccines however are not well defined. Nevertheless, with new product and process development approaches and analytical possibilities it is increasingly feasible to use the same approach for complex vaccine antigens.

There are numerous developments in the field of comparability and consistency of vaccine production and vaccine characterisation. These include concepts like analytical fingerprinting, process analytical technology and the development of *in vitro* functional assays.

Current developments in these fields can give us a better understanding of both the product as well as the production process. This integrative approach will result in optimal product quality (both potency and safety), production processes that are more robust and optimised with regard to costs, lower economical risk (less out of specifications and batch rejection) and reduced use of animals for routine release testing.

ID ABS: 263

Summary of recommendations of the ECVAM workshop on Three Rs approaches in the production and quality control of fish vaccines

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Due to the increasing importance of fish vaccines in aquaculture and animal welfare concerns associated with fish vaccine quality control, the European Centre for Validation of Alternative Methods (Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra, Italy) organised a workshop on "Three Rs Approaches in the Production and Quality Control of Fish Vaccines" in 2008. The workshop was attended by experts from academia, regulatory authorities, a scientific animal welfare organisation, and the fish vaccine industry. The main objectives of the workshop were a) to identify animal tests currently stipulated for the production and quality control of fish vaccines and highlight animal welfare concerns associ-

ated with these tests; b) to identify viable options to replace, reduce, and refine animal use for fish vaccine testing; and c) to discuss the way forward and give recommendations how these options may be realised without lowering the vaccine quality. The participants in the workshop agreed that efforts should be undertaken to replace the vaccination-challenge batch potency testing with tests based on antigen quantification or antibody response. Regulatory requirements of questionable scientific value and relevance for the quality of fish vaccines as the re-testing of batches produced outside of Europe or the double-dose batch safety test should be re-considered. As an immediate measure the design of the current animal tests should be evaluated and modified in the light of refinement and reduction, for example, the number of unprotected control fish in vaccination-challenge tests should be reduced to the minimum.

ID ABS: 275

Relation between *in vitro* growth inhibition test of leptospires and potency test in hamsters

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It was investigated in hamsters whether a correlation exists between the standard potency test of antileptospirosis bacterins and the *in vitro* leptospires growth inhibition test, in order to determine a threshold among neutralizing antibodies, which corresponds to the approval level of the vaccine in the potency test in hamsters. The assays were performed in male hamsters immunized with a commercial animal antileptospirosis bacterin in different dilutions.

The potency test by challenge was modified from the protocol of the United States Agriculture Department. The immunization protocol was based on two 0.25 ml doses of bacterin, pure and nine two-fold serial dilutions begun at 1:200, by subcutaneous route with a 15-day interval. The challenge was performed 15 days after the last immunization with a dose 0.2 ml (LD_{50}=108 cell/ml) of virulent *L. interrogans*, serovars Canicola or Pomona.

The bacterin was approved according to the international evaluation criteria. The mortality rates must be 20% in the vaccinated group and 80% in the non-vaccinated control group.

Hamsters immunized with the bacterin showed high titers of neutralizing antibodies in comparison to agglutinating antibodies. The comparison of the bacterin performance with the serovars Canicola and/or Pomona by the proportions of surviving animals in the challenge assay and the average of the neutralizing antibodies titers established that neutralizing antibodies' log10 titers were 1 [0.664; 1.336] to serovar Pomona and 1 [0.707; 1.293] to serovar Canicola, corresponding with the bacterin level of approval in the potency test.

ID ABS: 276

Pertussis, diphtheria and tetanus serological potency testing in a single animal model

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Whole cell pertussis (wP) vaccines are widely used in combinations with diphtheria (D) and tetanus (T) toxoids, but also with other vaccine components. wP vaccine potency is assessed by a mouse protection test (MPT) that inflicts severe distress on the numerous animals involved and gives highly variable results, making its replacement highly desirable. In this study, the pertussis serological potency test (PSPT), initially developed in mice, was transferred to the guinea pig (gp) model with the final goal of also testing diphtheria and tetanus potencies by using the same animal series. Two features of the serological response to wP vaccination were evaluated: 1) the overall antibody response, measured by a whole cell ELISA (PSPT-wC-ELISA), 2) the functional neutralizing antibodies to pertussis toxin (PT) as measured by the Chinese hamster ovary (CHO) cell assay.

The results showed that 1) the gp model can be used for wP serological vaccine potency testing, 2) comparable potencies were obtained in MPT and in the PSPT-wC-ELISA, 3) despite good repeatability and precision, the CHO cell test-based serologi-

cal assay did not generate results comparable to the MPT and highlighted significant differences in the ability of wP vaccines to induce neutralizing anti-PT antibodies. The gp sera produced during wP serological vaccine potency testing also generated a good dose-response curve for D and T. These preliminary data suggest that serological potency testing in the same gps might be a promising approach for batch release potency testing of the wP, D and T components of combined vaccines.

ID ABS: 279

Vaccine testing *in vitro* using the human artificial lymph node model (HuALN)

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The model of the Human Artificial Lymph Node (HuALN) is designed for immunofunctional and predictive immunotoxicological testing *in vitro*. Pharmaceutical proteins, vaccines or biologicals can be investigated for wanted and unwanted effects on the human immune system. The micro-organoid tissue culture serves as a model for induction or modulation of cellular and humoral immune responses. The controlled perfusion-system enables long-term culture for several weeks and provides the opportunity of long-term drug exposition or multiple and repeated dosings.

We will present recent data of the HuALN application for vaccine testing of commercial vaccine products against Hepatitis Viruses (HAV and HBV; Havrix™, Twinrix™) and viral protein preparations (Cytomegalovirus, CMV). T cell responses and shifts in the TH1/TH2 pathway are monitored by cytokine secretion profiles. The induction of humoral responses is demonstrated by B cell activation, plasma cell formation and antibody secretion profiles for IgM and IgG.

ID ABS: 334

Evaluation of a guinea-pig model for determining immunogenicity of vaccines containing Hib polysaccharide obtained by chemical synthesis

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Development of vaccines against invasive Haemophilus influenzae type b (Hib) diseases has been based on the fact that the capsular polysaccharide of the bacterium is the virulence factor and that antibodies against it are protective. For Hib vaccines no satisfactory biological assay is currently available. Quality control is therefore largely dependent on physico–chemical analyses. However, an immunogenicity model could helpful, mainly for testing new products. As new vaccine combinations containing Hib polysaccharide obtained by chemical synthesis have become available recently, we aimed to develop a guinea-pig model to contribute to the characterization of these vaccines. Groups of 8 guinea-pigs were immunized with a single high dose of Hib monovalent and combined vaccine formulations. Cellular and humoral responses to Hib were evaluated. Correlations regarding relevant physico-chemical and some other biological tests were also determined. Our results indicated the suitability of this model for evaluating the interference on Hib response of some antigens (Tetanus toxoid, whole-cell Pertussis, Hepatitis B). Its sensitivity was even higher than physico-chemical tests, and in general results were in agreement with clinical results. It was demonstrated that a guinea-pig model could be used to determine Hib response in vaccines.

ID ABS: 338

Development of an inhibition ELISA for potency testing of equine rabies immunoglobulin batches

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Rabies is a major public health problem. Post-exposure treatment of rabies includes the prompt use of human or equine rabies immunoglobulin in combination with administration of rabies vaccine. Currently, the activity of equine rabies immunoglobulin is controlled using a neutralization potency test in mouse. An inhibition ELISA was developed to replace the *in vivo* mouse virus neutralization potency test.

The inhibition ELISA method is able to determine the concentration of rabies virus-specific antibodies in the equine immunoglobulin preparation. The test is realized in 2 steps: neutralization of equine sera followed by ELISA detection. The neutralization step consists of incubating the equine sera in the presence of inactivated rabies virus. Then, the presence of remaining non neutralized rabies virus is detected by ELISA using specific monoclonal antibodies.

A good correlation is shown between this inhibition ELISA and the mouse neutralization test. Inhibition ELISA allows much faster testing times (two versus 30 days) and has a cost saving effect. These data demonstrate that the inhibition ELISA can be used to replace the mouse virus neutralization potency test, allowing the achievement of a 3Rs goal by moving from an *in vivo* potency assay to a full *in vitro* assay.

ID ABS: 374

Novel alternative *in vitro* method for determination of pertussis toxin activity in vaccines

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Bordetella pertussis toxin (PTx) is one major virulence factor of *Bordetella pertussis*, a Gram-negative bacterium, which causes pertussis (whooping cough). Immunisation is performed via so called Acellular Pertussis Vaccines (ACV), which contain as upmost important antigen PTx. To guarantee non-hazardous application for all recipients, successful detoxification has to be monitored for the absence of residual active toxin. Determination of toxicity has to be done by a lethal challenge animal test in mice. Beside high resources of animals, this test provides inconsistencies and therefore inevitable repetitions. Thus, an alternative testing method is needed.

For the estimation of residual active PTx a cell assay involving the respective ATP status is to be developed. PTx, an ADP-ribosyltransferase, transfers ADP-ribose to inhibitory G-proteins. Thereby it interferes in the signal transduction pathway, which leads to an increase in cAMP and to an accompanied decrease of ATP. As indicator for the activity of PTx the decreasing ATP level was implemented.

We investigated a PTx-cell assay, which consists of an incubation step and an examination step. Human peripheral blood mononuclear cells (PBMCs) are incubated with samples, and after one, five and 24 hours the somatic ATP level is measured. PTx causes a dose and time related decrease of ATP level. In contrast, heat-inactivated PTx is not able to induce this kind of reaction.

This test is similar to the *in vivo* situation of the PTx effect on human cells and shows extremely encouraging results.

ID ABS: 446

Comparative analysis of serological method, toxin binding inhibition test and lethal challenge for tetanus and diphtheria vaccine bioactivity control

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The bioactivity of tetanus vaccine is usually analyzed using lethal or paralysis challenge on animals (mice or guinea pigs). The European pharmacopeia (EP) describes two other methods with respect of the 3R rules: serological assays and ToBi. For diphtheria vaccine bioactivity control, dermic challenge of guinea pigs or lethal challenge of mice are the methods allowed by the

EP. However, other methods have been studied: a serological method, toxin binding inhibition (ToBi) and seroneutralization on Vero cells (Pharmeuropa Bio, 2004). In our lab, we developed serological and ToBi methods for tetanus and for diphtheria vaccines on guinea pigs. The objective of our study is to reduce and refine animal use; indeed, the same animals are used for the analysis of the two vaccines' bioactivity. The guinea pigs were immunized with a tetravalent vaccine and 42 days later they were anaesthetized and blood taken by cardiac puncture.

Furthermore, to validate our results on serum, the animals were then challenged with tetanus toxin and the morbidity evaluated five days later. We analyzed the immune response, the *in vitro* inhibition potential of antibodies and the *in vivo* protection by antibodies against the tetanus toxin. We can compare all the data from each individual animal and from groups of animals that have received different doses of vaccine.

Our results show that we have a quite good correlation between the different methods of vaccine bioactivity analysis. The combination of serological method and ToBi and perhaps seroneutralization on cells could replace lethal or dermic challenge.

ID ABS: 516

Cryopreserved whole blood cytokine release assay for determining endotoxin contamination of hyperimmune sera

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Introduction: Previous studies showed that the fresh whole human blood cytokine release assay is able to detect endotoxin contamination of hyperimmune sera, when it is diluted 1:10. Cryopreserved blood presents the advantage of avoiding blood collection every time it is needed to perform the assay. This study is licensed by the FIOCRUZ Research Ethical Committee under the number 368/07. Objective: This study aims to verify whether cryopreserved blood can be used for detecting endotoxin contamination of hyperimmune sera in the routine of quality control. Methodology: Fresh blood (10ml) was collected from 5 healthy volunteers into heparinized vials and pooled. A DMSO/Sörensen Buffer dilution was gently mixed with the pooled blood in a 1:1 proportion (blood:buffer). Blood was distributed into cryovials, left to stand for 15 to 30 minutes, and placed in a -70ºC freezer. After thawing, blood was incubated with spiked (O55:B5 *E. coli* LPS, 5 EU/ml) and non-spiked hyperimmune sera (anti-Bothrops snake venom, anti-rabies and anti-tetanus) and incubated at 37°C under a 5% CO_2 atmosphere. Cytokines (IL-1β and IL-6) were evaluated by ELISA (R&D Systems). Results: Non-spiked hyperimmune sera did not induce cytokine release, nor did undiluted spiked sera. When diluted 1:10, spiked sera induced cytokine release at least in the same amount as spiked 0.9% NaCl solution used as positive control. Conclusion: Results indicate that cryopreserved blood may be used for detecting endotoxin contamination in the routine of quality control of hyperimmune sera under sanitary surveillance.

ID ABS: 535

Promoting the 3Rs in a global vaccine company

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In the vaccine business, the typology of the use of animals is different from pharmaceutical companies: most of the *in vivo* tests are related to product characterization and batch release to ensure the safety and the efficiency of commercialized vaccines. Therefore, the use of animals related to production activities is performed under strict regulatory control. Any testing changes related or not to the 3Rs should be validated, and submitted to and accepted by all the national regulatory agencies. In R&D, investigators have more flexibility to develop and implement alternative methods.

The company commitments to the 3Rs are highlighted by major progresses; especially noteworthy is the reduction of animal use in the last decade. However, those improvements are not always shared with employees and external stakeholders. To implement a global 3Rs program by developing policies, committees and processes that focus on reduction, replacement and refinement is a way (i) to stimulate 3Rs initiatives, (ii) to promote the 3Rs efforts and (iii) to demonstrate the company commitments. The aim of the presentation is to share how 3Rs principles become a reality in a global environment.

ID ABS: 587

Mouse NVT: assessing the use of a transgenic mouse model as a suitable alternative for the release of oral polio vaccine

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Current international regulations require the oral antipoliomyelitic vaccine (OPV) to be tested for residual neurovirulence by employing the Monkey Neurovirulence Test (MNVT), which is carried out on non-human primates of the species *Macaca fascicularis*.

In the last years, several collaborative studies have been carried out under the guidance of the WHO in order to find alternative methods to MNVT, using *in vitro* (PCR) and *in vivo* (TgPVR21 transgenic mice Neurovirulence Test, mNVT) approaches.

At Novartis Vaccines & Diagnostics, Siena (Italy), 7 production bulks of OPV serotype 1 and 5 type 3 bulks have been tested on mice. Two different dosages of both vaccine and a homologous reference virus were inoculated intraspinally into mice. Cage allocation and inoculation sequence were randomized. Observation of mice for neurological signs of disease, such as paresis and paralysis, was performed daily for 14 days post-inoculation. The number of paralyzed mice inoculated with the vaccine was significantly lower than the number of mice inoculated with the reference virus. Data were compared with MNVT results, showing similarities.

The TgPVR21 transgenic mouse model is a suitable alternative to the MNVT for neurovirulence testing of live poliomyelitis bulk suspensions, as the transgenic mouse shows characteristic clinical manifestations similar to those seen in non-human primates.

PO19: Education and training

ID ABS: 40

Computer assisted learning as a refinement and reduction alternative

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Computer Assisted Learning has a long standing history in continuing education in laboratory animal science. Some excellent examples for such endeavours are the AALAS, CCAC or LAWTE web resources and the NORINA database. Here we report on a new tool in assisting specialist education in accordance with European legislation and in view of the 86/609/EWG and ETS123, respectively.

"Experimental Animal Work online" is a BMBF funded project of the BMBF priority "Methods to replace animal experiments". According to the German Animal Welfare Act, every person involved in working with animals is obliged to protect the animals' lives, to promote their well-being and to prevent pain, suffering or damage to these animals. The use of live animals for scientific procedures is ethically only justifiable if these principals are acted upon, but it is still required in safety testing and is a necessity in medical and basic research of the life sciences. Competent handling and proper scientific techniques as well as knowledge about ethics and legislation are crucial for animal welfare, and it is mandatory to ensure quality education in laboratory animal sciences before engaging in practical animal work. For this reason we developed a membership-free, non-fee internet based learning aid, with a zoomable anatomy section (rodents) and 35 videos, as well as information about legislation, ethics and the welfare of laboratory animals. The modular build allows it to be adaptable to different study purposes and target audiences, such as on-the-job training, advanced vocational training and university courses.

ID ABS: 85

Training courses on laboratory animal science: animal welfare is dependent on the education and training of researchers

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The present study shows the results of our experience of three courses in "Laboratory Animal Science" for researchers. They were carried out at the Fondazione Santa Lucia-Centro Europeo di Ricerca sul Cervello in Rome. Each course consisted of 40 hours of theoretical and practical training for graduates in scientific subjects. The aim of these courses was to contribute to broadening knowledge and to training the users of laboratory animals in Italian and European laws, the biology of the species used, zoonosis, main animal pathologies, alternative techniques, animal welfare, etc. Of 74 students who attended the courses, 68.92% (N=51) were graduates in biological areas, 14.86% (N=11) in veterinary areas, 9.46% (N=7) in medical and chemical areas and 6.76% (N=5) in psychological areas. The course evaluations demonstrated that the students realized that theoretical knowledge of laboratory animal science and practical skills are of great importance both for animal welfare and for the success of their future research involving animal experiments. Training programs for research personnel are discussed as a key resource that must be part of an effective animal care and use program.

ID ABS: 100

Simulating the effects of drugs on the ciliary motility of frog oesophagus

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Frogs are used as *in vivo* and *in vitro* models for demonstrating the effects of drugs to medical students. Cilia present in the frog oesophagus respond to cholinergic drugs, and hence the frog oesophagus is used to demonstrate their effects. Since a large number of frogs are killed for demonstration, it was decided to write a software program simulating the effects of drugs on the oesophagus to replace frogs used for teaching. The software displays a frog on a wooden board with its oesophagus dissected open and fixed. At the cephalic end of the oesophagus the user can place a poppy seed, which is moved caudally due to the motility of cilia present in the mucosal surface of oesophagus. The user should note the time taken for the seed to reach the end point fixed at the caudal end and this indicates the activity of cilia. The user can instil different drugs, such as acetylcholine, physostigmine and atropine on the oesophagus and observe the influence of these drugs on time taken for the seed to travel between start and end points. The results can be tabulated and analysed.

Live demonstrations of animal experiments are conducted for medical students mainly to reinforce what is taught in the pharmacology lecture classes. The same objective can be achieved with the software without sacrificing the frogs. The software includes tutorial and examination modules and can be used for demonstration as well as examination purposes. Thus the software can effectively serve as an alternative.

ID ABS: 122

Software controlled artificial animal models for teaching animal experiments

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Use of artificial models and mannequins is widely accepted in teaching the subjects of anatomy, surgery and pharmacology. Recent advances in computer assisted learning (CAL) methods have further revolutionized the demonstration of animal experiments in pharmacology. However, both artificial models and CAL methods have certain disadvantages as independent teaching methods. Hence, there is a need to develop better techniques which include the advantages CAL as well as artificial models.

In the present project an effort is made to synchronously use the artificial mannequins of rat and dog with a computer software program to develop a model that will fully simulate the wet laboratory invasive blood pressure experiment. The learner can carry out surgical procedure on this model and perform cannulation of blood vessels. Further, the effects of various drugs can be studied by injecting the relevant solutions through a patented cannula into the mannequin and observing the effects on the computer screen connected to the mannequin. This method of teaching pharmacological experiments is proposed to replace the invasive blood pressure experiments performed on live animals without compromising the teaching/learning objectives. The model will also help students to improve their surgical skills and perform the experiment in the same manner as in the wet laboratory experiment.

ID ABS: 123

DARENET: a novel technological platform to promote the use of the zebrafish model

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Initially, the zebrafish (*Danio rerio*) model was used as a model in developmental biology. However, in more recent years this model organism has come to the attention of the international scientific community in many other applicable areas in both basic and applied research. However, even though the areas of application and the scientific objectives of the different research centres and companies are quite varied, they all use the same model for research and development. Common procedures and methodologies should therefore be standardised, making it essential to introduce standard operating procedures (SOPs) in order to consolidate this model organism as an alternative method to other vertebrate model organisms such as mice, which have a higher cost in economic and ethical terms. In this regard, the European Union is promoting the use of alternative to vertebrate model organisms in all applicable areas. This is why one of the objectives of this technological platform is to promote the implementation of SOPs that ensure animal welfare for this model according to the 3Rs: Reduction, Refinement and Replacement. These include the development of protocols to evaluate the animal's welfare and to control its health, to establish microbiological and genetic standards for the animal and standardise anaesthesia, analgesia, endpoint criteria, etc. Likewise, another important objective of this platform is to increase research that uses this model organism in all priority areas such as health, biotechnology, and eventually, promoting the presence of this platform in private and public research centres and especially in large and small companies.

ID ABS: 146

Training programs designed to support and enhance the 3Rs in animal research

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In the United States, ensuring appropriate training is the responsibility of the Institution as stated in the Animal Welfare Act, the Public Health Service Policy and the Guide for the Care and Use of Laboratory Animals. Regardless of federally designated responsibility, programs of excellence recognize training as one of the pillars supporting animal research. Proper training in the humane care and use of laboratory animals for researchers, animal care staff and the veterinary staff is essential for the success of an animal care program. Training ensures animals are appropriately cared for, equipment functions and is used properly, safety

measures are adhered to, and research is conducted efficiently, effectively, and humanely. Additionally, animal welfare is greatly enhanced and animal numbers may be reduced when animal care and research techniques are performed by trained personnel. A robust training program will seek out ways to continually improve the way research is performed, optimizing resources and refining animal techniques. Three trainers from American academic institutions will describe a comprehensive animal care training program, current practices, successes and failures, and future training trends relevant to changing demands on an institution. Examples will be given as to how these programs support and enhance the 3 Rs in animal research. Additionally, this presentation will focus on alternative methods of meeting training needs for institutions not having their own training program or those wanting to refine how training is delivered.

ID ABS: 151

CAAT's humane science and toxicology certificate program

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The academic programs at the Center for Alternatives to Animal Testing (CAAT) at the Johns Hopkins Bloomberg School of Public Health educate students and professionals in the research field about alternatives, helping them gain a better understanding of the 3Rs and their role in improving the quality of science.

The Humane Science and Toxicology Certificate Program is central to CAAT's academic program. The Certificate curriculum consists of six courses designed to provide essential knowledge and skills in public health, the 3Rs as they apply to biomedical research, experimental design and analysis, and humane sciences – with an emphasis on the translational and policy implications.

The Certificate Program is open to anyone who holds an undergraduate or graduate degree in public health or the biomedical sciences, as well as to students in any degree-granting program at the Johns Hopkins University. Many of the courses are available through internet venues. CAAT intends to make the Humane Science and Toxicology Certificate Program available entirely on-line by 2010, making the Certificate Program more easily accessible to a wide audience in the business, legal and regulatory communities.

ID ABS: 152

Theory & practice of 3R conception in the educational process in Belarus

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The humanization of teaching in the life sciences is one of the educational tasks in ISEU, a university which was established in Belarus after the Chernobyl Disaster. The ISEU "Bioethics" course is the first university level course on this issue, and includes modules such as "Humane Treatment of Animals" and "Alternatives to Experiments on Animals". In the teaching of pathophysiology, according to the 3Rs philosophy, experiments on animals are replaced by video and computer programs. The optional course "Alternatives to the Use of Experiments on Animals" is based completely on work with educational computer programs. Co-operation between ISEU and both InterNICHE and the Russian Animal Rights Center VITA has supported such activity. The student organization EcoUni actively implements the ideas of humane education through the project "Person, Ecology, Bioethics". As part of the UNESCO project "Ecological Ethics in the System of Bioethical Education in Belarus" the following events have been organized: the international scientific and practical seminar "Humanization of Bio-Medical Specialists Education" (2006), the XV international conference "Human Ecology in the Post-Chernobyl Period" (2007), and the seminar "Ecological Ethics in High Education System of Belarus" (2008). Several books, manuals and films dedicated to humanization of education have also been published.

ID ABS: 181

Integration of alternatives to harmful animal use in the educational system of Ukraine: problems, progress, perspectives

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During the last 5 years, significant replacement of animal use in the education of natural science and medicine students has been achieved at department level at a number of Ukrainian universities. These examples and further anecdotal evidence suggest a broad decrease in animal use across the country brought about by a number of factors: Adoption in 2006 of legislation that supports student conscientious objection and regulates animal experimentation, including in education; encouragement to universities by the Ministry of Education to employ innovative educational methods, including computer technology; outreach and provision of information and resources by InterNICHE and other organisations, backed by signed agreements with universities to replace harmful animal use with alternatives; student demand for the modernisation and humanisation of teaching approaches; and growing awareness of the pedagogical, economic and ethical advantages of the use of alternatives. Further successful integration of alternatives into the educational system will be achieved as the following obstacles are overcome or removed: The low level of state funding for universities and the consequent shortage of computer equipment; the current scarcity of Ukrainian- and Russian-made alternatives and of translated Western software; the relatively high cost of some Western alternatives; conservative attitudes amongst older teachers and a tradition of animal experimentation in former Soviet countries; and the lack of initiative from some teachers in gathering information on alternatives and curricular transformation.

ID ABS: 185

Facilitate evidence based laboratory animal science by improving implementation of systematic reviews of animal experiments

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Researchers who perform animal experiments should present evidence that all reasonably available relevant knowledge has been adequately reviewed and is taken into consideration. Literature is an important source of information. In a survey among scientists it was demonstrated that the skills in searching for 3R information are limited. We have therefore implemented specialised training on searching literature in the FELASA category C courses in Nijmegen and participate in Amsterdam. Our experience from this specialised training and also from personal interviews with researchers is that not only searching for 3R information is complex. Also skills to perform a systematic search in biomedical databases and formulation of the specific research question for the best (animal) model need improvement. Systematic reviews (SR) are generally regarded as the highest level of evidence within medicine and should also be applied in animal research to improve scientific quality and animal welfare. In contrast to human clinical trails, where SR are standard and often even a necessary part of funding applications, SR are a relatively new approach in laboratory animal science. Besides improving the quality of the research, SR can also prevent duplication and enhance translational research. Guidelines, however, to perform SR of animal experiments are not yet available. To facilitate evidence based laboratory animal science by using SR of animal experiments we educate researchers in the design of animal experiments, develop guidelines for SR of animal experiments, and give specialised training to improve literature search skills.

ID ABS: 187

Applying the Three Rs principle in Korean veterinary medical school

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The Institute for the 3Rs of Konkuk University, the first institute of its kind in Korea, has been applying the Three Rs principles; Replacement, Reduction and Refinement in Korean veterinary medical schools. First, we developed an "Animal Blood & Body Donation Program" to reduce the numbers of animals purchased and purpose-bred for education. Second, we are practicing several animal alternative programs, such as computer simulation programs, animal models and innovative equipments to replace the use of live animals in veterinary laboratory classes. Third, we opened a website to provide resources and information on how to apply the 3Rs and ethical consideration in education, especially providing the methods of refinement to educators and students. We also held an international joint symposium together with the Korean Association for Laboratory Animal Science. Five international professionals were invited and presented their experiences. The attendees were provided with opportunities to witness the educational alternative programs provided by InterNICHE and meet the speakers in person during the booth presentation session. The Korean government implemented a new Animal Protection Law from 2008 including 3Rs principles mandated to each institution which uses animals and a new Laboratory Animal Law is effective from March 2009. The institute is playing a leading role for animal welfare education, sharing resources with the Korean veterinary medical schools and other institutions in Korea.

ID ABS: 234

More is less: reducing animal use by raising awareness of the principles of efficient study design and analysis

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Good experimental design and the appropriate use of statistical tests are corner stones of high quality scientific research. This is especially important when the experiments involve the use of living animals to ensure that their use is necessary, that harms are minimised and that as few as possible are used to obtain statistically sound data that meet the objectives of the study. One way to raise awareness of the importance of efficient study design and analysis is to provide training courses. It is apparent that many scientists have little formal training in the basic elements of experimental design and statistics, and also that they have difficulties in finding and retrieving useful training material on these subjects. In addition, they are often unable to access the necessary in-house expertise of a suitably-qualified statistician. Training provides an ideal opportunity to facilitate dialogue and enhance the application of experimental design and statistical analysis to animal experimentation thereby improving: a) animal welfare; b) the amount of information obtained from a given number of animals; and c) the quality of biomedical research and testing. This paper reports the views of participants at three training schools with reference to why they felt that attendance was necessary and how effective they felt the experience had been. An assessment of the success of the training is given. Implications of the participant feedback are discussed and considerations for future training events are noted.

ID ABS: 242

Importance of students' involvement in humanization of education

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Students have a right to humane education. Personal initiative of students plays an important role in education humanization. The results of questioning medical students and student biologists show that 52% of respondents consider that experiments on animals are unnecessary and that there are other methods of acquiring equivalent knowledge. All respondents think that teachers should inform students about the possibilities of humane alternatives usage, 70% of students would be able to protest against experiments on animals. To inform students and young scientists about possibilities of humane education, the creative group of students EcoUni developed the optional course "Alternatives to experiments on animals". The tasks of the course include analysis of animal usage expediency in historical and modern perspectives of the experiment, ethical treatment of laboratory animals, familiarization with the possibilities of humane education and science, which are achieved by alternatives usage, evaluation of legal regulation relevance in responsible treatment of laboratory animals, determination of importance to make students realize their rights to an education without cruelty.

ID ABS: 285

Scientists' attitude to the 3Rs

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Even under the present extensive regulation and supervision of animal use, researchers' individual responsibility is still decisive for the implementation of the 3Rs. Training in laboratory animal science is intended to raise researcher awareness, but its effect on scientists' attitudes has not been systematically assessed.

Sixty-one participants in three FELASA Cat C courses in Portugal – most of whom already worked with animals – were surveyed through a self-administered two-part questionnaire (one before the introductory lecture presenting the 3Rs and the other immediately after). Questions mainly covered the 3Rs and their application, attitudes towards animal use and the ethical review of animal experiments.

At the start of the courses only 26% of the participants could name the 3Rs. For their own research field, most participants identified the use of animals as absolutely necessary (41%) or very important (47.5%) and most expected no (29%) or some (62.3%) change over the coming 50 years. Faced with a potential Reduction-Refinement conflict, 59.5% considered it ethically preferable to use 20 mice than conducting 20 tests on the same animal. Of respondents with own research experience, 27% reported already having applied the 3Rs. If scientifically valid and practically possible, and if suggested by a laboratory animal scientist, respectively, were identified as the most convincing arguments for adapting research to incorporate the 3Rs.

To assess any long-term effects, the same respondents will be surveyed one year after each course, and some of this data will already be available by the time of the WC7.

ID ABS: 317

Embalmed swine for education and training in surgical techniques

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To optimize animal use for surgical training, we present our experience at the University of São Paulo with embalmed swine. Thirty two anesthetized swine (Thiopental [180 μg/kg/min] and Fentanyl [0.08 μg/kg/min]), received heparin (2.500 UI) after being used in advanced surgical trainings by residents and euthanasia was performed with anesthesia overdose and super-

saturated solution of potassium chloride intravenously. The vascular system was then flushed using saline solution through the left common carotid artery before the perfusion of a modified Larssen solution (100 ml/kg). The left jugular vein was used for the circulatory system drainage. Embalmed corpses were preserved at a temperature of -20ºC. When needed the swine were defrosted to ambient temperatures for 48 hours. The embalmed models have been tested with great success for the following surgical exercises: suture of cutaneous remnants; tenorrhaphies; surgeries of the spine; access to the cranial socket; pulmonary resection, bronchus anastomosis; gastric procedures; digestive endoscopy, mucosal resection and dissection, for NOTES training; liver and biliary vesicle surgeries, laparoscopic cholecystectomy; critical interventions of trauma. In conclusion, sharing and reuse of the *ex vivo* swine embalmed models optimized the effective utilization and improved the quality of medical education and research using alternative models.

ID ABS: 332

Animal use basics: teaching and training in Italy

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Training of experimental staff is an ethical need, as it is necessary to know the real benefit that scientific experimental studies on animals can result in. It is also a need for scientific knowledge, because it allows us to understand how small changes in the physiology of animals may affect the experimental data, and since 1986 it has become a legislative requirement in Europe. In some countries several government structures and some associations promote training courses of the personnel involved with animal use in the procedures.

In Italy training is mostly episodic, and there is still nothing planned and institutionalized, even though it is possible to highlight a growing interest in recent years. Trying to institutionalize something more programmatic, a bill on the education and competence of various skills, suggested by FELASA, involved in biomedical research using animals was proposed but it failed.

In Italy there are currently two post-graduate schools for the science and medicine of laboratory animals, organized by two universities, some advanced courses on laboratory animals, also organized by universities, and some more theoretical than practical courses organized by public or private research organizations without a common program. Moreover, some faculties include in their degree a subject on the proper and ethical use of laboratory animals.

Observing the Italian situation, the authors propose a strategy to improve teaching and training for people who care and use animals for scientific purposes. Moreover, they agree that students who attend scientific and biomedical faculty have to receive basic instruction in animal use in research as a possibility of using conscientious objection.

ID ABS: 346

Veterinary students' perspectives on alternative methods used in teaching: results from a questionnaire in two European universities

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Although the Three Rs approach in research and education is encouraged, little is known about its effectiveness from the students' perspective, especially in veterinary education, where practical teaching involving animals is fundamental.

Here, we present the results obtained from a questionnaire developed to assess the effectiveness of alternative methods in the veterinary curriculum. The questionnaire was given to students attending 5th year veterinary medicine at two European universities: Milan and Thessaloniki.

The main objectives of the study were to: a) evaluate alternative methods related to learning objectives, b) determine the "value" of alternative methods and their importance as a compulsory or optional approach in the veterinary curriculum, c) establish priorities regarding the use of alternatives in practical classes, d) determine risks and/or benefits of using alternatives in veterinary education and e) encourage participation of the students in a working group responsible for the development of additional alternative methods that could be used in veterinary education.

Overall the results indicate that teaching based on alternative methods was well accepted by veterinarian students on practical, economical, learning and ethical grounds. Furthermore, suggestions were made for expansion of this approach to postgraduate training and to continuing veterinary education courses.

ID ABS: 351

Humane endpoints in laboratory animal experimentation: a website on humane endpoints in rodent experiments

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A humane endpoint can be defined as "the earliest indicator in an animal experiment of (potential) pain and/or distress that, within the context of moral justification and scientific endpoints to be met, can be used to avoid or limit pain and/or distress by taking actions such as humane killing or terminating or alleviating the pain and distress". Humane endpoints are nowadays advocated as an efficient strategy to limit the number of experiments raising the highest level of ethical concern.

The website "Humane endpoints in laboratory animal experimentation" is meant for improving awareness and knowledge of humane endpoints. It can be used as a portal/platform for those who work professionally with laboratory animals and is relevant for education and training purposes.

The website is an extended version of the CD-ROM "Humane Endpoints in Laboratory Animal Experimentation", which was issued in 2005. Nowadays more than 2,000 copies have been distributed so far in almost all parts of the world. The website includes chapters on normal behaviour of a mouse and rat, pain and distress, pathology, recognition of general clinical signs as well as clinical signs typical for a number of specific biomedical research areas (e.g. cancer research), law and legislation and information on applying humane endpoints. The website will be partly open to the public; general information about humane endpoints will be accessible to everyone, but more specific information and material like images and video clips, forum, training material will only be accessible to registered persons. The website will be launched at the end of 2009.

ID ABS: 361

Replacement of animals in education in Gujarat, India

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In the state of Gujarat, India, there has been increasing replacement of university level dissection and animal experiments, reflecting efforts to modernise and make humane the education and training of students. Nominees from the Indian government agency that controls animal experimentation, the CPCSEA, some of whom are also involved in animal protection organisations, are increasingly rejecting applications for animal-based practical classes. Examples of replacement at several universities are described, along with details of the regulations, outreach and training, provision of alternatives, and cultural and economic realities that have helped create a context for successful curricular transformation in Gujarat.

ID ABS: 392

Developing training courses for meeting the information requirements of the US animal welfare act

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The 1985 amendments to the US Federal Animal Welfare Act (AWA) included a requirement that investigators consider alternative methods whenever animals are proposed for studies that may involve more than slight or momentary pain and distress. To assist the regulated community in meeting this new mandate, in 1993, the Animal Welfare Information Center (AWIC) developed a workshop with the goal of teaching researchers, information providers, veterinarians, and animal care and use committee members how to conduct effective literature searches for alternatives. This paper outlines the decision process AWIC took in creating the workshop so that other facilities can develop their own courses based on the AWIC model. It de-

scribes the steps taken in identifying the target audience, developing course goals, and distinguishing relevant databases and online resources. The difficulties associated with demonstrating the importance of multi-database searching and teaching how to create useful search strategies will be discussed. Also mentioned are how to decide on the best teaching format and identify supplemental articles that will support the workshop's educational goals. Tips such as catering to different audience members, directing searchers toward locally available and relevant resources, and creating a link between information providers and scientists will be addressed. Finally, the paper discusses the importance of learning about and incorporating new databases and websites, software programs, and alternative testing methods into the coursework when teaching how to search for reduction, refinement, or replacement options.

ID ABS: 393

Live cadavers as an alternative model for laboratory surgical training

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Laboratory surgical training in both human and veterinary surgery is important in refining skills and preparing residents and young surgeons for the operating room experience. Often live, healthy animals are used for this propose. Here we describe an alternative model for veterinary and human surgical training utilizing ethically-obtained cadavers specially prepared as follows: A portion or a whole cadaver can be used. The major vessels are cannulated and connected to artificial blood reservoirs. The arterial side is further connected to a machine that provides pulsating pressure. A pressure of 120 mm Hg is applied to the arterial side, and a pressure of around 15 mm Hg is applied to the venous side. Serum bags work well as blood reservoirs, and pressure bags are used to apply and adjust the pressure. With this arrangement, the artificial blood will fill and circulate inside the vessels, providing a cadaver specimen that can bleed and arteries that can pulsate. All kinds of surgical procedures can be applied to this model, including endoscopic and endovascular procedures. Trainees can make skin incisions and suture the incision site, dissect soft oozing tissues, ligate and coagulate bleeding vessels, and practice vascular anastomosis or intestinal anastomosis, transplantations, angiograms, etc. Implementing this model into the teaching curriculum will help eliminate the harmful and fatal use of animals for surgical training in both human and veterinary practice.

United States Patent No US 6,790,043 B2. Sep 2004

ID ABS: 452

Replacement in Mexico: the vision and strategies of the Center for Animal Alternatives in Education (CAAE) program

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The Center for Animal Alternatives in Education (CAAE) program was established in 2008 to facilitate full replacement of harmful animal use at the University of Guadalajara in Mexico. Beginning with a survey of over 1700 health science students and an analysis of laboratory manuals and teaching objectives, CAAE has built a platform for discussion and designed a strategy for humane innovation within the university and beyond. In collaboration with InterNICHE, CAAE has provided information and other resources to teachers and students. It hosts the InterNICHE Mexican Alternatives Loan System, a library of software, mannekins and simulators, and has co-organised local and national events. In physiology, workshops have promoted the use of software and self-experimentation apparatus. In veterinary anatomy, the education of teachers has brought about partial replacement, with the remainder to be achieved through the use of software and ethically sourced animal cadavers. A body donation program has been established, with cadavers to be sourced from the university clinic and other veterinary practices. CAAE and InterNICHE also aim to replace the annual use of 2000 dogs in surgery practice in medicine. A surgery alternatives seminar has been organised to provide training for teachers and professionals in Aboud's Method, which uses perfused cadavers for "live" surgery practice, and to demonstrate the POP-trainer simulation device for laparoscopic surgical skills acquisition. With support from the university administration, a consensus in favour of replacement has been built, and the use of alternatives is increasingly recognised as a tool to enhance education and training.

ID ABS: 455

Replacement strategy for a class on histological techniques with animals in southern Brazil

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Study of animal ethics and replacement methods for classes with animals is recent in Brazil, but an increasing number of publications and researchers are dedicated to the field, including the research "Replacement strategies for research and classes with animals". Firstly, we follow a class on histological technique within a vocational course in biotechnology, which uses a rat (*Rattus norvegicus*) for students to learn how to prepare histological samples. As a strategy, rat tissues were replaced by necropsied formaldehyde-fixed dog tissues. The teacher mentioned that she felt uncomfortable using animals and considered that such a method "solved the problem" (sic.). She said she would no longer use animals in her classes in order to avoid creating negative feelings concerning their deaths. She also said that some students had complained about not using live animals, but they understood that the aim of the class was to learn how to make histological samples of necropsies and biopsies, which was achieved. A student that took part in the same class, but using the traditional method, mentioned that he felt sad and embarrassed, and he considered the use of fixed tissues a good alternative. Another student said, regarding classes with animals: "I thought it was horrible!! (sic.) I didn't like to see the animal suffer before dying." In that work, we used the principle of replacement, according to the 3Rs – reduce, refine, and replacement (Russel & Burch, 1959) – the only one that creates conditions to abolish the use of animals in scientific practices.

ID ABS: 478

Alternatives to animal dissection in teaching veterinary anatomy

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Teaching veterinary anatomy involves the use of animals which are embalmed carcasses. In the past, an adequate number of animals were used for this purpose. However, restrictions are imposed by CPCSEA, SPCA and Government of India on the use of animals for laboratory experiments and this has affected the teaching programme in veterinary anatomy. Though it is not possible to completely stop the use of animals for practicals, we could reduce their number by using advanced technology available in the field, like use of CDS on animal dissection, coloured organ specimen and mounted skeletons. This helped a lot in reducing the use of animals for dissection. Although cadaver dissection is superior to video demonstration, it is apparent that students can learn veterinary anatomy by both methods. The dissected specimens are preserved system-wise in separate containers. A beautiful museum of self explanatory live models of the visceral organs of different species of animals has been developed in this hall. These museum specimens are suitably labelled, and hence it became easy for students to understand the comparative anatomy of animals. To create interest among the student to learn this subject with enthusiasm, our department is trying hard to evolve various kinds of teaching aids, such as multimedia computer assisted learning on dissection, organ specimen, blow up charts, lesson folders and mounted skeletons of animals. There were no significant differences in students performance noticed when using these alternatives to animal dissection.

ID ABS: 498

Is an academic absurd a substitutive method to teach surgical techniques?

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Since 2000 the College of Veterinary Medicine, University of São Paulo, surgical technique classes no longer use living animals for student training. Ethical cadavers are difficult to obtain and thus it is important to reuse preserved specimens. Dogs and cats that died in our veterinary hospital of causes apart from infectious disease or zoonosis were preserved. To preserve ani-

mals' cadavers we used modified Larssen solution containing 100 ml of 10% formalin, 400 ml of glycerine, 200 g chloral hydrate, 200 g sodium sulphate, 200 g sodium bicarbonate, 180 g sodium chloride and 2 l of distilled water. One part of the above stock solution was mixed with three parts of distilled water to prepare the working solution using a blender at room temperature. After fixation and between each class use, each cadaver was stored in a bag in a walk-in freezer at -16°C to -20°C.

Each cadaver was used 4-6 times, once for each surgical training laboratory, without emitting decomposition odors and keeping texture, color and consistency of tissues like skin and muscles similar to those of live animals. Different procedures were performed by the students.

The students' feedback on the use of preserved cadavers was that 96.6% of students were in favour of the use of cadavers for surgical training; in 95.1% students' point of view the ideal class would be an initial training on cadavers followed by classes with animals admitted to the veterinary hospital. This preservation technique provides acceptable cadaver quality and tissue handling for use in surgical instruction.

Acknowledge: FAPESP

ID ABS: 504

Dying to learn: The use of companion animals in U.S. colleges and universities

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Although Americans consider dogs and cats as household pets, many are harmed and killed for teaching and training purposes despite the availability of effective alternatives.

Based on a review of 92 U.S. public colleges' and universities' Institutional Animal Care and Use Committee's (IACUC) 2005-2007 records, 52% are using live or dead dogs and cats, and 26% are using live dogs and cats in harmful teaching exercises in undergraduate life science, veterinary, and medical education. A separate survey of university biology departments indicates that over half of the respondents are using cat cadavers to teach anatomy/physiology. In specific cases, IACUCs are failing to provide effective oversight to minimize animal use and suffering in education as required by the Animal Welfare Act (AWA), even though federal resources for training exist to enhance the use of alternatives.

Sources of dogs and cats for education include Class A and Class B dealers (Random Source and Biological Supply companies). A review of United States Department of Agriculture (USDA) 2005-2007 inspection reports reveal repeated violations and inhumane treatment, yet these dealers continue to sell thousands of dogs and cats annually to universities for use in education. Many of these animals were former pets obtained from pounds and shelters.

A growing number of universities, however, are changing their policies and replacing harmful animal use with pedagogically sound alternatives. Detailed examples include Educational Memorial Programs (EMPs), Shelter Medicine, Surgical Simulations, and Virtual Dissection. Templates based on successful Student Choice Policies, and No Random Source Animals Policies are also provided.

ID ABS: 533

Preclinical microsurgery training aiming at reduction and replacement

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At the end of 2004 a team composed of academic scientists and professionals (veterinarians and surgeons) started to work on a project on alternative methods for education and training in laboratory animal science (named the 3Rs in Education & Training project). The main focus had been on the development of new alternatives to the animal's use for research and education, mainly on early training in microsurgical techniques in accordance with the 3Rs principles (Refinement, Reduction, Replacement). Main targets were students, surgeons, veterinarians, laboratory animal technicians, pathologists and scientists.

The project was composed of:

1) the handbook "Anatomy of laboratory animals (Rodents and Lagomorphs)"
2) the "Microsurgery Interactive Course" on DVD

3) the device Hydraulic Trainer for preclinical microsurgery
4) the handbook "Textbook on the anatomy of swine in preclinical research".

The Microsurgery Interactive Course (a multimedial support for basic surgical knowledge and skill) and the Hydraulic Trainer for preclinical microsurgery (a simulator featuring similar to *in vivo* preclinical models) were specifically developed for preclinical microsurgery, targeted at the surgeons' early training phases.

In 2007-2008 the 3Rs project had been a key educational tool in post-degree national academic courses (Masters) and microsurgery training courses. The DVD and the Hydraulic Trainer (prototype) had been used for hand-surgery, plastic reconstructive surgery and neurosurgery courses in addition to other alternative educational tools (videoclips, rodent cadavers). Specific features and details of the "Microsurgery Interactive Course" and the Hydraulic Trainer prototype will be illustrated.

ID ABS: 554

Alternative to animals in the teaching of pharmacology laboratory

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Over the years, the pharmacology laboratory course has served as a supportive course for pharmacology lecture courses in institutions. It provides the students with an opportunity to verify the information presented in the lecture courses. A thorough understanding of scientific concepts in pharmacology should include actual exposure to the experimental determination of some of the frequently used parameters. These laboratories provide students with opportunity to work with live experimental animals and tissue preparations. Currently, this is no longer happening in most institutions where pharmacology is taught. For some years now, the pharmacology laboratory is being deleted from the curriculum in most colleges without any replacement. We offer here a review of some alternatives to the use of animals that will enhance student comprehension of pharmacological concepts during pharmacology laboratory. These alternatives include but not limited to simulations, computer modeling and other emerging technologies. Isolated tissue preparations, purified enzymes and in some cases a good cat cardiovascular experiment could be recorded and presented over time to students. This will significantly reduce the number of animals needed to teach this laboratory. The unit of information presented in the laboratory is meant to enhance the students' understanding of the materials presented in the lecture courses and therefore should be encouraged and continued.

ID ABS: 559

Animals in the laboratory: alternatives in new education from the point of view of animal welfare experience in Peru

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The housing guidelines for laboratory animals are laid out in Appendix A of the European Convention for the protection of laboratory animals. Article 5 of the Convention and Articles 5a and 5b in the Directive state the housing conditions for laboratory animals must be appropriate to their health and well-being and that any restriction on the extent to which an experimental animal can satisfy its physiological and ethological needs shall be limited to the absolute minimum. But in Latin America the regulations on the use of animals in experimentation are limited. Ricardo Palma University in Peru has developed a replacement program for the use of animals in different areas of science teaching with the goal of creating a new professional ethical way of thinking and respect for animals. In addition a wellness program for animals that are still used in laboratories has been established. This experience is important but must be backed by a legislative plan.

ID ABS: 561

The impact of education and training alternatives on animals, society, research and testing

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The impact of education and training alternatives is usually assessed in terms of acquisition of knowledge, skills and attitudes. While such assessments usually demonstrate the superiority of alternatives over harmful animal use, the level of assessment is often limited to standard teaching and training objectives. By recognising and looking beyond these limitations, the acquisition of knowledge, skills and attitudes from both the acknowledged curriculum and the hidden curriculum can be investigated. The resulting awareness can guarantee a more effective education and training; the cancellation of negative lessons from the hidden curriculum; and the aspiration to achieve a higher degree of knowledge, of practical and critical thinking skills, and of emotional and ethical literacy. Such knowledge, skills and attitudes can significantly impact on the choice of career. A fully humane education and training will positively impact on the student, the trainee, and the animals no longer used as tools; but equally important, it will facilitate a reconnection of professional practice to the roots of biology and medicine. A career in animal experimentation is not compatible with biology as the study of life, and with medicine as the practice of caring and healing.

ID ABS: 562

Animal free: the international provision of low cost and no cost alternatives

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The replacement of dissection and animal experiments in education and training continues to gain momentum due to the pedagogical, ethical and economic advantages of innovative and humane tools and approaches. The rate of replacement can be increased through effective information provision, outreach and demonstrations, access to and training in alternatives, and sharing experiences of development and implementation. An important catalyst for progressive curricular change is the provision of low cost and free alternatives. This is particularly true in countries and universities with financial limitations. As part of its commitment to making alternatives more accessible, InterNICHE has funded through its Humane Education Award the production of new software alternatives in anatomy, physiology and pharmacology that are open source or freeware. It has also negotiated with producers to encourage the international sharing of products that were formerly sold or limited in their availability. The production of freeware and the specific agreements made with producers have made available a range of alternatives that are open source or available free or at low cost. International projects to distribute such alternatives direct to teachers through the InterNICHE network of national contacts, partners and other collaborators have brought about direct replacement and the enhancement of education and training. Examples of specific alternatives, distribution projects and replacement will be given.

ID ABS: 564

Catalysing change in the curriculum across Latin America

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Significant outreach and distribution of alternatives across Latin America has supported the replacement of harmful animal use in education and training. A 6-week series of seminars addressing change in the curriculum was held across Bolivia, Peru, Brazil, Argentina and Mexico. Organised by InterNICHE and partner organisations and universities, up to 6 full-day seminars were organised at universities in each country. Smaller meetings with campaigners, teachers and professional bodies comple-

mented the seminars. All events included spoken presentations, demonstrations and free trial of a wide range of alternatives. Speakers included InterNICHE experts and teachers, students and lawyers who are involved in replacement activity from the host countries. Partner organisations were empowered through the process of planning and execution of the seminars, and the seminars helped to identify and provide support to others who are progressing humane education initiatives. Information resources and freeware alternatives were widely distributed, and translations of material into Spanish and Portuguese continue. Data on the current situation concerning animal use and alternatives, including laws and regulations, was also gathered from each country. The seminars succeeded in raising awareness and generating interest in replacement alternatives, and received significant media coverage. To continue the hands-on access to alternatives provided at the seminars, InterNICHE Alternatives Loan Systems have been established in Mexico, Peru and Brazil, and alternatives donated to selected universities will promote humane education through example. Further national-level conferences, training and distribution of alternatives are being organised.

ID ABS: 589

How should animals be studied?

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The diversity of the forms, physiology, developmental biology, genetics and the evolutionary history of animals and plants grossly sets the styles of experimental studies (both past and current ones) to understand the molecular, cellular cascades of dynamics of these organisms. Experimental biologists are expected to follow certain guidelines (institutional, personal, ethical, or otherwise) in order to safeguard the routine life support systems to be operative while the experimental tenures are on. This scheme would likely provide a moderately tolerable amount of data on the organisms under stresses (both exogenous and endogenous), ablations, implantations, herniated situations, grafting, etc. Historically, of course, the strategy of using, for example, carbon particles, colloidal iron, etc. has been replaced with applying radio-labelled substances in order to measure the degree of uptake or absorption by a particular organ or parts thereof. However, colloidal gold is even today routinely used in studies in histochemistry. This paper will document some of the earlier experiments of Trembly on hydra and also of Anna Bidder on the digestive physiology of cephalopods and others to indicate the finer tunings of experimental biology. Usually, we tend to forget the components of mind, pain and sorrow of wild animals. In the Darwinian birth bicentennary year (1809-2009), while accepting the evolutionary unity or bondage among the single celled to multicellular organisms, our concerns for the optimum care for organisms should be sincere and institutionally binding.

ID ABS 803

The Doerenkamp-Zbinden foundation's chairs on alternatives to animal experimentation in research and education

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The DZS foundation is a Swiss-based foundation that has dedicated its activities and support to the development and promotion of alternatives to animal experimentation in biomedical research and education according to the well-known 3R principle (replacement, reduction and refinement) in the field of biomedicine. The foundation has undergone four eras of existence and development. In the last era, which started in 2004, the foundation's course has been strongly streamed into the direction of promotion of replacement and reduction alone. The last era of the foundation can also be called an era of chairs on alternatives to animal experimentation. Realizing the importance and the role of chairs on alternatives to animal experimentation the foundation has, up to now, focused on establishing endowed chairs at several universities. The first chair named "Doerenkamp-Chair for Innovations in Animal and Consumer Protection" was installed in 2003 at the university of Erlangen (Germany) by the foundress H. Doerenkamp personally. This chair has been installed for the period of 5 years and is now in a prolongation phase of 2 more years. It has been mainly focused on refinement and reduction by imaging techniques in biomedical research. In 2006, a "Foundation-Professorship for *In vitro* Methods for the Replacement of Animal Experiments" was installed at the Uni-

versity of Konstanz (Germany). The exceptionally good start of these chairs motivated the foundress to encourage the decision to place the emphasis of the foundation's work on the establishment of further university chairs. Since then the following chairs have been established:

In October 2008 at Utrecht University (The Netherlands) the "Doerenkamp-Zbinden Professorship for Alternative Methods in Toxicology" was installed. The chair will be financed for six years. In January 2009 in co-operation with the Egon-Naef Foundation the DZS established the "Doerenkamp-Naef-Zbinden Professorship on Alternative Methods to Animal Experimentation" at the University of Geneva (Switzerland). The chair will be financed for six years. Also in January 2009 the "Doerenkamp-Zbinden Endowed Chair for Evidence-based Toxicology" was established at the Johns Hopkins University, Baltimore, USA. This chair has been permanently installed for as long as the Johns Hopkins University exists. In July 2009 a contract for 5 years was signed with the Bharathidsan University, Tiruchirappalli/Tamil Nadu in India to found a "Mahatma Gandhi-Doerenkamp Center for alternatives to the use of animals in life science education" with a Gandhi-Gruber-Doerenkamp chair.

PO20: Animal use policies

ID ABS: 2

Corporate social responsibility and laboratory animals

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Protection of laboratory animals is not an exclusive government task. Businesses and organisations should also take responsibility in this matter. Corporate Social Responsibility (CSR) offers us a sound framework within which the particular responsibility towards laboratory animals can be modelled. Corporate ethics with respect to people, planet and profit are at stake. However, animal testing is not covered, whereas it can surely be considered a social issue. For this reason, the Dutch Society for the Replacement of Animal Testing has initiated a round table discussion with key players from corporations, academia and not-for-profit organisations to ensure that policy on laboratory animals is encompassed in CSR.

An initial result of the talks is the Transparency Code, whereby organisations undertake to report on their animal testing policy, according to set guidelines. Organisations adhering to the code agree to report on numbers of animals and species used, research goals, as well as their policy regarding alternatives to animal testing. The next step would be to report on their policy and to evaluate its implementation.

The round table is presently elaborating on a dialogue between stakeholders, another essential element in advancing CSR. Supply chain responsibility will also be addressed. An organisation that outsources its animal testing or buys products that have entailed animal testing, must likewise assume responsibility.

This new approach in the debate on animal testing opens up innovative ways to achieve a reduction of animal testing. Cooperation between companies, academia and animal welfare organisations yields new insights to the benefit of laboratory animals.

ID ABS: 68

Employing the European seabass (*Dicentrarchus labrax*) in tests requested by Directive 67/548/EEC (REACH) for optimizing the use of test animals

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The REACH Directive (EC n.1907/2006) contains many regulations, which in accordance with the Global Harmonization System (GHS) discipline the labelling of the hazard factor of chemical substances put on the European market. Furthermore, the Directive requires the classification of chemical substances through the collection of ecotoxicological data. This kind of data can be obtained by tests with algae, crustaceans and fish species as specified in Annex V of Directive 67/548/EEC and integrated in the regulation CE 440/2008, Part C: "Methods for the determination of ecotoxicity". The four methods implicating fish use different species, which not only complicates the laboratory management but also implies an excessive use of test organisms. During the last 10 years both ISPRA and ARPAER have worked with the European seabass (*Dicentrarchus labrax*, L. 1758), an eurialine species often used in aquaculture, which therefore, and considering our experience, could be an appropriate test organism for application of the REACH Directive. This species offers various advantages, like its constant annual availability and a good tested sensibility for many reference toxicants (D.Lgs. 152/06, D.D. 23/12/02, Manual ICRAM-APAT concerning marine sediments). We propose to use the European seabass as the only species for all the requested chemical substance tests with fish, because this will most importantly reduce the number of organisms needed by about 50% and moreover facilitate and simplify the laboratory work required. In order to carry out the REACH regulations, ISPRA and ARPAER are preparing an experimental project on performing the requested ecotoxicological tests only on the European seabass.

ID ABS: 75

Cosmetic use of Botox

I. Ruhdel, U. Gross and R. Kolar

Animalwelfare Academy – German Animal Welfare Federation, Neubiberg, Germany

Botox is the brand name of botulinum toxin, the most potent poison known produced by bacteria. It acts by blocking nerve impulses to muscle fibers, paralysing them. Botox is increasingly used as a local cosmetic treatment for relaxing facial muscles, which temporarily smoothes the overlaying skin. Each batch of bacterial poison is tested, not locally but systemically, on about 100 mice in a classical LD_{50} test (lethal dose-response killing 50% of animals). The suffering of the mice is extreme: paralysis, impaired vision and death by suffocation after 3 or 4 days.

The European *Pharmacopoeia* accepts three alternative testing methods (one animal-free and two which reduce the suffering), but still the LD_{50} test is used. As a cosmetic, Botox is licensed independently from Botox for clinical use. In spite of the EU cosmetic animal testing ban manufacturers continue to use the LD_{50} test on mice even for Cosmetic Botox without restriction, ostensibly because Botox is injected subcutaneously.

In addition to facts on Botox testing, the poster will reflect the discussions of the international expert meeting on alternatives in Botox testing, which will take place in April 2009 at the German Federal Institute for Risk Assessment.

ID ABS: 97

The 3Rs – the next 50 years

K. Reid

EWLA/Eurogroup for Animals, Brussels, Belgium

Since the 3Rs of replacement, reduction and refinement were developed exactly 50 years ago, they have become a "household term" and a definite driving force to improve welfare of animals used in laboratories all over the world and they contribute to a better life for millions of animals.

Now we look at the future, the next 50 years, and the commitment required to reach the ultimate goal of replacement. Where will we be in 2059? What will we have achieved? What is required is more emphasis on alternative research and implementation, constructive debate involving all stakeholders and importantly, a change of mindset to embrace the idea that it is possible to accomplish scientific improvements and safety testing without reliance on animals. Everything is possible!

ID ABS: 125

Regulation increases people's willingness to support the use of animals in research

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Different countries have adopted different regulatory systems to protect research animals, all with the goal of maintaining public accountability. Whether the regulatory system affects public acceptance of the use of research animals has never been evaluated. This study used an interactive, web-based survey to test the effect of different levels of regulatory oversight on people's (n=592) willingness to accept the use of animals in research. Three different research scenarios were presented in the survey: non-invasive research using normal pigs, invasive research using normal pigs and non-invasive research using genetically modified (GM) pigs. All research scenarios were presented in a regulated (i.e. including independent ethical review and site inspection) or unregulated environment. Most (82%) participants agreed to animal use in non-invasive/non-GM research. For invasive and GM research, regulation increases the support from survey participants. For example, 61% of participants did not support the use of pigs in unregulated invasive research, but 52% of these initially negative votes switched to a "yes" when asked if they would permit this animal use under a regulated oversight system. These results show that the use of animals in non-invasive/non-GM research enjoys support from survey participants and that this support declines when research is invasive or involves GM subjects, but that the decline in support is reduced when the animal use occurs under a regulatory oversight system. Our study highlights some concerns around invasiveness and GM, but also illustrates that people trust regulatory bodies to address these concerns, while simultaneously enabling scientific progress.

ID ABS: 174

The Israeli Animal Experimentation Law: insights from 15 years of legal campaigning

T. Lousky

InterNICHE Israel, Bat-Yam, Israel

The Israeli Animal Experimentation Law (IAEL) was legislated in 1994, based substantially on the US Animal Welfare Act and the European Commission Directive 86/609. The IAEL includes the 3Rs principle, and defines the process by which animal use in research, testing, the production of biologicals, and education and training is regulated. This regulatory process includes the National Animal Experiments Council (NAEC), which supervises over 50 Internal Ethics Committees (IEC) in various academic and industrial institutes. The NAEC is also responsible for developing detailed regulations and for accepting and authorising alternatives. Since it has been enacted, the IAEL has been frequently criticised by animal protection organisations. Claims have been made that the NAEC and the IECs are inherently biased, that alternatives are not given due consideration, and that animal experiments are almost automatically authorised. Several lawsuits and appeals to the Israeli Supreme Court have been filed. This work discusses the IAEL, the criticism against it, and some of the legal campaigns initiated since its enactment.

ID ABS: 193

Dialogue: a commitment to communicate about animal experiments

M. Kuil-van Nederpelt

Dutch Society for the Protection of Animals, The Hague, The Netherlands

In the Netherlands animal experiments are regarded as a necessary evil. It is a sensitive subject of which the general public is hardly aware. Openness and transparency about the use of animals and the objectives could increase the awareness. Unfortunately, many research institutions and scientists are not willing to be open about their work for fear of being attacked and threatened by aggressive radical persons and animal rights groups.

The Dutch Society for the Protection of Animals, the biggest animal welfare organisation in the Netherlands and a member of Eurogroup for Animals in Brussels, is of the opinion that only

by dialogue among the stakeholders – including indirectly the general (interested) public –, a serious and respectful debate about animal experiments is possible. This opinion is also supported by many other animal welfare organisations in Europe and other countries all over the world.

Replacing, reducing and refining animal experiments can only be accomplished when there is respect for each other and when there is trust, hope and commitment that by working together problematic issues can be solved. Together we should communicate to the public our concerns about societal health and safety issues, scientific developments and animal welfare improvements and show them that we are working towards phasing out animal experiments. Dialogue is the key way to do that.

ID ABS: 199

Trends in animal experimentation and 3R research in the Netherlands: a survey

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During the last two decades, the debate around animal experiments has heated up. Although in the past and now animal experiments have been and still are important for scientific progress, medical needs and the economy, the resistance against animal experimentation has grown increasingly. To overcome this dilemma the Dutch government has recognized that more in depth knowledge about focus and strategies in 3R research is crucial. This poster will show the results of two running studies that will help to develop a policy that can react proactively to trends in animal experimentation and 3R alternatives. The first aims at describing current and future trends in animal experimentation and 3R alternatives. The second study focuses on prioritising areas of 3R research by revealing current innovations in 3R alternatives research and strategies. To fulfil the aims of the above-mentioned studies, a survey was performed in the Netherlands amongst researchers, representatives of licensees of animal experiments, laboratory animal experts, relevant professional organizations and NGOs, biotechnological companies and bio-technicians.

ID ABS: 200

Revision of EU Directive 86/609: current developments

R. Kolar, I. Ruhdel and U. Gross

Animal Welfare Academy – German Animal Welfare Federation, Neubiberg, Germany

Since the adoption of the EU Directive 86/609/EEC in 1986 important progress has been made in science and new techniques. Additionally the demand of the public for a better protection of laboratory animals increased as shown by recent opinion polls. A first draft for a new Directive was published by the Commission in November 2008.

The proposal of the Commission already includes some provisions that would improve current animal welfare standards for laboratory animals within the EU as the broadening of the scope to basic research and training experiments, a compulsory authorisation procedure including ethical evaluation for all animal projects, a retrospective assessment of some projects, more impetus on the funding of 3Rs methods and the establishment of national reference laboratories. Disappointing is the lack of a ban on the use of non-human primates. It looks like the European Parliament will try to weaken the Commission's proposal at the first reading (plenary vote expected in May 2009), especially with regard to the authorisation system, the use of non-human primates and the scope.

Together with its umbrella organisations, Eurogroup for Animals and the European Coalition to End Animal Experiments, the German Animal Welfare Federation will continue its lobby and campaign activities throughout the legislative procedure to reach the best protection for laboratory animals possible.

ID ABS: 205

JaCVAM statement on new alternative to animal testing

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The Japanese Center for the Validation of Alternative Methods (JaCVAM) has a framework for peer review and regulatory acceptance of alternative methods. After JaCVAM has received a request for peer review from a researcher or developer, the JaCVAM steering committee meets to deliberate on the proposed methods. Upon the receipt of permission for peer review, JaCVAM organizes the oversight committee in order to evaluate a new test method. Based on the report and references prepared by the oversight committee, a peer review panel evaluates a new or revised test method. The members of the oversight committee and the peer review panel assigned to evaluate a new test method are selected by the JaCVAM steering committee. JaCVAM and its steering committee have a regulatory acceptance board for new or revised methods. This board reviews new or revised test methods based on the reports of the peer review panel and prepares a report and statement on the test method for the regulatory agency.

In 2008 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the non-commission members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statement:

1) Vitrolife-Skin, a 3-dimensional cultured skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the OECD testing guidelines No. 431 on *In Vitro* Skin Corrosion: Human Skin Model Test.
2) LLNA (Local Lymph Node Assay) -DA can be used for distinguishing between sensitizer and non-sensitizer chemicals within the context of the OECD testing guideline No.429.

ID ABS: 223

Editorial policies of scientific journals regarding the use of animals – a survey of current practice, and proposals for good practice

M. Jennings, N. Osborne and K. Westwood

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The role of scientific journals in disseminating information on good practice in animal research has been reported in a variety of fora including the scientific literature. The inclusion of this information facilitates the uptake and implementation of the 3Rs, contributing to the development of more humane science. In addition, journals have a role in helping to address the ethical issues integral to the use of animals in research and testing, and in stimulating informed discussion of such issues.

We have recently carried out a survey of journal policies to see how well they fulfil the roles described above, with a view to identifying and developing good practice. A statistically representative sample (304/1444) of journals that published papers and/or articles involving the use of animals, between 2006 and 2007, was taken and the policies were then analysed against a number of criteria relevant to animal welfare, ethics and the 3Rs. The results of the survey will be described briefly in this presentation. We subsequently developed "principles of good practice" and a summary of these will also be presented.

The principles have been well received, and we are now collaborating with journals and publishers regarding their inclusion within editorial policies.

ID ABS: 265

International accreditation to support application of animal policies

J. Guillen

AAALAC International, Pamplona, Spain

There are several mechanisms that in different combinations may serve to control and ensure the application of animal care and use policies at research institutions: i) the specialists on laboratory animals working at the institution (veterinarians, biologists, animal technicians, etc); ii) the ethics/animal welfare committees, either at institutional or government level; iii) the

official inspectors from the competent authorities; iv) the application of Quality Systems, like ISO and GLP; and v) the application of a specialized and independent scheme of assessment and accreditation like AAALAC International.

This evaluation and accreditation is a peer review process performed by specialists in all the areas of laboratory animal science who form the Council on Accreditation. These external specialists assess whether the institution complies with the applicable animal care and use recommendations, policies and regulations, identify deficiencies and recommend how to solve them. The periodicity of the accreditation system engages institutions in a continuing improvement process, linking animal welfare and quality of science. The different policies and regulations around the world and the diverse research environment make the application of performance standards, which can be more easily harmonized, indispensable to carry out assessments of animal care and use at the international level.

ID ABS: 268

Nonhuman primate use under scrutiny

U. Gross, I. Ruhdel and R. Kolar

Animal Welfare Academy – German Animal Welfare Federation, Neubiberg, Germany

The use of primates is increasing in Europe; over 11,000 of them, mostly macaques, are used every year. Opinion polls show growing opposition of European citizens to primate use in laboratories, while research organisations are fighting any restriction on primate use. However, the more critical attitude towards primate experiments is finding its way to licensing authorities. In Zurich, Switzerland, two license applications for primate experiments in brain research were rejected recently. In Germany primate experiments in brain research have been rejected in Berlin, Munich and Bremen. In all cases cost to the animals was balanced against the expected benefit for humans. This resulted in the decision that the hypothetical benefits did not outweigh the suffering of the macaques and marmosets, and thus the experiments were not ethically justifiable. In Zurich and Bremen, researchers did not accept the verdicts and went to court. No final decisions have been made so far.

A review of ethical evaluation in licensing procedures has been long overdue. This poster provides information on the rejected experiments, recent developments of the court cases, the lines of argument of all parties and proposed regulation on the use of primates in the draft EU directive on the protection of animals used for scientific purposes.

ID ABS: 282

Cumulative suffering – how can it be assessed?

K. Garrod, K. Ryder and D. Anderson

Animal Scientific Procedures Inspectorate, Home Office, London, UK

The proposed revisions to EU Directive 86/609 will require the severity of procedures applied to animals for a scientific purpose to be classified as mild, moderate or substantial or unclassified. Assessing the severity that a scientific procedure will cause to an animal can be particularly difficult when animals undergo several procedures and when the nature of the procedures means that the animals may have to be kept in less than ideal housing conditions. For example, animals used for behavioural neuroscience experiments may have to undergo several lengthy operations over the course of many months or years, have their bodies restrained and have their heads immobilised for several hours a day for many weeks at a time. Access to food or water may be limited during periods of work. Single housing for prolonged periods of time may be needed for husbandry or scientific reasons. Each individual procedure, taken in isolation, is likely to cause no more than moderate severity if done in the most refined way, but the cumulative effect will need to be assessed in order to determine the overall severity of the combined procedures. Published papers on assessing severity can help identify what criteria should be considered but do not provide definitive advice on what constitutes mild, moderate or substantial suffering in such circumstances.

The poster will describe how UK Animals Scientific Procedures Inspectors have assessed cumulative suffering both before and during the course of a series of procedures using case examples.

ID ABS: 283

Unclassified, mild, moderate, substantial severity – what do they mean?

K. Ryder, K. Garrod and D. Anderson

Animal Scientific Procedures Inspectorate – Home Office, London, UK

Since 1986 UK legislation has required the severity of procedures applied to animals for scientific purposes to be classified as mild, moderate, substantial or unclassified. This classification is used to limit the amount of pain suffering distress and lasting harm that can be applied to an animal and to provide an overall description of the "average" suffering that an animal would experience when being used in a particular scientific programme of work. This allows the welfare costs to the animals to be weighed against the benefits likely to accrue from their use. The proposed revisions to EU Directive 86/609 introduce these concepts into European legislation for the first time.

The poster will describe how these classifications have been interpreted by the UK Animals Scientific Procedures Inspectorate and provide examples of procedures that can fall into the different categories and procedures that may fall into two or more different categories, depending on how they are used and what scientific or humane end points are applied.

ID ABS: 289

The current regulatory regime on laboratory animals in the Brazilian biomedical research arena

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The aim of this presentation is to describe the current regulatory regime on laboratory animal experimentation in the Brazilian biomedical research arena and to shed light on the issues representing potential challenges to policymakers as regards regulation implementation. The use of laboratory animals in research, testing and education is a complex and controversial issue addressed by antivivisection organizations, animal welfare groups, law makers, researchers, producers, veterinarians and politicians internationally. On March 7, 2008, Science magazine published an article on the Brazilian scientific community's battle against a series of local attempts to ban animal experimentation and the hope for a federal law addressing animal research that would put a stop to such local bans. As presented in the Science magazine article, a federal bill addressing laboratory animal experimentation was introduced in 1995 by Chamber of Deputies member Sérgio Arouca. In the mid-2000s, as opposition to the use of laboratory animal testing intensified, researchers lobbied harder to get the bill to a vote. After 13 years of lingering in the Chamber of Deputies and the Federal Senate, the Brazilian Federal Law on Animal Experimentation (Law 11794) was finally passed. Law 11974 regulates the care and use of laboratory animals in teaching and research in accordance with the 3Rs principles of humane experimental technique. However, it places less emphasis on alternatives than was previously treated legislatively and is expected by the Animal Protection Societies, for example banning the use of laboratory animals for scientific or didactic purposes when alternative or substitutive experimentation methods exist.

ID ABS: 297

Draft proposal for a Brazilian centre on alternative test methods

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Several initiatives are currently underway in Brazil to foster the creation of centres dedicated to alternatives to animal testing. In 2008 Dr. Sá-Rocha organised a meeting with Brazilian regulatory authorities and the major stakeholders in the field of testing to foster discussions on the process of funding, development and validating alternative methods in Brazil. Dr. Presgrave published a scientific article on "The need for the Establishment of a Brazilian Centre for the Validation of Alternative Methods". Dr. Nunes and Prof. de Carvalho prepared and presented a proposal for the creation of a Centre for the Validation of Alternative Methods in 2008 to the Brazilian National Agency of Health Surveillance (ANVISA). Furthermore, also in 2008, a new legislation was adopted in Brazil regarding the use of animals for scientific purposes ("lei Arouca"), in which Dr. Rivera was involved. It establishes, amongst other provisions, the task to monitor and evaluate the introduction of alternative methods. However, the promotion and information on existing alternative methods to the large Brazilian scientific community are not foreseen within such legislation.

In order to streamline the different activities undertaken and to consider the current and novel Brazilian needs related to alternative test methods, a new joint proposal is under discussion by those involved in the initiatives which took place so far and with the collaboration of Dr. Eskes from ECVAM as an independent moderator. Indeed, ECVAM has been involved in the initiatives on various occasions already.

ID ABS: 298

The ECVAM retrospective validation study on cytotoxicity- and cell function-based *in vitro* assays for eye irritation

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European legislation strongly calls for alternatives to replace animal testing. In major validation studies conducted in the 1990's, no single method was found suitable to fully replace the Draize eye irritation test. However, some alternatives showed good reproducibility and reliability.

In 2006, ECVAM initiated the retrospective validation of four cytotoxicity and cell function based assays: Neutral Red Release (NRR), Red Blood Cell test, Fluorescein Leakage (FL) and Cytosensor Microphysiometer (CM). The study was coordinated by an international Validation Management Group (VMG) including ICCVAM-NICEATM and JaCVAM observers. Existing data were collected and compiled according to ECVAM Principles of Weight-of-Evidence Validation. The resulting Background Review Documents (BRDs) for each assay were structured according to the ECVAM Modular Approach to Validation and underwent independent quality control. The VMG evaluated the four BRDs and made recommendations concerning the suitability of the assays to be used in Bottom-Up and Top-Down Approaches for Eye irritation Testing Strategy proposed in a 2005 ECVAM expert meeting. The CM protocol INVITTOX 102 modified and the FL-INVITTOX 71 were recommended to discriminate severe irritants from other classes. Furthermore, the FL-INVITTOX 120 and the NRR INVITTOX 54 and PREDISAFE were recommended to discriminate non-irritants from other classes. These assays are currently under Peer Review by ESAC. The ultimate goal is to combine validated *in vitro* assays for eye irritation according to their performances and applicability domains, to develop the most suitable testing strategy to classify substances according to their eye irritation potential and to ultimately replace the Draize rabbit eye test.

ID ABS: 310

The ethics committee's functions for animal experimentation in Lithuania

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In Lithuania there are 7 institutions which use laboratory animals for teaching and research. The number of used animals is decreasing: about 15,000-17,000 vertebrate animals were used in 1995-1997, and only 4,000-5,000 during the recent years. This can be related with reorganization of scientific research institutions and their programmes (eliminating or involving animals); researchers having the possibility of buying standardized animals; the possibility of applying alternative methods; improved qualifications of researchers; training of personnel. Today in Lithuania we have in force legal acts that regulate the use of laboratory animals for experimentation or other scientific purposes. The Ethical Committee (EC) for the use of Laboratory Animals represents a governmental establishment (State Food and Veterinary Service, SFVS) and was founded ten years ago. The main objectives of EC are to implement the requirements of European Conventions, EU and national legislation covering vertebrate animal breeding and use for research, and to put forward a proposal for SFVS on licenses regarding experiments involving animals. Two nongovernmental organizations serve the same purpose, i.e. Lithuanian Association for the Protection of Animals and Lithuanian Laboratory Animals Science Association. The Ethical Committee collects and analyses the data of experiments which are carried out and propagates alternative testing methods for research and teaching.

ID ABS: 316

Animal experiment refined: first Brazilian regulation on animal experiments

T. de Astrogildo e Tréz

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The very first law on animal experimentation was approved recently in Brazil, and is now part of a roll of legal instruments that profile the government's attitude regarding animal use in experiments. The law 11.794/08 establishes a new legal reality that at the same time starts a questionable legitimacy concerning not only the animal experimentation issue also its context of approval, will orientate new possibilities of conduct to Ethics Commissions, researchers and representatives of animal protection societies. This work aims to analyze critically the implications that this law brings to the Brazilian reality. The link between this law and the concepts of Russell & Burch's Reduction, Refinement and Replacement is identified and questioned. The conclusion is that the body of the law emphasizes the refinement of animal experiments and gives small importance to reduction and replacement. The implication is discussed around concepts of humanitarianism and animal welfare.

ID ABS: 320

Improving implementation of 3Rs knowledge – which way to go?

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Implementation of the 3Rs contributes not only to animal welfare but also to the scientific quality of the research. Accessibility of current knowledge of the 3Rs is needed to optimally apply the available 3R alternatives. Huge effort has been put into developing 3R databases and making 3R information available in bibliographical databases, and expertise on the subject has grown. However research among biomedical scientists (Leenaars, *ATLA*, 2009) has shown that knowledge of 3R databases and skills to search in these databases is limited.

As a follow up, we are conducting a national survey to get more insight into how knowledge of available 3R alternatives is being obtained and used in the Netherlands. Scientists, AEC members and animal welfare officers of Contract Research Organisations (CROs), industry and universities are being asked

how current 3R knowledge is obtained and implemented in practice, how they evaluate these activities and what are barriers and opportunities for optimal implementation.

The results of this study will lead to new insights into common practice and new strategies to improve implementation of available 3R knowledge. Preliminary results from pilot interviews with local researchers, animal welfare officers and members of the animal experiment committees show that investigators are aware of the moral importance but not of the possible scientific value of the 3Rs for their own research.

ID ABS: 530

Veterinarians' roles in improving the use of alternatives in the life sciences

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Veterinarians are uniquely positioned to assist in the development, acceptance, and implementation of alternatives to animal use in the life sciences.

Trained in systems biology and medicine, research veterinarians are involved in the conduct and evaluation of *in vivo* bioassays, and are well-suited to help develop alternatives. Actively engaged in developing animal models of human and animal diseases, veterinarians should also participate in the validation of new methods and work to gain their acceptance and implementation.

Veterinarians play a critical and trusted role in the broader use of animals in the life sciences and can be instrumental in shifting societal perceptions of acceptable use. Central to the 3Rs is duality of respect for the utility of animals and their welfare; veterinarians can extend these principles beyond laboratory settings.

Veterinarians have multiple opportunities to educate scientists, educators, corporate and non-profit personnel, policy makers and the public in the scientific and ethical merits of embracing alternatives. Laboratory animal veterinarians ensure animal welfare by providing health care and advice, training in good practices, and counsel scientists about selection of appropriate models for research. They may assist policy makers in developing standards that appropriately ensure consideration of alternatives. In educational settings, veterinarians can teach proper respect for animals, appropriate care to ensure welfare, and guide educators and the public who make use of animals in the classroom, science fairs or exhibits. Veterinarians in private clinical practice can educate the public about the importance of adopting a 3Rs mindset, while embracing the value of biomedical research.

ID ABS: 551

Attitudes towards genetic modification and the number of animals used in experiments

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Reducing the number of animals used in an experiment is a vital approach to achieving reduction, and experts within the scientific community are typically responsible for the determination of these numbers. In many countries total numbers of animals used in all experiments are publicized for members of the public to view; rarely are they privy to the numbers of animals used within individual experiments. Using an online survey and an experimental approach, we probed the attitudes of the public towards different numbers of pigs used in 2 different types of research to reduce agricultural pollution or to improve organ transplant success in humans. Each survey varied from 10, 100, to 1,000 pigs and varied by whether or not the research involved genetic modification (GM). Public support for animal use declined rapidly when the research required the use of GM animals. In contrast, the number of animals required for experiments had less effect on support. For example, 82% of participants supported the use of 10 pigs for the study to improve transplant success; this support declined to 57% when research required 100 or 1,000 pigs. Some participants commented that a study with just 10 animals seemed too small to be scientifically valid. These results suggest public acceptance of animal use is less affected by the number of animals required than the type of research and the nature of the animals used.

ID ABS: 570

Evaluation toward the reduction, refinement and replacement of laboratory animal procedures: thoughts on some encounters with Dr. Enrique Lelo De Lar

E. De Larrea Reyes

Universidad Nacional Autonoma de Mexico, Mexico

A variety of encounters with Dr. Enrique Lelo de Larrea Reyes over the last 25 years are reviewed in relation to their significance in terms of progress toward the reduction, refinement and replacement of animal procedures in toxicology and toxicity testing. Included are the work of the International Conferences on Practical In *Vitro* Toxicology and the foundation of Toxicology in Vitro, the work of an Institute of Medical Ethics working party on issues raised by animal experimentation and alternative approaches, the need for *in vitro* assays for chemical carcinogenesis based on the transformation of human cells, the problem presented by the human hazard potential of thousands of chemicals already in use before modern regulations for the registration of new chemicals came into force, and the importance of testing strategies with a focus on the integrated use of non-animal computer-based and *in vitro* test systems.

ID ABS 809

Rehabilitation of macaque monkeys retired from neuroscience research

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Macaque monkeys are commonly used in biomedical research. Some reach "humane end point" while others are healthy enough to continue life.

However, as opposed to ex-laboratory chimpanzees, much less effort is put into retiring and rehabilitating these monkeys.

This paper will describe our ten years experience in rehabilitating macaque after neuroscience research, based on 70 macaque monkeys (*macaca fascicularis* and *macaca mulatta*) retired up to date. The monkeys arrived separately during the years and are now living in groups created in the sanctuary ranging from 3-13 individuals.

First, establishing the conditions for retirement is discussed as an important factor for overcoming initial barriers for retirement.

Next, the success of the rehabilitation process will be addressed using three main indices for assessment: the physical condition of the monkey, the degree to which social skills have been gained through rehabilitation and the occurrence of stereotypical behavior.

Some limitations of retirement and rehabilitation of macaque will be put forward.

Theme 3: Progress in life science domains

Breakout Sessions

Session BS21: Skin and eye toxicity I

Contribution of post-validation activities in the area of *in vitro* reconstructed human epidermis (RhE) skin irritation tests to the process of OECD acceptance

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After a prevalidation of five *in vitro* tests to predict skin irritation, two promising assays, the EpiSkin and EpiDerm, based on Reconstructed human Epidermis (RhE), were further refined and a common skin irritation test (SIT) was developed. The SIT was then evaluated with 20 prevalidation test chemicals, verified by another 35 chemicals and finally entered the ECVAM Skin Irritation Validation Study (SIVS). While both RhE models showed acceptable reproducibility in the SIVS, only EpiSkin showed a balanced sensitivity/specificity leading to the recommendation as stand-alone replacement of the rabbit test by ECVAM's Scientific Advisory Committee (ESAC). In contrast, the EpiDerm provided a lower sensitivity, and was recommended for use in a tiered strategy.

To increase sensitivity, the EpiDerm SIT was modified by prolonging chemical exposure from 15 to 60 min. This modified EpiDerm assay was evaluated by testing 52 SIVS chemicals in one laboratory and then validated by blind testing of 20 Performance Standard (PS) Reference Chemicals (RC) in four laboratories. At about the same time, another RhE model, SkinEthic -RHE, was evaluated by testing 39 chemicals and then validated by blind testing using the 20 RC chemicals in three laboratories. Also here, The exposure time was optimized to 42 min to represent the intrinsic properties of the test method. Both assays were endorsed by ESAC in 11/2008 as stand-alone replacement of the rabbit test.

The OECD draft Test Guideline for *in vitro* skin irritation testing based on RhE is based on the three validated assays, the EpiSkin, modified EpiDerm and SkinEthic-RHE. Furthermore, an equivalent EU test method (B.46) was accepted for inclusion into the EU Test Method Regulation 440/2008. The Performance of the three assays under the Globally Harmonized classification System (GHS), adopted by the EU in December 2008, were retrospectively confirmed and endorsed by ESAC. Consequently, also the original PS, based on the previous EU classification system had to be adapted to GHS. Meanwhile, a study on testing of colorants and a further PS-based study employing a Japanese RhE (LabCyte) have been completed. At presently, the inclusion of all four RhE models into the Draft OECD TG is under evaluation.

An iterative approach to complete regulatory acceptance

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The cosmetic legislation imposes validated Replacement methods in the risk assessment process of ingredients and finished products and foresees strict testing and marketing bans. REACH, on the contrary, envisages the use of "suitable" 3R-alternatives for hazard assessment and screening purposes.

Therefore, during the development and validation of alternative methods, the most stringent legislative requirements should be taken on board. Unfortunately, this principle has not always been respected.

For local toxicity, a number of validated Replacement alternatives exist for testing of skin corrosion (TER, EpiSkin, EpiDerm), skin irritation (EpiSkin, modified EpiDerm, SkinEthic) and phototoxicity (3T3 NRU PT). The LLNA, however, is a validated Refinement/Reduction alternative for skin sensitisation that still can be used (outside Europe) until 2013.

A reality check on the acceptance and incorporation of these alternative methods in cosmetic dossiers was set up by establishing a databank containing all safety data present in the publicly available SCC(NF)P opinions, made between 2000 and 2009. These concern potentially harmful cosmetic substances including UV-filters, preservatives, colorants and in particular hair dyes.

Careful analysis of these data revealed critical points that need more attention in the near future, namely (i) verifying the applicability domain and (ii) conducting post-acceptance monitoring as part of the optimisation process of recently validated methods. Measures are being taken now to develop better interactions between regulatory (actual SCCS) and validation authorities (ECVAM) starting from the setting up of lists of relevant reference compounds for new validation studies. This should assist in better future acceptance.

Building and tuning *in vitro* skin toxicity protocols is driven by tissue model structure and test-application constraints

J. Cotovio

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Many reconstructed tissues are available today as sound models for *in vitro* skin irritation/toxicity studies. The complexity of the structural elements combined with multiple cell-to-cell, cell-to-substrate, and cell-to-medium interactions will influence the key fundamental capacities and behavior of the tissues. Therefore, these models may be able to mimic some of the inter-individual variability existing in human skin. Skin irritation Prediction Models (PM) defined through already validated test-methods in EU (i.e. EpiSkin™, EpiDerm™, SkinEthic™-RHE) and regulatory recommendations for further use will have a certain impact on possible upcoming new candidate test methods. Recommendations quoted in guidelines intended to fix the sphere of operation for future developments and the adequate choice of models, parameters and PM. However, it is expected to find reasonable flexibility in well targeted predefined points, allowing new test-methods, adjusted to new test systems, to develop. Adaptations should necessarily play with specific model's constraints (biomechanical resistance, packaging and environment...) and specificities (cell type, metabolism, barrier function, aging...). Furthermore, test-substance specificities are also part of the necessary adaptations (physicochemical properties...). Finally, constraints related to the protocol itself are usually linked to timings (exposure and incubation steps), spreading test materials, rinsing issues and the endpoint used (standardization, robustness, transferability). Obviously, all adaptations have to combine some of these constraints in order to fit in with main recommendations while keeping dynamic processes. The presentation will address some of these issues and will give examples of adaptations on skin models currently commercialized and/or purpose made which may possibly impact endpoints and protocols.

Mechanistic assays for *in vitro* prediction of skin sensitization: integrating biological and chemical measurements in a holistic approach

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The skin sensitization reaction to small molecules is a complex immunological process, yet it is relatively well understood at the molecular level: In order for a molecule to be a sensitizer it must (i) gain access to the viable epidermis, (ii) react with skin proteins, (iii) the modified proteins need to be presented by MHC molecules on dendritic cells, (iv) in parallel the molecule must activate a "danger signal" in keratinocytes and/or dendritic cells to initiate migration of dendritic cells to lymph nodes and finally (v) the dendritic cell must activate proliferation of hapten-specific T-cells in the lymph node. Given the complexity of this process, there is a paradigm widely held within the scientific community that no single *in vitro* assay can reliably predict sensitization potential of chemicals. Instead, a battery of tests will be needed. In this talk, progress made on the development of (a) bioavailability models, (b) dendritic cell activation models, (c) protein reactivity models, (d) other cellular models reflecting the "danger signal" induction and (e) T-cell activation are briefly reviewed. Detailed results are then presented on our current simplified test battery: By combining (i) data from peptide reactivity as a purely chemical measurement and (ii) cell based data on the induction of the Nrf2-KeapI-ARE signalling pathway, a good prediction can already be made, as we have shown with a set of >100 chemicals of known sensitization potential. The relevance of these tests in the simplified battery for the *in vivo* situation will be discussed.

In vitro phototoxicity studies with reconstructed human skin equivalents

H. Kandarova

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Phototoxicity is an acute reaction, which can be induced by a single treatment with a substance and subsequent exposure to ultraviolet or visible radiation. For hazard identification, the regulatory accepted *in vitro* test method is the 3T3-NRU PT (OECD Test Guideline 432). However, toxicologists need also *in vitro* assays that will give indication of risk, which is not feasible with 3T3-NRU PT.

The use of 3D reconstructed human skin equivalents (RHSE) has been proposed since the early 90's as a possible solution to determine phototoxicity of topically applied substances. Since the RHSE closely resemble structure of human epidermis, i.e. possess functional stratum, barrier properties and are metabolically active, they allow for testing of bioavailability of the test substances in the skin and thus enable realistic estimation of their toxic potential. Another advantage is that parameters of commercially produced RHSE as e.g. EpiDerm, EpiSkin, SkinEthic or EST-1000 can be precisely controlled and thus long-term reproducibility achieved.

ECVAM pre-validation study has been so far competed for EpiDerm phototoxicity assay, and although none of the existing methods is as yet formally validated, the phototoxicity tests with RHSE are used in the cosmetic and pharmaceutical industry to 1) evaluate the relevance of positive results obtained in the 3T3-NRU PT, and 2) to estimate non-phototoxic doses of phototoxic compounds in formulations (e.g. in topically applied pharmaceuticals). Several research groups investigated potential of RHSE to estimate phototoxic hazard and risk. The presentation will review existing findings and will discuss the usefulness of 3D RHSE in phototoxicity testing.

ID ABS: 173

Assuring safety without animal testing: skin allergy case study

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Assuring consumer safety without the generation of new animal data is currently a considerable challenge but one that we believe is ultimately achievable. Skin allergy (sensitisation) is an important consumer safety endpoint for home and personal care products and an endpoint where animal data (e.g. mouse local lymph node assay data) are often needed to inform risk assessments. We are currently evaluating a conceptual framework for skin sensitisation risk assessment that does not require the generation of new animal data.

At present we are generating data using four non-animal "lines of evidence" to establish their ability to characterise the sensitisation potential of novel ingredients when applied alone and/or in combination. The risk of an allergic response occurring following skin exposure to an ingredient is known to be dependent on many factors in addition to the inherent sensitisation potential of that ingredient (e.g. how the product is used, where it is applied, and individual susceptibility to developing allergic skin disease). In order to address this, we have developed a prototype model for probabilistic risk assessment of skin sensitisation in order to better understand and predict the factors that drive the prevalence of skin allergy in a consumer population. Our ultimate aim is to ensure that the inherent uncertainty around these factors (e.g. exposure, diversity within populations) is more explicit within our new risk assessment.

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

Session BS22: Skin and eye toxicity II

ECVAM Bottom-Up/Top-Down testing approach: testing strategy to reduce/replace the Draize test and validation/regulatory acceptance of *in vitro* assays: current status

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To reduce and/or replace the Draize test, testing schemes combining strengths of particular *in vitro* assays were proposed during a 2005 ECVAM expert meeting. The testing scheme proposes, based on expected irritancy of the test substance, a Bottom-Up approach, beginning with test methods that accurately identify non-irritants, or a Top-Down approach, beginning with test methods that accurately identify severe irritants before progression of further *in vitro* testing. Furthermore, as its core activity, ECVAM participated in the retrospective validation of and has peer reviewed scientific validity of four organotypic assays, and undertook retrospective validation of four cell function/cytotoxicity assays. The BCOP and ICE organotypic assays were ICCVAM and ESAC endorsed as scientifically valid for identifying severe irritants, and OECD Test Guidelines are under adoption. NRR, FL and CM cell function/cytotoxicity assays were recommended by an ECVAM Validation Management Group for identification of non-irritants or severe eye irritants in the Bottom-Up/Top-Down approaches. These assays are under ESAC peer review. Finally, a joint ECVAM-COLIPA prospective validation study was initiated in 2008 to evaluate

two Reconstructed human Tissue assays to discriminate non-classified materials from eye irritants, based on the proposed test strategies. The ultimate goal is to combine validated *in vitro* assays, based on their performances and applicability domains, to define the most suitable testing strategy to classify substances for eye irritation potential and ultimately replace the Draize test. This presentation will discuss the proposed testing scheme and provide details of the validation/regulatory status on *in vitro* assays for use in this scheme.

ECVAM validation drives expanded EpiOcular™ applicability domain for EU legislation relevant test articles: successful international pre-validation

P. Hayden, Y. Kaluzhny, H. Kandarova and M. Klausner

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Recently implemented EU legislation including the Cosmetics directive and the REACH directive has heightened the need for *in vitro* ocular test methods. In response, the European Centre for Validation of Alternative Methods (ECVAM) eye irritation task force in 2007 requested that submitters, model developers and companies involved in EpiOcular™ pre-validation studies work toward expanding the test chemical applicability domain (AD) of the model. The EpiOcular model producer (MatTek Corp.) therefore undertook development of an expanded EpiOcular AD protocol. This protocol uses a single exposure period: 30 minutes with a 2-hour post-exposure incubation (liquids) or 90 minutes with 18-hour post-exposure incubation (solids). A single cut-off in relative survival is used for classification: <60% = irritant (I) (R36 and R41); >60% = non-classified (NC). Tissue survival is determined by the MTT assay. 76 materials including alcohols, hydrocarbons, amines, esters, and ketones were evaluated. A prediction model discriminating ocular irritants and non-irritants was developed which resulted in 94.4% sensitivity and 67.5% specificity. This protocol was subsequently evaluated in 2007/2008 by the European Cosmetics Association (COLIPA) in a multilaboratory study. Twenty coded chemicals were tested in 7 laboratories (4 EU and 3 US). Overall, 298 independent trials were performed, demonstrating 99.7% agreement in prediction (NC/I) across the laboratories. Coefficients of variation for the % survival of tissues across laboratories was generally modest (<16%) except where tissue survival values were low. Using these data, a formal submission was sent to ECVAM (2008) in support of the EpiOcular protocol entry into a formal validation study.

Optimization and pre-validation of an *in vitro* test strategy for predicting ocular irritancy using the SkinEthic™ Human Corneal Epithelial (HCE) model protocol

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Prediction of eye irritation potential of test substances by means of *in vitro* methods is a crucial point for industries in the current context of Cosmetic Directive and REACH legislation. For this purpose, a test method, using the SkinEthic HCE Reconstructed Human Corneal Epithelium has been developed to predict chemicals classified as eye irritants (GHS Cat 1 and 2) versus non-classified. An optimized SkinEthic HCE protocol including two specific exposure time assays (10 min and 1 hr + 16 hrs post-exposure incubation) was established for a set of more than 100 chemicals. Relevance (predictivity) and reliability (reproducibility) of the HCE test method for ocular irritation of chemicals were evaluated using a 50% cell viability cut-off prediction model. Based on the established ECVAM pre-validation recommendations, both intra- and inter-laboratory reproducibility were assessed, followed by the evaluation of the test method's predictive ability to discriminate irritant (classified) versus non-classified chemicals. This work demonstrated for a set of 90 chemicals, that the test method performances can be appreciably increased by measuring chemical reactivity of test substances as a prerequisite (reactivity categorization) for orientating them towards the most adapted exposure treatment time of the test method. Using a wide chemical-physical diversity of test substances, the results have demonstrated the usefulness of the test strategy for eye irritancy hazard identification with this test method. The prediction model of the SkinEthic HCE test method has now been established and is currently undergoing formal ECVAM validation in a prospective validation study.

Prospective validation study of *in vitro* reconstructed human tissue assays for detection of acute eye irritation

S. Freeman

Farino Consulting, on Behalf of Validation Management Group, Surrey, UK

In vitro Reconstructed human Tissue (RhT) test methods using the EpiOcular™ and SkinEthic™ HCE human corneal models are currently undergoing formal validation with ECVAM through conduct of a prospective validation study. Pre-validation studies with both test methods have served to optimise protocols, refine prediction models and provide multi-laboratory assessment. Through this work these test methods have been shown to predict eye irritant versus non-classified substances with a high degree of accuracy, approximately 80%. The prospective validation study is being conducted to assess the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of these test methods with a challenging set of coded test substances for which high quality *in vivo* data are available. As such, the study is being conducted in accordance with international guidelines (OECD) for validation and acceptance of new or updated test methods for hazard assessment and also according to the ECVAM Modular Approach to Validation. The primary goal of the validation study is an evaluation of the ability of the RhT test methods to reliably discriminate substances not classified as irritant to the eye from all other hazard categories including moderate (GHS Cat 2, R36) and severe (GHS Cat 1, R41) irritants. The ultimate goal is to incorporate these test methods into a tiered testing strategy, to include additional assays, that will facilitate appropriate classification and labelling of novel substances according to the GHS system. This presentation will provide a status update on the RhT assays validation study.

Combining alternative test methods to predict eye irritation potential: a practical approach based on data mining techniques

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Predicting the full range of eye irritation potential of test substances using individual current *in vitro* test methods is still a challenge. Using a testing strategy such as the Bottom-up/Top-down approach may overcome this problem. For this approach it is important to identify *in vitro* assays that are highly sensitive or highly specific. Data used for analysis were from publicly available sources on selected *in vitro* eye irritation assays with corresponding Draize eye irritation data. Different data mining techniques (discriminant analysis, support vector machines, classification trees, k-nearest neighbors method) were applied to these data to increase sensitivity or specificity of the *in vitro* assays. Briefly, for each test substance an outcome variable (GHS-classification assigned based on Draize data) was defined that is predicted based on a set of predictors (*in vitro* test methods endpoints). Prediction models were evaluated in term of classification errors using a training and testing set or cross-validation. The relation of each individual *in vitro* method with the *in vivo* Draize eye test was also explored to establish the relation of *in vitro* endpoints with the different tissue scores from the *in vivo* test (i.e., corneal opacity, iritis, conjunctival redness, conjunctival chemosis). This analysis may support indication of applicability domain of individual *in vitro* assays and establish relevance of *in vitro* prediction models with respect to *in vivo* eye irritation classification. Use of data mining and analysis techniques in the context of a testing strategy will be illustrated with some real data practical examples.

Measuring depth of injury (DOI) in an isolated rabbit eye irritation test (IRE) using biomarkers of cell death and viability

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While DOI is a mechanistic correlate to the ocular irritation response, attempts to measure DOI in alternative tests have been limited to qualitative histopathologic assessment by veterinarian pathologists. The purpose of this study was to determine whether DOI could be measured objectively by fluorescent staining for biomarkers of cell death and viability using an *ex vivo* IRE test. A panel of seven materials characterized by *in vivo* DOI were selected that caused slight (5% SDS and Tween 20), mild (10% acetic acid and sodium hypoclorate), moderate (cyclohexanol and parafluoranaline) and severe (8% sodium hypdroxide) irritation. Materials were then tested using a modified IRE test with 3 hours recovery and then processed for cyrosectioning and staining using a TUNEL assay to detect cell death, phalloidin to detect intracellular f-actin and DAPI staining to detect nuclei. Control eyes showed intense phalloidin staining of the corneal epithelium and stromal keratocytes but no TUNEL labeling. In general, eyes treated with mild, moderate and severe irritants showed regions of TUNEL labeled epithelial and keratocyte nuclei with no phalloidin stain overlying phalloidin stained, undamaged cells. DOI measurements showed that slight irritants damaged <40% of the epithelium, mild irritants damaged >50% of the epithelium and superficial stroma, moderate irritants damaged the anterior 20-30% of the stroma and the severe irritant damaged >80% of the stroma. Regression analysis between *ex vivo* and *in vivo* DOI showed a significant ($p<0.001$) correlation ($r=0.929$). These data suggest that fluorescent staining of biomarkers can be used to objectively identify damaged/undamaged cells.

ID ABS: 142

Development of new alternative method for eye irritation test using gel embedded culture method

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To evaluate the eye irritation potential of chemicals, we developed a new alternative method, the SIRC-embedded gel assay. This method is a cytotoxicity assay with SIRC (rabbit corneal cell line) cells, based on a gel-embedded culture technique using the collagen gel. For the SIRC-embedded gel preparation, SIRC cells were cultured three-dimensionally in the collagen gel. Twenty-eight materials with two concentrations, 100% and 10% dissolved in physiological saline or mineral oil, were examined by the SIRC-embedded gel assay. The test materials were applied in the rings put on to the SIRC-embedded gel. After exposure for a certain time, the cell viability of SIRC cells was assessed by MTT assay. The test materials were categorized as an irritant or a non-irritant group according to the obtained cell viability. The comparison of the irritation classification in SIRC gel with the *in vivo* score was conducted. The corresponding, sensitivity and specificity rates of irritation classification between the SIRC-embedded gel assay and the *in vivo* score were more than 85%. This investigation demonstrates that the SIRC-embedded gel assay is a highly useful alternative method to evaluate the eye irritation potential of a wide range of chemical substances.

* presenting author

Session BS23: Systemic toxicity and target organs

Biokinetic considerations in the use of *in vitro* systems for estimating systemic toxicity

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The use of *in vitro* toxicity data for the risk assessment of a chemical highly depends on the relevance of the *in vitro* derived data and the possibility to use these data in an *in vitro-in vivo* extrapolation (IVIVE). A prerequisite for this extrapolation is the interpretation of the concentrations of the compound found to have effects in the *in vitro* system for its relevance in the *in vivo* context. This requires knowledge of the biokinetic behaviour of the chemical. Physiologically based biokinetic (PBBK) models are essential tools in this context. If one wants to avoid the use of *in vivo* data to build PBBK models, the parameterization of the models will need to be based on non-animal data. Physico-chemical properties of the compound, e.g. its lipophilicity, can be used to estimate a number of parameters, such as absorption and partitioning over tissues. Other parameters, e.g. metabolism or the ability to be transported over barriers, can be measured in *in vitro* systems.

On the basis of these data, biokinetic models can be built that can be used to perform a reversed dosimetry, i.e. a translation of the concentration in the *in vitro* system to a dose (or an exposure scenario) *in vivo*. Examples of the application of this approach will be presented.

How to evaluate the target organ toxicities of cyclosporines? Lessons learned from *in vitro* models applied in drug development

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Cyclosporine A (CsA) is an immunosuppressive drug widely used in organ transplantation as well as in the treatment of autoimmune-mediated diseases. In patients, CsA causes mild reversible side effects mainly in kidney and liver, which can be easily managed by dose adjustment. The CsA-induced side effects were qualitatively and quantitatively different in animals and man. Thus, the development of CsA follow-up candidate drugs and the extrapolation their potential side effects from animal data to man represented a serious challenge. In order to understand better the relevance of the animal findings for man, as well as getting an insight into the mechanisms underlying the observed target organ toxicities, tailor-made kidney, vascular system, liver and brain *in vitro* models were developed. These models were organ-specific and demonstrated structural, physiological and functional integrity similar to the *in vivo* situation. The presentation will summarize the lessons learned and explain how to apply these *in vitro* models to address specific cyclosporine-mediated safety issues during the drug development phase.

Evaluation of organ-specific toxicity with integrated discreet multiple organ co-culture (IdMOC) system

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IdMOC is a novel technology developed to model the complex, *in vivo* human or animal. Primary cells representing key organs: for example, hepatocytes (liver); renal proximal tubule cells (kidney); airway epithelial cells (lung); vascular endothelial cells (blood vessel); neuronal cells (nervous tissue) can be co-cultured in IdMOC as physically-separated (discrete) but interconnected (integrated) cultures. IdMOC employs a wells-in-a-well concept, with the individual cell types in separate inner wells which are interconnected by an overlying medium. Treatment of an IdMOC culture therefore allows the multiple cell types to be treated by a common medium, allowing metabolites to be formed and to interact with all co-cultured cell types. After treatment, each cell type can be analyzed individually (e.g. quantification of MTT metabolism or cellular ATP content) for viability. Using animal or human cells, IdMOC serves as an alternative to an animal or human for the evaluation of xenobiotic distributioin, metabolism and toxicity.

In vitro models to study pulmonary absorption and clearance of drugs and (nano)particles

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The human body has developed sophisticated systems to protect the fragile pulmonary epithelial structures against inhaled particles and to inhibit their translocation into the systemic circulation.

Even if the tightness of the pulmonary barrier and the two major clearance mechanisms in the lung – macrophage mediated clearance and mucociliary clearance – are indispensable to life, they represent a real obstacle for the therapeutic application of advanced aerosols.

Cell culture systems of pulmonary epithelial cells can be used for the prediction of drug or particle transport; however the experimental setup has to be chosen carefully. Submersed cell culture and the application of the drug in solution or suspension, is not realistic for pulmonary drug application, where dry aerosol particles are landing on the slightly wetted lung surface. Besides cell culture in air interface conditions and air interface deposition, the aerosol should be deposited on the epithelial surface with devices allowing the simulation of the human inhalation manoeuvr.

We developed two different aerosol deposition devices to simulate the bolus inhalation of a medical aerosol and the subsequent deposition of the aerosol particles on air interface cultivated pulmonary cell cultures. The devices are compatible with all commercial available DPI's, and deposit the inhalation relevant aerosol fractions by sedimentation forces or impaction on air interface cultivated Calu-3 cells.

With the aid of an embryonic chicken trachea model and mouse alveolar macrophages the interactions between the clearance mechanisms and nano- to micronsized particles were investigated. In contrast to mucociliary clearance, a significant effect of particle size on macrophage uptake and clearance was found.

Molecular mechanisms of toxicity in human renal epithelial cells

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"Omic" approaches such as DNA microarrays allow the possibility to more quickly unravel molecular mechanisms of compound induced cellular toxicity. In this study we examined the effects of sub-cytotoxic doses of the nephrotoxins cadmium chloride and diquat dibromide in a human proximal tubular cell line, HK-2.

Utilising genome-wide DNA microarrays (Affymetrix HGU 133 plus 2), we uncovered a surprisingly large overlap between transcriptomic changes caused by both compounds. A subset of these genes was likely to be under the transcriptional regulation of Nuclear erythroid 2 p45-related factor 2 (Nrf2). Nrf2 is

* presenting author

a redox-sensitive basic leucine zipper transcription factor that is involved in the regulation of many antioxidant genes such as heme oxygenase-1 (HO1) and NAD(P)H dehydrogenase, quinone 1 (NQO1). Silencing Nrf2 by siRNA decreased the expression of NQO1 and HO1 due to both cadmium chloride and diquat dibromide exposure.

This study demonstrates that the Nrf2 pathway plays an important role in proximal tubular cell survival following exposure to certain toxins. Since toxic compounds often elicit their deleterious effects through the build up of free radicals and oxidative stress, this pathway may be of major relevance to the prediction of toxicity *in vitro*.

ID ABS: 485

Characterization of toxicological effects on the energy metabolism after exposure to endocrine disrupting compounds

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Endocrine disrupting compounds are classically defined based on their effects on estrogenic, androgenic and thyroidal related endpoints. However, recently it has been suggested that other pathways of the endocrine system could be influenced by environmental pollutants, such as for instance the energy metabolism. Compounds that affect energy homeostasis cause fundamental problems (e.g. diabetes, obesity, etc.) and thus could have profound consequences at higher levels of biological organization. The aim of our research is to study the effect of a variety of compounds on the energy metabolism and to unravel the molecular mechanisms that underlie the disturbance of fundamental metabolic pathways.

Focusing on pancreas and liver, this study investigates, by means of a rat pancreas (INS-1 832/13) and a mouse liver (Hepa-1c1c7) cell line, if pollutants are capable of disrupting energy homeostasis and in particular insulin secretion and functioning. In practice, cells are exposed to a series of compounds, followed by (1) measurement of insulin secretion and (2) expression analysis of genes known to be transcriptionally regulated by insulin. Based on these endpoints, interesting chemicals are selected for which the molecular mechanism of disruption is unraveled by microarray analysis. Additionally, this research attempts to shed a new light on the concept of endocrine disrupting compounds by using *in vitro* models in a toxicogenomics approach.

Session BS24: Genotoxicity and carcinogenicity

Re-defining *in vitro* genotoxicity tests for better performance – a report from the International Workshop on Genotoxicity

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In vitro genotoxicity tests are widely accepted screens to judge whether a compound may act as a genotoxic carcinogen. Over the past 30 years, these tests have been engineered for high sensitivity to the extent that such assays yield many results at odds with data on genotoxicity *in vivo*, carcinogenicity in rodents or mechanistical considerations on human risk. In August 2009, the International Workshop on Genotoxicity will discuss the following topics in various expert groups to enhance the performance of in vitro genotoxicity tests:

1. Suitable Top Concentration for Tests With Mammalian Cells: Following the proposal for testing of pharmaceuticals for genotoxicity to reduce the top concentration from 10 to 1 mM,

clarification is needed whether the same recommendations can be applied to non-pharmaceutical compounds. In this context, the experts also look at redefining suitable measures of cytotoxicity.
2. Photogenotoxicity Testing Requirements: Recent publications suggest that *in vitro* photogenoxicity tests may be prone to pseudo-positive effects (e.g. "pseudophotoclastogenicity"). Hence, revisions to conducting such tests are needed and guidance recommendations have to be changed.
3. *In vitro* Test Approaches with Better Predictivity: Better test systems to improve the predictivity of *in vitro* tests are needed for risk assessment. This group will review current studies investigating whether certain cell types are more susceptible to giving irrelevant positive results *in vitro*.
4. Use of historical control data for the interpretation of positive results: The presentation at the World Congress will report on the recommendations given by the expert groups on these topics.

Genotoxicity testing *in vivo*: strategies to reduce the number of animals in mandatory regulatory studies

P. Kasper

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Animal studies are still an essential part in the assessment of genotoxicity of pharmaceuticals, both to complement *in vitro* tests in basic testing and to follow-up positive findings from basic *in vitro* studies. A draft revision of the ICH genotoxicity guideline (ICH S2R1) is proposing several changes that have the potential to significantly reduce the number of animals used for genotoxicity testing. A major impact on reduction in animal usage is expected from the new recommendation to integrate *in vivo* genotoxicity assessment, e.g. the micronucleus test (MNT), into existing repeat dose toxicity (RDT) studies. This approach would supersede the currently requested stand-alone animal genotoxicity studies. Another proposal is combination of the *in vivo* MNT and *in vivo* comet assay in one study to cover systemic as well as local genotoxic effects (site of contact and target organ for toxicity). This approach would ensure obtaining the maximum information from one animal study and should ideally also be integrated into RDT studies. However, there is not yet sufficient experience in particular with integration of the comet assay. A collaborative pharmaceutical industry research initiative is currently under way in order to investigate issues of sensitivity and specificity as well as feasibility in relation to integrating multiple genotoxicity endpoints into RDT studies.

The revised ICH guideline also reinforces already existing reduction options, as these have not been sufficiently utilized in the past, such as the use of one sex only and possibilities of omitting positive control groups.

Cell transformation assays: current status

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The potential for a compound to induce carcinogenicity in humans is a crucial consideration when establishing hazard and risk assessment of chemicals and pharmaceuticals. To date, the standard approach to assess carcinogenicity at a regulatory level is the 2-year bioassay in rodents. With the entry into force of the new European chemical legislation REACH and the 7th Amendment to the Cosmetics Directive, a need for alternatives to full animal methods has been acknowledged. Several *in vitro* alternatives have been developed for predicting carcinogenicity. In vitro genotoxicity tests measure only one mechanism involved in carcinogenicity, induction of genetic damage. In contrast, *in vitro* cell transformation assays (CTA) have been shown to involve a multistage process that closely models some stages of *in vivo* carcinogenesis and have the potential to detect both genotoxic and non-genotoxic carcinogens. Regulatory agencies have been reluctant to adopt this assay in their safety testing schemes, one of the reasons being the lack of formal validation.

With that as a basis, the European Centre for the Validation of Alternative Methods (ECVAM) coordinated a formal validation of the CTA, involving for the first time laboratories from Japan, the US, and Europe. The validation assessment addressed issues of protocol standardisation, within-laboratory reproducibility, test method transferability, between-laboratory reproducibility, and preliminary predictive capacity of three CTA variants: CTA with SHE cells at pH 6.7 and pH 7, and CTA using Balb/c 3T3 cell line. The results of this validation study and the conclusions by the Validation Management Team will be presented during the meeting.

The NEDO project on carcinogenicity

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The development of novel alternative *in vitro* test systems has been requested to screen currently used existing chemicals for carcinogenicity. A sensitive cell transformation assay (CTA) for tumor initiators as well as promoters has developed using v-Ha-ras-transfected BALB/c 3T3 cells, i.e. Bhas 42. This system is able to sensitively examine many more chemicals compared with an original BALB/c 3T3 CTA in a short period. Bhas 42 cells show initiated cell-like characteristics by an incorporation of the v-Ha-ras gene, consequently they sensitively respond to tumor promoters.

So far we have tested over 100 chemicals, including carcinogens (initiators and promoters) and non-carcinogens using the Bhas 42 CTA. The assay has shown good performance: concordance of 72%, sensitivity of 71%, specificity of 75%, positive predictivity of 84% and negative predictivity of 60%. Particularly, it has detected many Ames negative carcinogens in the promoter assay. An important aim of the NEDO project is that the Bhas 42 CTA is adopted as an international standard. For this purpose, the validation studies were started in 2007 and the results of the prevalidation study have proven the reproducibility, applicability and relevance of the Bhas 42 CTA. The international validation studies are ongoing. Meanwhile, a method using 96 well plates has been developed to be utilized in a HTP system. From the mechanistic viewpoint, we are trying to screen marker genes involved in cell transformation induced with carcinogens to develop a further rapid method using a luciferase reporter-gene assay system.

ID ABS: 444

The use of *ex vivo* human skin for safety testing

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EU legislation, including REACH and the 7th Amendment to the Cosmetics Directive, marks the need for replacement of animal tests regarding safety evaluation of compounds. For those compounds that reach the viable layers of the skin following dermal application or systemic exposure, reliable *in vitro* models are urgently needed. Fresh *ex vivo* human skin was used to evaluate the genotoxic potential of compounds, because this tissue is considered closest to the human situation with respect to e.g. dermal absorption and metabolism.

Human skin tissue is obtained from cosmetic surgery, and skin membranes are cultured in an air-liquid interface on Netwell™ inserts. In this system skin is viable up to 5 days, based on lactate production, histomorphology and proliferating keratinocytes (Ki-67 and BrdU expression). Compounds can be applied via the topical route or basolateral side, simulating "systemic" exposure.

The model is successfully used for the assessment of potentially (photo)genotoxic effects. DNA damage was detected by using the Comet assay and micronucleus test. Several compounds with a known (photo)genotoxic potential demonstrated a dose-related and reproducible response. Currently additional test compounds, including compounds that need metabolic activation or activation by UV light, are being investigated in order to extend the suitability of the model. Furthermore, this method may reduce the number of false positives obtained from standard *in vitro* genotoxicity assays. Results so far indicate that the *ex vivo* human skin model can be a relevant alternative method for the safety evaluation of chemicals, such as genotoxicity, and therewith improve translation to humans.

ID ABS: 27

In vitro test, image analysis and statistical classification: towards an integrated approach for human carcinogenicity testing

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The Cell Transformation Assay (CTA) is a promising *in vitro* method used as predictor of human carcinogenicity. Neoplastic phenotype is monitored in suitable cells by foci formation, showing typical transformed morphological features. Transformed cells having acquired all malignant characteristics form tumours in susceptible animals. The classification of transformed foci, performed

by a trained human expert, relies on light microscopy scoring. Three types of morphologically altered foci have been described: Type I, characterized by partially transformed cells, Type II and Type III, considered to have undergone neoplastic transformation. The method of visual classification, although widely accepted, is time-consuming and, in some cases, prone to subjectivity, leading to possible over/under-estimation of carcinogenic potential of chemical or physical agents. Due to these disadvantages, the CTA is not accepted yet by regulatory authorities.

Herewith we refer to a recently developed method to objectively measure features of foci that have been associated with cell transformation. The method is based on: 1) image analysis of foci microscopy images, 2) extraction of morphological descriptors and 3) statistical classification of said descriptors.

Image analysis is performed in a quantitative way and aims at the extraction of foci features, such as texture and structure that characterize the transformed phenotype. The statistical analysis of morphological descriptors provides the information needed to perform the automatic classification of transformed foci. The integrated *in vitro-in silico* approach provides an improvement of the CTA towards full automation.

Fondo di Ateneo per la Ricerca (FAR UniMIB 2006) is acknowledged for partial funding to C.U.

Session BS25: Reproduction, development and fertility

Establishing an extended 1-generation study at the OECD level – successes and challenges

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A protocol to evaluate the potential developmental and reproductive effects of test chemicals has been developed by the Life Stages Task Force of the ILSI/HESI Agricultural Chemical Safety Assessment (ACSA) Technical Committee. Since the original publication, several international groups have provided public comment on conducting the test. The extended one-generation reproductive toxicity test is now under consideration as a potential test guideline. The protocol uses a flexible approach that is markedly different from the current multigenerational guidelines. It encourages the use of toxicokinetics when setting the doses, evaluates more than one rat per sex per litter in the F1 offspring and does not necessarily require mating of the F1 to produce an F2 (F1 mating may be triggered by the presence of effects in the P0 and developing F1 rats). A number of additional reproductive endpoints and neuro- and immunotoxicity cohorts are included. The ACSA protocol was developed emphasizing scientifically appropriate methods using toxicological endpoints and exposure durations that are relevant for risk assessment. Compared to existing testing strategies, the proposed approach uses substantially fewer animals, provides additional information on the neonate, juvenile and pubertal animal, and includes an estimation of human exposure potential for making decisions about the extent of testing required. In this paper, the evolution of the protocol since the 2006 publication is discussed. These changes reflect the collective input of a U.S. expert panel of government and industrial scientist convened in 2007 and discussions of an OECD expert group held in Paris, France (October, 2008).

The ReProTect framework program: new innovative approaches for evaluating fertilization, implantation and prenatal development

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Reproductive toxicity testing will have the highest impact on animal use and costs for regulatory safety testing under REACH. Integrated testing strategies using *in vitro* tests for prediction of reproductive toxicity are urgently required. A new integrated project called ReProTect (www.reprotect.eu) was started in 2004 funded by the European Commission within the 6th Framework Program for research, assembling 33 European partners from academia, SMEs, governmental institutions and industries. The aim of ReProTect is to develop or improve *in vitro* assays for their integration into a testing strategy, aiming to provide detailed information on the hazard of compounds to the mammalian reproductive cycle. In ReProTect the reproductive cycle was broken down into its biological components in order to define and further develop *in vitro* systems able to assess a chemical's hazard in the areas of: male/female fertility, implantation and prenatal development. Furthermore, innovative methods, including proteome analysis, QSARs, and microarray technologies, have been implemented for specific endpoints. More then 20 different tests are now available, reflecting various toxicological endpoints which measure effects on spermatogenesis, folliculogenesis, germ cell maturation and fertilization, steroidogenesis, the endocrine system, the pre-implantation embryo, placentation, uterus function, and embryonic development. More than 100 peer-reviewed reproductive toxicants have been tested. Scientific information on the presumed mechanism of the various test chemicals has been collected and SOPs for each of the tests have been produced. An independent statistical analysis for evaluating the intra/inter-laboratory variability of the tests, sponsored by ECVAM, has been completed for most of the tests.

Assessment of the embryonic stem cell test and application and use in the pharmaceutical industry

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Our group uses the spontaneous differentiation and growth of unrestrained mouse embryonic stem cells to predict the likely embryofetal toxicity of drug candidates. We used the ECVAM predictive assay as a starting point for our efforts at improvement. These efforts included the use of different analytic tools (Mahalanobis distance and random forest statistical models), the use of measures of gene expression, and responses to oxidative stress. This talk describes that work and leads up to the model we are currently using, which includes measures of gene expression, several IC_{50} values, as well as the slope of the dose-response curve at the IC_{50}. By adding many more compounds to those initially tested by ECVAM, we are more confident about the limits of the assay and its strong and weak points. The talk will describe these strengths and weaknesses, as well as how the test is used to help development teams make decisions.

Three-dimensional gonocyte-sertoli cell co-culture: characterizing systems based omic responses with *in vivo* testis gene expression profiles

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Gonocytes exist in the neonatal testis and represent a transient population of male germ-line stem cells. It has been shown that stem cell self-renewal and progeny production is probably controlled by the neighboring differentiated cells and extracellular matrix (ECM) *in vivo* known as niches. Recently, we developed an *in vitro* three dimensional gonocyte/Sertoli cells co-culture model (3D-SGC) with ECM and demonstrated that this culture system creates an *in vivo*-like niche and supports germ-line stem cell functioning within a 3D environment.

In this study, we applied microarray-based gene expression to examine the effects of 13 chemicals including known *in vivo* male developmental toxicants (DTs) and non-developmental toxicants (NDTs) in 3D-SGC. We observed that treatments with DTs induced significantly greater dose-dependent morphological changes, decreases in cell viability and increases in cytotoxicity than NDTs treatment. We found a significantly greater number of genes changed in the DTs versus NDTs treatment. Similar gene expression patterns (GEP) within the DTs or NDTs group were observed in a non-supervised cluster analysis. The GEP that resulted from our *in vitro* 3D-SGC were consistent with the results from an *in vivo* exposure study reported by Liu et al. (2005). Our systems-based GO-Quant analysis found significant alterations in cell cycle, phosphate transport, and apoptosis regulation with DTs but not with NDTs. In comparison with our cytotoxicity results, the global gene expressional analysis reveals a much more distinct pattern between DTs and NDTs response. In summary, our observations demonstrated that this germ-line stem cell-based model, which captures both the 3D organization and multicellular complexity of the target system, may be useful in identifying responses to developmental reproductive toxicants. (EPA R826886, NIEHS 1PO1ES09601, R01-ES1063, P30 ES07033 and SOT CP Grants, CAAT and HSUS/P&G Alternatives Award)

ID ABS: 357

Modification and evaluation of the chicken embryotoxicity screening test (CHEST) as *in vitro* test system for embryotoxicity

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Developmental and reproductive toxicity are of very high concern and currently have to be assessed in resource intensive animal studies. In this study the potential of the chicken embryotoxicity screening test (CHEST) to predict the embryotoxicity of exogenous chemicals was evaluated. The CHEST is performed using fertilized hen eggs during early developmental stages, which by legislation are not regarded to be sentient. Therefore, the CHEST can be considered as a replacement method. In contrast to other embryotoxicity assays (e.g. whole embryo culture), the CHEST is easier to conduct and does not require the use of embryonic stages from mammalian species. We have tested fifty substances, representing teratogens (e.g. retinoic acid, hydroxyurea, valproic acid, thalidomide), pro-teratogens (e.g. albendazol, cyclophosphamide, acetylaminofluoren) and non-teratogens (e.g. aniline, urea, isopropanol). The results show an excellent correlation with the teratogenic properties of these substances *in vivo*. Therefore, the CHEST assay could either be a possible replacement for *in vivo* testing or could be used as part of testing strategies as an early tier test. Within our department, the assay has proven to be a very valuable screening test in early compound development. In summary, this assay has the potential to significantly reduce the number of animals used for embryotoxicity testing.

ID ABS: 28

Evaluating teratological findings from alternative developmental toxicity assay used in conjuction with traditional *in vivo* rodent studies

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Evaluating teratological data from alternative developmental toxicity assays in conjunction with *in vivo* mammalian studies can be difficult. Establishing a weight of evidence for a prioritization process is critical to the successful use of these assays. To illustrate this process, we provide a case study with an Abbott compound that compares *in vivo* developmental toxicity results with results from the alternative developmental toxicity assay FETAX (Frog-Embryo-Teratogenesis-Assay–Xenopus). In the *in vivo* study pregnant rats were administered the test article via oral gavage from GD 6-17. Fetuses were subjected to external, visceral and skeletal examinations. Edema, diaphragmatic hernia and bent long bones of the fore/hind-limbs and bent scapula (all skeletal malformations), were seen among fetuses in the treated groups. In the FETAX model, the test material induced craniofacial edema and abnormal medial mytotome development resulting in distention of the gut. Results from limb development test indicated that the test material did induce lesions in the osteoprogenitor tissue of the elongating hind limb which would have ultimately developed into the femur. The effect was most marked in the full hind-limb development assay, indicating that the insult occurred during the final stages of limb-bud organization. Hind-limb-bud micromass cultures exposed to the test material demonstrated that the organization of the osteoprogenitor cells was disrupted. A high degree of similarities in the malformations induced in both systems suggest the utility of *Xenopus* to evaluate developmental toxicity of discovery level compounds and to establish a weight of evidence to utilize these assays in the prioritization process.

Session BS26: Disease models

In vitro models of skin cancer using human cells and tissue

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BCC is the commonest skin cancer in humans and accounts for between 15% and 20% of all carcinomas. Development of BCC is associated with constitutive activation of the Sonic Hedgehog (SHh) signalling pathway, through mutational inactivation of the Ptch1 tumour suppressor gene, primarily mediated by the Gli transcription factors Gli1 and Gli2. Currently there are no characterised BCC cell lines and so mouse models are widely used. In an attempt to develop *in vitro* models that can be used to understand the role of Gli transcription factors in BCC we have developed a range of cell culture models. These include retroviral transduction of Gli1 and Gli2 into human keratinocytes and through targeted knockdown of the PTCH tumour suppressor protein. In addition we have established cultures of stromal fibroblasts from BCC as well as primary cultures of BCC keratinocytes using stromal cells as feeders. We have now developed novel three-dimensional models of BCC. These models involve co-culture of BCC derived fibroblasts with Gli over expressing of PTCH knockdown keratinocytes. These cells are grown together in organotypic cultures that mimic human skin and allow us to investigate cell migration and invasion. Such models allow us to investigate in more detail oncogenic mechanisms of SHh signalling and we have now translated these models across to investigating these pathways in prostate and breast cancer.

ID ABS: 38

A cell-based chronic wound bioassay to replace unnecessary animal experimentation

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As the population ages chronic, impaired wound healing is affecting more and more people and represents a major financial drain on Health Care providers. In vivo chronic wound animal models have been reported however, they fail to accurately model human chronic wounds. This investigation aims to develop an *in vitro* chronic wound bioassay for chronic venous leg ulceration. Chronic venous leg ulcer (CWF) and patient-matched normal (NF) fibroblast strains were retrovirally infected with a human telomerase (hTERT)/puromycin construct. Population doubling levels indicated that, compared to primary (non-immortalised) CWF and NF, the hTERT infected fibroblasts had escaped replicative senescence and formed immortalised cell lines. Affymetrix^{TM} microarray analysis of the CWF-hTERT with the NF-hTERT identified 15 genes that were significantly up- or down-regulated in CWF (validated by QRT-PCR). Upstream promoter regions of 13 genes of interest and a housekeeping gene have been identified by database searches/sequence analysis, amplified and cloned into the promoterless reporter vector pZsGreen1-DR. Reporter constructs have been transiently transfected into human NF and CWF cell lines as proof of principle and levels of gene expression have been quantitated. These cells will form the basis of a low cost, highly reproducible fluorescent reporter cell-based bioassay which, it is envisaged, will be utilised in the discovery of novel therapeutics which have the potential to alter chronic wound healing and, in turn, replace unnecessary animal experimentation.

Acknowledgements: the Dr Hadwen Trust, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the Laboratory Animal Science Association (LASA).

Refining clinical care in animal disease models

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Much emphasis has been placed on the development and validation of replacement and reduction alternatives. Seemingly less emphasis has been placed on refinement alternatives, even though animal models are currently extensively used throughout biomedical research. More emphasis on the refinement of animal models is required until suitable replacement alternatives are accepted. Refinement of animal disease models presents a vast range of opportunities from selection of the most appropriate species or strain to the use of acclimation and enrichment techniques, supportive care, and humane endpoints. This presentation will summarize the various aspects of refinement applicable to animal disease models and explore the possibilities for enhanced clinical care of animal research subjects as disease models. Providing supportive care in the research setting has the potential to not only improve the quality of life for the individual animals but also the quality of scientific results from these studies and represents a more comparable disease model for the clinical situation. In this way, refinement of animal disease models can immediately and directly improve animal welfare and indirectly reduce the numbers of animals required while we wait for the ultimate goal of replacement alternatives.

Efforts to refine psychiatric disease models

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According to WHO estimations, psychiatric diseases represent approximately 20% of debilitating clinical conditions in humans and have a tremendous social and economical impact. Despite much progress having been made towards a full understanding of the etiology and biochemical basis of these diseases, many processes still remain unknown. Given the ethical and safety implications limiting human trials, models are useful tools to study patho-physiologic mechanisms, to identify molecular targets and to explore new potential therapies. Non-animal models available so far do not mimic the complexity of processes driving these diseases; only living organisms can enable the verification of any hypothesis under highly controlled conditions, by using genetically homogeneous subjects. However, the strength of these models is proportional to the genetic and

neuro-anatomic homology with the human being necessary to enable the exploration of the shared cerebral functions and efficacy of new therapies. Models are applicable, for example, to diseases like depression, anxiety. For other diseases, like *schizophrenia*, animal models are used in an integrated testing strategy, including also trials where human healthy volunteers are used as an "animal model" of cognition impairment in a sleep deprived condition. From a critical appraisal of some examples, it will be evident that only models based on complex organisms are nowadays suitable to study psychiatric diseases and that the implementation of new technologies contributes to make these models more predictive.

Session BS27: Environmental science

Recent advances in developing alternatives for acute and chronic fish toxicity tests using fish cell lines and embryos

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In the environmental risk assessment (ERA) of chemicals, PNECs (predicted no-effect concentrations) are derived from exposure concentrations that do not cause adverse effects on organisms. Assessment factors are applied to cover uncertainties (e.g. species differences). This procedure is a mandatory part of regulations for the registration of industrial chemicals, pesticides, biocides and pharmaceuticals and results in a large number of acute and chronic tests using fish, the major group of vertebrates required in ERA. The environmental relevance of acute toxicity tests has been questioned, but they are considered as a base set of toxicity data in various regulations. Alternative approaches using cell lines, fish embryos, QSARs or a step-down procedure have been proposed to replace acute toxicity tests with adult fish. Recent promising research that tries to overcome major limitations using fish cells and fish embryos is highlighted. The development of alternative test systems for chronic fish toxicity is an even more challenging task. Since any toxic effect will originate from an initial molecular interaction, the identification of these early interactions using systems biology approaches may provide the key for the development of alternative test systems. As an example, gene expression analysis in fish embryos is presented. This approach will allow unravelling the mode of action and may provide useful information beyond environmental risk assessment of chemicals, such as for the development of QSARs or for the prediction of human health effects.

Integrating non-vertebrate toxicological information as a substitute to vertebrate environmental testing

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A variety of approaches is currently being explored to reduce dependence on *in vivo* aquatic vertebrate tests. The greatest effort is placed on fish, as this group constitutes the greatest number of animals used for hazard identification, classification and labeling, and environmental risk assessment. This talk will review several approaches that have been successfully applied including use of inter-species correlation estimation (ICE) and leveraging algal and invertebrate data prior to Step-Down ap-

proaches. ICE is a collection of regressions summarizing sensitivity relationships between pairs of species tested against a wide range of chemicals and modes of action. The nature of algae and invertebrate to fish extrapolation, as well as fish to fish, algae to algae, and invertebrate to invertebrate relationships will be explored, as these trends are fundamental to the concept of cross-species toxicity predictions. Step-Down approaches have been advocated to reduce *in vivo* fish testing by prioritizing results from algae and invertebrate assays. This gives the opportunity to assess how often fish testing is truly needed. The vast majority of work in this realm has been conducted using acute toxicity, the most rudimentary of toxicological tools. Progress in other areas, such as chronic toxicity, endocrine disruption, and bioaccumulation, are hampered by more complex biological, ecotoxicological, and methodological issues in addition to expansion of taxonomic coverage to other vertebrates used in environmental testing (e.g., amphibians, birds). Goals for future progress associated with these endpoints will be presented and a path forward described.

Use of mode and mechanism of action information to support *in silico* prediction of eco-toxicity

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Models for eco-toxicity, to replace existing animal tests, are considerably stronger when based on modes and mechanisms of toxic action. This is particularly important with regard to *in silico* (quantitative) structure-activity relationships (Q)SARs). The prediction of the mode and mechanism of action, and the development of mechanistic QSARs, are intimately linked and generally better developed for acute as opposed to chronic eco-toxicity. Chemicals can be assigned to a particular mode or mechanism by a number of structural methods e.g. the Verhaar rules. From this information, mechanistic models can be developed and used successfully. Assignment of chemicals to mechanisms and modes of action also allows for the grouping of chemicals into categories and hence read-across of effects. Grouping according to mechanistic criteria allows for more structurally diverse, relevant and robust categories than from structural analogues alone. From these groupings, there is high confidence in the QSAR prediction of the toxicity of certain modes, e.g. narcosis. To support these efforts, further information, toxicological data and mechanistic understanding is required from experimental studies. The use of omic technologies to provide fingerprints of mechanisms and/or *in vitro* results to support mechanistic classification would both support model development and provide confidence to assignment of modes of action. Well designed experimentation would also assist in the definition of the structural domains of the mechanisms.

The universal biomarker concept: using non-mammalian alternatives to protect the environment and human health

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We now recognise an intimate relationship between the status of the environment and human health. Diagnostic biomarkers/ other alternative methods are widely available for both clinical and environmental assessment. Some of these tools have a shared technological base (i.e. essentially the same *in vitro* assay used in either a clinical or ecotoxicological setting); but, more importantly, some of the underlying biochemical responses to toxic substances are universal in a range of invertebrates, fish, livestock and humans. It is time to put aside the perceived barriers to integrating the health of humans and the protection of the environment by moving forward with: (i) an evidence based acceptance of *in vitro* assays as surrogates for both wildlife and human health, (ii) exploitation of the common scientific technology of non-invasive alternatives so that toxicological monitoring of exposure or effect can be truly integrated for both humans and ecosystems, and (iii) change how we manage human health and chemicals in the environment with a central shared laboratory with common tools and language that can be used by all the many regulatory agencies involved. The universal biomarker approach is much more than an alternative to mammalian testing and exploits the fundamental mechanisms of toxicity in cells and the ubiquitous nature of some enzymes, transporters, cellular peptides/chelators, and pollutant responsive genes. The concept is illustrated with reference to metal toxicity and novel chemicals such as nanoparticles. This approach challenges our current thinking, but can provide a calibrated and informed picture of biological effects in organisms ranging from microbes to man.

Development and regulatory acceptance of alternative OECD approaches and test guidelines for the protection of the environment and human health – an international perspective

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In recent years, alternative approaches/test methods have been developed by the OECD Chemical Programme, including the (Q)SAR Application Toolbox and alternative Test Guidelines (TG), for the assessment of chemicals for protection of the environment and human health.

The first version of the (Q)SAR Application Toolbox was released in 2008 for free download (1). The main feature of the Toolbox is to allow the user to group chemicals into toxicologically meaningful categories and to fill data gaps by read-across and trend analysis. Further developments of the Toolbox aim at extending its functionalities, ensuring that the categories approach for filling data gaps works uniformly for all discrete organic chemicals and for all regulatory endpoints.

The OECD Test Guidelines Programme (2) is responsible for developing alternative Test Guidelines; alternative TGs for skin and eye corrosion/irritation testing, acute oral and inhalation toxicity testing have been adopted, in addition to introduction of refinement and reduction in several updated TGs. A fish embryo toxicity test is undergoing validation as a potential replacement for the acute fish toxicity test (TG 203).

The Endocrine Disruption Testing and Assessment Task Force (EDTA) of the TGP deals with the development of Test Guidelines for the testing of chemicals with potential endocrine disruptive properties. Three Validation Management Groups for mammalian, eco-toxicity and non-animal testing, respectively, manage the validation of new test methods to populate the 5-level EDTA Conceptual Framework (3). Validations at the TGP follow the validation and regulatory acceptance criteria described in Guidance Document No. 34 (4).

URLs:
(1)[www.oecd.org/env/existingchemicals/qsar]
(2)[http://www.oecd.org/department/0,3355,en_2649_34377_1_1_1_1_1,00.html]
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ID ABS: 301

The ILSI-HESI project on animal alternative needs in environmental risk assessment

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The ILSI Health and Environmental Sciences Institute (HESI) established a project in 2009 on Animal Alternatives in Environmental Risk Assessment (AA-ERA) following a successful two-year emerging issues assessment of the topic. The early stages of this work included the execution of an international workshop on the fish embryo test as a potential alternative to the standard OECD 203 fish acute toxicity test. The workshop, held in 2008, involved 41 participants from academia, government, and industry representing 10 countries. A successful foundation for international and cross-sector collaboration emerged and led to the formal establishment of this HESI project. The project intends to provide a forum for discussion and action plans to resolve pressing needs regarding animal alternatives for environmental toxicology. The intial scope of work will be presented in this poster along with the workshop conclusions. Due to the success of previously established efforts to address acute fish toxicity alternatives, the AA-ERA project has expanded to identify future needs and posssible avenues to address extrapolation of *in vitro* to *in vivo*, chronic fish toxicity, and consideration of *in vivo* alternatives for detection of endocrine-disrupting compounds. Sub-teams have been established to define work portfolios and a kick-off workshop is being discussed to facilitate awareness, planning, and commitments from participants and organizations. Additional participants are sought and interested individuals are encouraged to contact the authors.

ID ABS: 337

Environmental predictors of US county mortality patterns on a national basis

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A growing body of evidence has found that mortality rates are positively correlated with social inequalities, air pollution, elevated ambient temperature, age and availability of medical care. This study develops a model to predict the mortality rates for different diseases by county across the US. The developed model is then applied to predict changes in mortality caused by changing environmental factors. A total of 1,510 counties from the continental US, excluding Alaska and Hawaii, were studied and divided into five geographical regions. A subset of 252 counties from the 1,510 counties was chosen by using systematic random sampling and these samples were used to validate our model. Principal component analysis and step-wise regression analysis were used to estimate the linkage between environmental pollutants, socio-economic factors, social capital, weather and median age to explain variations in county-specific mortality rates for heart disease, cancer, stroke, chronic obstructive pulmonary disease (COPD) and all causes combined for five regions of the country. Generally the estimated models fit adequately for all diseases for all regions. The model also adequately predicted risks for the 252 validation counties. This study suggests the need for a better understanding of the pathways through which these factors and mortality are related at the community level, thus providing policy-makers at local, state, and federal levels with more explicit and feasible targets for policy intervention at the community level. Predictions of changing mortality with changing climate are used to illustrate the predictive value of the model.

Session BS28: Animal welfare science

When do mammalian young become sentient?

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Published literature and studies from our laboratory show that neurological development in mammalian young at birth ranged from being exceptionally immature (e.g. newborn marsupials), through moderately immature (e.g. newborn cats, dogs, mice, rabbits, rats) to mature (newborn cattle, deer, goats, sheep, horses, pigs, guinea-pigs). In all cases, brain electrical activity indicates that, under normal circumstances, none of these young exhibit consciousness before birth. This is the case with exceptionally and moderately immature young, because their neurological development is not adequate to support consciousness until several months and several days, respectively, after birth. Neurologically mature newborns do have the capacity for consciousness before birth, but this is usually prevented by the operation of a number of neuroinhibitory mechanisms that are unique to fetal life. After birth these newborns exhibit consciousness within minutes or hours. The evidence for this and the implications for safeguarding the welfare of fetuses and newborns of these different mammals in experimental settings will be discussed.

International perspective on animal welfare

M. Rose

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The notion of animal welfare is captive to the complexity of the relationship between humans and other animals. Animal welfare can be seen as a judgement about both an animal's experiences as well as a benchmark that defines the actions and duties of human beings towards other animals; a judgement that is honed by cultural, social and personal values and beliefs. Thus legislation and government policies that refer to animal welfare will vary between countries, reflecting cultural needs, values and expectations.

Controversy over the use of animals in research is longstanding and marked by robust public debate. In these circumstances, many countries have enacted legislation seeking to promote the public good by recognizing and seeking to balance the competing claims of animal welfare and the potential benefits to society from such research activities.

Although in the legislative context, notions of animal welfare may differ, research is not constrained by national borders; it is a global activity. Consequently, in recent years there have been a number of initiatives whereby common ground has been sought so as to develop a harmonised, international framework to promote the welfare of animals used in research. In these endeavours, Replacement, Reduction and Refinement have been the unifying, guiding principles.

This paper will provide an overview of these international initiatives. Through several case studies, the seminal role of animal welfare science to inform the development and implementation of such activities will be argued and potential barriers to achieving an international approach will be discussed.

Animal husbandry, standardization and scientific validity

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Laboratory rodents housed in barren cages develop abnormal behaviours and other signs of poor welfare that can be attenuated by environmental enrichment. However, concerns have been raised that enrichment disrupts standardization, thereby reducing the precision and reproducibility of animal experiments. We tested these concerns and found that the welfare of laboratory mice can be improved by environmental enrichment without reducing the precision and reproducibility of experimental results. In fact, concerns that enrichment would disrupt standardization are based on a flawed concept of standardization. It is based on the true finding that experimental results vary depending on environmental conditions, and on the false belief that standardization will "spirit away" such variation. This has been referred to as the "standardization fallacy". Albeit counterintuitive, the standardization fallacy explains why the reproducibility of experimental results decreases with increasing environmental standardization. Indeed, we recently showed that environmental standardization increases the variation between replicate studies and the rate of false positive results, whereas systematic environmental variation (heterogenization) reduces both variation between replicate studies and the rate of false positive results. Importantly, this increase in the external validity and reproducibility of animal experiments can be achieved without the need for larger sample sizes. Therefore, animal experiments should be refined in two ways. First, barren cages should be replaced by enriched cages to improve animal welfare and, second, environmental standardization should be replaced by environmental heterogenization to improve scientific validity. This will advance both animal welfare and animal research in the best of meanings of the 3R concept.

Enrichment, chronic stress and its impact on research data

J. Garner

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Behaviour is what makes an animal an animal, and almost every aspect of animals has evolved as a consequence of behavior: the digestive system is dependent on feeding behavior; the reproductive system upon mating behavior; the skeleto-muscular system exists to allow locomotive behavior; even the immune system is intimately interwoven with behavior. The major role of behavior is to allow animal to control their environment, and to survive in environments that are physiologically inhospitable, or even lethal. Consequently a lack of control over even innocuous stimuli can induce widespread and devastating stress responses. Biologically relevant enrichments provide animals the mean to control stressors in the environment with suitable species-typical behaviors. Thus properly designed, and experimentally ratified enrichment, should render animals more normal and less variable. Conversely, barren environments, which lack any meaningful control for the animal are likely to induce widespread stress-mediated changes in animals, and such animals will be abnormal and of little relevance as research models. For example, experimental vaccines may be ineffective in barren housing, but extremely effective under enriched conditions, as a product of stress-induced immune suppression in barren housing. As such, the call for enriched housing to show that it is no different from barren housing is nonsensical – instead the burden of proof should be on barren non-enriched housing to show that animals housed therein are not abnormal. Using mice as an example, this talk will review these concepts, and illustrate the central role of the animal's natural history, sensory world, and behavior (the animal's point of view) in designing and validating enrichments. In particular, real world examples will be discussed, where failing to take the animal's point of view has lead to the adoption of "enrichments" or "refinements" that actually impair wellbeing; where taking the animal's point of view provides superior solutions to wellbeing issues; and where suitably enriched animals are demonstrably more "normal" and yield superior research data.

Less pain, same gain: combining science and welfare

D. Morton

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One of the objectives of the Three Rs was to combine good science with good welfare, and not to cause avoidable suffering. Or as Russell and Burch put it in the introduction to The Principles of Humane Experimental Technique: "…to analyse methods to diminish inhumanity in experimentation". This has been referred to as the Humanity Criterion: "If we are to use a criterion for choosing experiments, that of humanity is the best we could possible invent." Humanity can be promoted in various ways,

the most obvious being the total replacement of animals in research, education and testing. However, at present, this is not possible and so the other two Rs need to be better developed, particularly that of Refinement.

Refining experiments requires the basic step of being able to recognise when animals are suffering, for without that step nothing is actioned. Once potential suffering is suspected, or suffering such as pain and distress is observed, strategies can be developed to determine its cause, its avoidance, and its treatment. This will involve better training of all those who are involved in the care and use of animals. Improved experimental design strategies can be devised to make the research more humane. These may include: the use of early indicators of success and failure in meeting the scientific objective; pre-painful, predistressful, surrogate and humane endpoints; and ethical proportionality. Examples of these approaches will be given.

ID ABS: 538

Planning for refinement and reduction

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Experiments using laboratory animals do not normally occur as isolated "one-off" studies, and there is often considerable scope for decreasing overall severity and reducing the number of animals used by careful planning and design in the context of the complete programme. Experimental design texts usually provide no guidance on how to design an individual experiment to minimise severity, and are silent on how to organise a sequence of experiments. A well-planned programme should allow early recognition of important adverse effects and choose routes to achieving an experimental goal which involve least severity. Examples of how good planning and design have had a major impact on animal welfare as well as reducing animal usage will be presented. Realising that it would be useful to collate the various ideas to help researchers in this area the FRAME Reduction Steering Committee has developed a flow chart for planning a typical research programme with a sequence of experiments. The steps in this will be discussed and the potential impact on refinement and reduction of widespread implementation of its principles evaluated.

ID ABS: 239

Improving the quality of publications on animal experiments to make systematic reviews possible

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Systematic reviews (SR) and meta-analysis are regarded as the highest level of medical evidence by evidence-based medicine professionals. Although performing SRs is standard practice in clinical studies, this is not yet the case in animal experiments. Executing SRs in the animal experimentation field will prevent unnecessary duplication of animal experiments and thus unnecessary animal use and time loss. In addition, a SR on animal experiments will improve the interpretation of scientific results that have already been published, through which scientific quality will improve and patient safety will be optimized. SRs must therefore become the standard practice when doing animal experimentation.

Unfortunately, it is currently not possible to do a systematic evaluation of published literature, because of the variable study designs and different animal species being used. Moreover, there is often a significant lack in scientific quality and in the necessary details reported. Our presentation will discuss ways to improve the quality of scientific literature concerning animal experimentation and suggest ways to solve the problems in performing SRs. We have developed a complete publication checklist, encompassing all the necessary elements. Moreover, guidelines for performing systematic reviews and meta-analysis for animal experiments in specific research topics are being developed.

ID ABS: 163

Applicability of alternative approaches in food and feed risk assessment at the European food safety authority

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The founding Regulation of European Food Safety Authority (EFSA) requires the Authority to contribute to a high level of protection of human life and health and to take account of animal health and welfare. Most of the food and feed risk assessments conducted by EFSA require experimental data. EFSA's Scientific Committee has been reviewing the status of the science and possibilities for applying replacement, reduction and refinement of animal tests in the following areas of risk assessment: toxicokinetics, acute and local toxicity, genotoxicity, repeated dose toxicity, reproductive toxicity, carcinogenicity and ecotoxicity. Current EFSA approaches implementing the 3Rs will also be explained, such as tiered testing approaches, which have been developed for food contact materials, and the application of the threshold of toxicological concern for flavouring substances. Another approach is the implementation of the qualified presumption of safety, which will lead to a significant reduction in the need for animal testing in the assessment of microorganisms. Based on this review, the Scientific Committee has made recommendations for implementing better animal welfare within EFSA's activities.

Recognising that the Commission and Member States have responsibility for agreement of new testing methods, there is a need for good communication between EFSA and the corresponding Commission Services that lead on these areas. EFSA can be informed of the latest developments on the validation and acceptance of new testing methods and can help in their implementation. Communication on implementing the 3Rs with other EU agencies dealing with chemical risk assessment will also be important.

Session BS29: Immunology

Introduction to the immunology session

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Driven by a variety of new technologies, our knowledge and understanding of the immune response, both as protector of health and as cause of disease, has been expanded.

In this session, this progress will be discussed for a number of immune-mediated diseases and for protective immune responses. The discussions will address the potential of the current knowledge and understanding as basis for the development of alternatives to animal experiments.

Innate immune mechanisms in contact dermatitis and the resulting responses

S. Martin and P. Esser

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Allergic contact dermatitis is a chemical-induced inflammatory skin disease that is mediated by T cells reacting to chemical contact allergens. A key event in this disease, both in the sensitization and elicitation phase, is the induction of inflammation. The molecular mechanisms by which chemicals induce skin inflammation are poorly understood. It is becoming clear that contact allergens activate innate immune and stress responses, and analogies with infections are observed. Recent studies begin to elucidate the pathways of activation of the innate mechanisms involved in the allergic sensitization process and the complex interplay of innate immune and stress responses. The progress made in basic research in this field has great potential to exploit this knowledge for the development of better treatment strategies for allergic contact dermatitis as well as for the development of *in vitro* assays for contact allergen identification and potency assessment to replace animal testing.

Analyzing immune mechanisms underlying multiple sclerosis

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MS is the most common disease of central nervous system demyelination, and is associated with extreme erosion in quality of life and reduced life-expectancy; there is an urgent need for new treatments. The disease involves inflammatory damage to the myelin sheath, in time leading to death of those nerves and irreversible disability. The damage is believed to be caused by inflammatory attack by T lymphocytes. Since it is clear that healthy individuals also harbor autoimmune, anti-myelin T cells, much research has focused on elucidating those conditions that are associated with the breakage of functional 'self-tolerance' and resulting disease. It is inevitable that in a complex, multi-system disease such as MS, much research emphasis has been placed on animal models. Since May 1935 when Thomas Rivers first described a rhesus monkey model of experimental autoimmune encephalomyelitis (EAE) mimicking some aspects of MS, there have been more than 10,000 publications on the induction of EAE in diverse species including, rhesus monkeys, macaques, rabbits, guinea-pigs, rats and mice. While many of the exciting new therapeutics for MS currently in phase II and III trials come directly out of the EAE studies, there are also a number of caveats. For example, the mouse models may under-estimate the importance of some cell-types such as CD8 cells and NK cells. Furthermore, many co-stimulatory molecules are implicated by knockout mouse studies in skewing of the immune response and a resulting impact on disease susceptibility. However, the translation of these findings to the human clinical context has been difficult. We have developed a number of *in vitro* approaches to look both a the function of human patient cells, and at the modulation of immune molecules such as B7 family members using siRNA approaches

From the 3Rs of animal studies to the 3Rs of the MIMIC system: representative, reliable and reproducible

W. Warren

Vaxdesign Corporation, Orlando, USA

A challenge is the translation from test systems (animal or cell culture) to human immunology. The successful transfer between human biology and traditional test systems requires an intricate understanding of disease pathogenesis and immunological responses at all levels; innate, adaptive and functional. This talk will provide data on the development of the biomimetic *in vitro* MIMIC system to accurately assess drug, biologic, particle, and vaccine candidates/formulations.

The MIMIC system is modular; the first module simulates the innate responses via antigen presentation and inflammatory responses; the second simulates the adaptive responses of antigen specific T and B cells; and a third module is a functional assay that uses the products of the other two modules together with the pathogen to measure the effect of the immune response on the disease. These modules reproduce the conditions that exist in a human body, such as the spatial segregation of immune cells and the temporal dynamics that bring different immune cells together in a sequential order to accurately reflect interactions in a lymph node.

We will show *in vitro* models able to assess the immunogenicty of particle formulations; immunotoxicology of biologicals, *in vitro* inflammatory sensor to study ageing-related diseases, cross-reactivity and cross-protection relationships for influenza vaccination; and infectious disease models.

ID ABS: 387

Search for biomarkers to evaluate immunotoxicity of chemicals and to develop cell-based screening methods

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We are always exposed to enormous numbers of chemicals, which potentially impact our immune system. In such cases, dendritic cells (DC) sense these chemicals and trigger or modulate immune response. Epithelial cells in the route of entrance of chemicals affect DC function. Finally, T cell responses themselves may be modulated by chemicals. In this study, to discover the common response patterns in DC, epithelial cells, or T cells that are stimulated with different chemicals, we stimulated human monocyte-derived DC (MoDC), normal human epidermal keratinocytes (NHEK), and T cells with 5 different chemicals that affect our immune system (2,4-dinitrochlorobenzene (DNCB), $NiCl_2$, formalin, diesel exhaust particles (DEP), $HgCl_2$) and analyzed their effects on mRNA expression by these three cell types using DNA microarray and real-time PCR.

We selected immune-related genes among those either up-regulated or down-regulated by each chemical treatment from three independent experiments. Finally, we found that DC augmented IL-8 and PBEF1 by 5 chemicals, and C5AR1, CXCL3, INHBA, and ZEB1 by 4 chemicals. NHEK augmented IL8 and HMOX1 by 3 chemicals. T cells augmented CXCL3 and GADD45A by 3 chemicals, while they down-regulated IFNg and IL-4 by 5 chemicals, and IL12B by 4 chemicals. By real-time PCR, we confirmed the response of IL1A, IL1B, IL4, IL6, IL8, TNFa, PBEF1 and HMOX1 mRNA. These data suggest that several chemicals share some common mechanisms to affect our immune system irrespective of their chemical structure. In addition, these genes can be considered candidate biomarkers to predict the immunomodulatory activity of chemicals.

ID ABS: 521

Uromodulin exhibits pro-inflammatory properties in an *in vitro* model of whole blood activation and neutrophil migration

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The urinary protein, uromodulin, possesses diverse functions, such as protection of the kidney against stone formation and protection against ascending urinary tract infection. Recently it has also been shown that uromodulin has immunomodulatory properties. Uromodulin is synthesised in the cells of the thick ascending limb (TAL), apically sorted and secreted into the lumen, and is found in high amounts in the urine. However, uromodulin is also found in low quantities in the blood, which can increase to much higher levels upon renal tubular damage.

In order to better understand the interaction of uromodulin with the immune system, we investigated the potential of uromodulin to induce pro-inflammatory cytokine release from human whole blood cells. Additionally we investigated the effects of uromodulin on neutrophil adherence and migration across renal epithelial monolayers.

Uromodulin induced a dose dependent release of TNFα, IL-1β, IL-6 and IL-8 in human whole blood cells. Both neutrophil adherence and transepithelial migration was significantly higher in uromodulin expressing LLC-PK1 cells than in non-uromodulin expressing LLC-PK1 cells.

These experiments demonstrate that uromodulin can interact with and activate circulating immune cells and that uromodulin facilitates neutrophil migration.

Session BS30: Neuroscience

Overview of DNT: problems, approaches for minimizing animal use and maximizing data collection

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There is considerable concern that chemical exposures are contributing to the increasing incidence of neurodevelopmental diseases in children. However, most chemicals have not been evaluated for their potential to cause developmental neurotoxicity. Relying solely on the existing guidelines to address current and anticipated future regulatory demands for DNT of the thousands of chemicals for which there is little to no DNT data would incur unacceptable costs in terms of animals and person-years. Developmental neurotoxicity testing (DNT) is therefore perceived by many stakeholders to be an area in critical need of alternatives to current animal testing protocols and guidelines. The goal of this short introductory presentation is to provide the audience with an overview of the opportunities and challenges for developing alternative testing protocols and integrating the 3Rs into DNT.

Human neural progenitor cells for DNT testing

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Current developmental neurotoxicity (DNT) testing in rodents *in vivo* possesses two major obstacles: the requirement of large amounts of animals and the uncertainty of extrapolating the data to humans. To reduce animal consumption and tackle species specific differences, we have established a human *in vitro* model for DNT based on normal human neural progenitor cells (hNPCs) and their murine correlates (mNPCs, E16), which are both cultured as proliferating neurospheres.

On poly-D lysine/laminine coated surfaces, NPCs migrate radially out of the sphere and thereby differentiate into the major neural cell types of the brain. Thus, cell proliferation, migration and differentiation, which are key steps in brain development, can be assessed. Moreover, hNPCs react with Ca^{2+} signaling to diverse stimuli. Comparing these endpoints between the different species, we found that murine neurospheres proliferate faster, migrate initially at a higher speed but over a much shorter period of time and mature more rapidly during differentiation than human spheres. Furthermore, they have an overall shorter lifespan than human cultures. These data suggest that the *in vitro* cultures mirror the gross developmental differences of mice and humans. First testing of positive and negative test compounds which are known to interfere or are not associated with brain development indicate that e.g., IC_{50} values for inhibiting proliferation, migration and differentiation for methylmercury are in the nanomolar and for paracetamol, an agent known to exert liver toxicity, in the millimolar range.

More test compounds are investigated and will give us information on the validity of this promising system.

In vitro models to study cell-cell interactions that influence developmental neurotoxicity

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The nervous system is by no means homogenous, and a variety of cell types exist each with different roles. It is now well established that one cell type can exert significant influences on other cells. To better mimic the *in vivo* situation, where different cell types are in close contact and interact with each other, *in vitro* co-culturing systems may be used. Astrocytes foster the differentiation of hippocampal neurons, causing a significant increase in neuritogenesis. This effect appears to be mediated by permissive factors (e.g. fibronectin) released by astrocytes. By targeting astrocytes, a neurotoxic metal such as manganese can affect the differentiation of neurons. This effect involves generation of oxidative stress in astrocytes which in turn decreases the expression and release of fibronectin. The neuritogenic action of astrocytes is increased by neurotransmitters such as acetylcholine. By targeting muscarinic receptor signal transduction pathways, at the level of phospholipase D, ethanol inhibits the neuritogenic action of astrocytes. Astrocytes also stimulate synaptogenesis as evidenced by an increase in pre- and postsynaptic proteins and in synaptic puncta; this effect is also inhibited by ethanol. Astrocytes can also protect neurons against neurotoxicant-induced apoptotic cell death. For example, neurotoxicity of polybrominated diphenyl ethers toward cerebellar granule cells is significantly decreased by astrocytes, which provide neurons with glutathione (GSH) as cellular defense toward oxidative stress. A reduced level of GSH, caused by various genetic polymorphisms in GSH synthesizing enzymes, increases susceptibility of neurons and hampers the ability of astrocytes to provide protection toward neurotoxicity.

A genetic model system for studying effects of mutations and toxins on developing neurons *in vitro*

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Cultured neurons from developing brains of *Drosophila melanogaster* (fruit fly) manifest an endogenous morphogenesis program directing the construction of complex neurite arbors. This provides a normal, or wild-type, starting point from which to identify effects of single-gene mutations, drugs, and environmental neurotoxins. In addition, the system holds the promise of allowing systematic study of gene-X-environment interactions affecting neuronal viability and morphology. We first present the genetic demonstration that deficiency of the actin-bundling protein fascin disrupts neurite trajectory. We then show how this dramatic, quantifiable mutant phenotype allowed us to conduct a novel screen for drugs that could block tumor invasion and metastasis and for drugs that might improve neuron function in children with certain developmental brain disorders. Further, we identify distinct morphological neurotoxic phenotypes that are readily detected *in vitro*. In particular, we will present provocative data showing that a commonly prescribed class of drugs, associated with reversible cognitive dysfunction in some individuals, causes a specific, reversible neuronal morphogenesis defect *in vitro*. This drug effect can be induced in normal neurons, but is enhanced by a genetic mutation. In summary, we have used an invertebrate model to provide first-line screening for mutant phenotypes, beneficial drug effects, neurotoxic defects, and gene-X-environment interaction relevant to human health and disease. This strategy should make follow-up mammalian studies more focused and efficient, reducing the number of animals needed.

In vitro high throughput analyses of cellular processes critical for neurodevelopment

W. Mundy

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Current approaches to developmental neurotoxicity testing rely on the use of animals, cost millions of dollars and take years to complete for a single chemical. New screening assays are needed that can rapidly and efficiently identify chemicals of potential concern (hazard identification) and provide information on mechanisms and pathways. These approaches would focus further animal testing to the most relevant chemicals and endpoints, significantly refining and reducing animal testing. For developmental neurotoxicity testing, *in vitro* cell cultures may be useful as model systems for chemical screening. While *in vitro* systems cannot fully replicate the complex interactions in the developing brain, neuronal cultures can recapitulate many neurodevelopmental processes such as cell proliferation, differentiation, growth, and synaptogenesis. *In vitro*, cell-based assays for these cellular events have been proposed as one approach for high throughput screening of chemicals for developmental neurotoxicity. We have evaluated primary neurons, neuronal cell lines, and human neural progenitor cells as models for cell proliferation, neurite outgrowth, and synaptogenesis. Assays for these events were based on automated epifluorescent microscopy and image analysis (high content screening; HCS). Assays were performed in 96-well plates which allowed testing of multiple chemicals across a wide concentration range. The ability of these assays to detect changes was determined using a "training set" of chemicals with known effects *in vitro*. The results demonstrate that HCS assays can rapidly quantify chemical effects *in vitro*, and distinguish between effects on neurodevelopmental endpoints and nonspecific changes in cell health. (This abstract does not necessarily reflect USEPA policy.)

ID ABS: 105

Gene expression as a sensitive endpoint to evaluate developmental neurotoxicity induced by pesticides

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The major advantage of primary neuronal cultures for developmental neurotoxicity (DNT) testing is their ability to replicate the crucial stages of neurodevelopment. In our studies using primary cultures of cerebellar granule cells (CGCs) we have evaluated whether the gene expression relevant to the most critical developmental processes, such as neuronal differentiation (NF-68 and NF-200) and functional maturation (NMDA and GABAA receptor), proliferation and differentiation of astrocytes (GFAP and S100β) as well as presence of neural precursor cells (nestin and Sox10), could be used as an endpoint for *in vitro* DNT testing.

The expression of these genes was assessed after the exposure to various pesticides (paraquate, parathion, dichlorvos, pentachlorophenol and cycloheximide) that could induce developmental neurotoxicity through different mechanisms. All studied pesticides significantly modified the expression of selected genes related to different stages of cell development and maturation of neurons and/or glia. The most significant changes were observed after the exposure to paraquate and parathion (down regulation of mRNA expression of NF-68 and NF-200, NMDA and GABAA). Similarly dichlorvos affected mainly neurons (decreased mRNA expression of NF-68 and GABAA receptors) whereas cycloheximide had an effect on both neurons and astrocytes as significant decrease in the mRNA expression of both neurofilaments (NF-68 and NF-200) and astrocyte marker (S100β) was observed. Our results suggest that toxicity induced by pesticides that target multiple pathways of neurodevelopment can be identified by studying gene expression and could be used as a sensitive endpoint for initial screening to identify the compounds with a potential to cause developmental neurotoxicity.

Extra Breakout Sessions

Session EB8: Status report on ACuteTox

Overview of the ACuteTox project and multivariate analysis of the *in vitro* data

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ACuteTox is an integrated project, funded by the EU 6FP, with the aim to develop and pre-validate a simple and robust *in vitro* testing strategy for the prediction of human acute systemic toxicity of chemicals. Previous validation studies show that basal cytotoxicity data gives a good estimate of the acute systemic toxicity for about 70% of chemicals tested. ACuteTox aims to improve the prediction capacity to a level sufficient for regulatory purposes. The approach taken in ACuteTox was to identify outliers from 97 reference chemicals tested using 6 basal cytotoxicity assays and for which human and animal data are collected from literature. The 28 outliers were evaluated in order to introduce further parameters such as absorption, distribution, excretion, metabolism and specific organ toxicity (haemato-, neuro-, nephro- and hepatotoxicity). *In vitro-in vivo* modelling of human LC_{50} values and LD_{50} values for rat were performed using different combinations of 75 *in vitro* assays used to test the outliers and 29 non-outliers.

The results showed that small batteries of few *in vitro* tests give better predictive capabilities compared to subsets with more *in vitro* variables or individual variables, both for models based on LD_{50} rat (R2=0.59, Q2=0.57) and LC_{50} human (R2=0.71, Q2=0.69). The variables contributing to the best models were mainly basal cytotoxicity tests. Target organ specific tests only improved the correlation slightly. Based on these results, it was concluded that further statistical analysis of the data is needed in order to select methods for the testing strategy.

Statistical analysis of the ACuteTox data: challenges and statistical approaches

A. Kopp-Schneider

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The ACuteTox project aims at developing a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used nowadays for regulatory purposes.

In the ACuteTox project, 57 chemicals were tested in 71 *in vitro* assays. Dose-response experiments were performed and characteristic values such as the EC_{50} were derived. Not every assay-chemical combination was tested, and for many assay-

chemical combinations more than one laboratory performed more than one experiment. The first challenge in the statistical analysis is to derive a meaningful summary characteristic value if more than one experiment is performed, especially if not all the experiments show a clear dose-repsonse relationship. If none of the experiments show an effect the largest concentration tested is reported and indicated as a "censored" observation. An algorithm will be presented for summarization of characteristic dose-response values for various situations, depending on the type of characteristic value used in the experiment.

The second issue in the statistical analysis of the ACuteTox data is the identification of a subset of assays that is able to classify the given set of chemicals into the 5 GHS categories. Statistical classification methods are usually based on complete data sets. The classification task for ACuteTox involves a mixture of uncensored and censored observations as well as missing data and, therefore, the classical statistical methods for classification and variable selection cannot be used for application to the ACuteTox situation.

Appropriate modifications of these methods will be presented.

Session EB9: Status report on ToxCast

ToxCast: using high throughput screening to identify profiles of biological activity

R. Kavlock

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ToxCast, the United States Environmental Protection Agency's chemical prioritization research program, is developing methods for utilizing computational chemistry and bioactivity profiling to predict potential for toxicity and prioritize limited testing resources (www.epa.gov/ncct/toxcast). This presentation will provide an overview of the rationale, design and status of ToxCast, while the accompanying presentation by Judson will discuss the data analysis. In Phase I, our proof-of-concept component, we have focused upon evaluating chemicals with an existing, rich toxicological database in order to provide an interpretive context for the high through put screening data. This set of 320 reference chemicals, largely food use pesticides, represents numerous structural classes and phenotypic outcomes. Bioactivity data is derived from a broad spectrum of more than 500 readouts from biochemical assays, cell-based phenotypic assays, and model organisms. ToxCast is part of a larger government effort (Tox21) being conducted jointly by EPA, the National Toxicology Program of NIEHS, and the NCGC that is obtaining high throughput screening data on more than 2,000 chemicals, with plans to expand to nearly 10,000 chemicals in 2009. This is an abstract of a proposed presentation.

ToxMiner: relating ToxCast bioactivity profiles to phenotypic outcomes

R. Judson

US EPA, Research Triangle Park, USA

One aim of the US EPA ToxCast program is to develop predictive models that use *in vitro* assays to screen and prioritize environmental chemicals for further evaluation of potential toxicity. One aspect of this task is the compilation, quality control and analysis of large amounts of *in vitro* and *in vivo* data to develop predictive models or signatures. We have developed a computer system called ToxMiner which combines a database and statistical analysis tools to carry out these tasks. The ToxMiner database, which is one component of the larger EPA ACToR (Aggregated Computational Toxicology Resource, http://actor.

epa.gov) holds *in vitro* assay data generated by the ToxCast program, *in vivo* animal data, gathered through the EPA ToxRefDB effort (http://www.epa.gov/ncct/toxrefdb) and related biological information on genes and pathways. The ToxMiner statistical tools can find univariate associations between *in vitro* and *in vivo* data and can produce machine learning predictive signatures. We demonstrate the use of ToxMiner by showing examples of signatures for whole animal toxicity from cancer, developmental and reproductive endpoints.

Disclaimer: The United States Environmental Protection Agency through its Office of Research and Development funded and managed this research, and reviewed and approved this publication. Reference to specific commercial products or services does not constitute endorsement.

Session EB10: Status report on Sens-it-iv

The current knowledge about the biological processes that occur when tissue is exposed to sensitizing materials

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This presentation gives an update on how Sens-it-iv has contributed to the expansion of knowledge about human cells and cell lines, and their responses to selected compounds.

The precision-cut-lung-slices technology has identified DC of the alveolar compartment as major target cells of inhaled substances. Comparative *in vitro* co-cultures revealed essential requirements for direct EC-DC interaction. Catalogues of primary EC and DC, as well as cell lines, were established. The myeloid DC line MUTZ-3 exhibited the most *in vivo*-like properties. For skin EC, work is focusing on primary human keratinocytes and the NCTC2544 cell line, while a final candidate for lung EC remains to be defined. EC-DC cross talk is addressed in various culture systems. Since sensitizer-specific read-outs are observed in non-separated EC-DC co-cultures direct EC-DC contact might be essential. Genomic, proteomic and kinomic analysis, and CD-antibody array studies were used to establish "signatures" of activation markers. Langerhans cells (LC) or the corresponding MUTZ-3 derived MUTZ-LC cells migrate towards the recombinant chemokines CXCL12 or CCL5 if activated by sensitizers or irritants, respectively. Effective methods, indicative of sensitization for allergic contact dermatitis (ACD), were developed to assess proliferative *in vitro* stimulation of naïve human T cells with chemically modified DC. The involvement of innate immune responses in the induction of ACD opens ways to define additional targets for the development of *in vitro* skin sensitizations assays.

The acquired information was implemented and has resulted in 7 assays currently subjected to further development and refinement.

PCLS: from learning about *in vivo* to reduction and replacement

A. Braun

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Occupational asthma is one of the most common lung diseases in developed countries. Risk assessment for potentially sensitising chemicals is mostly performed in animal models. With an increasing public demand to limit the number of animals used in respiratory research and to reduce the distress to the animals, several models have been developed. Human PCLS are an *ex vivo* model, in which all relevant cell types are present in their natural position. We use PCLS to test for modifications of local immune responses, assessing a variety of immunological endpoints.

Human PCLS were prepared from lung lobes of cancer patients. Tissue was exposed to LPS, dexamethasone, respiratory and contact allergens. Viability of PCLS was determined with WST-1, LDH and LIVE/DEAD staining for confocal microscopy. Cytokine contents were detected with Luminex technology and ELISA.

Employing LPS and dexamethasone we were able to show that the inflammatory response in PCLS resembles the *in vivo* situation very closely. Repeated stimulation with LPS (intra- and inter-assay variances <20%) showed that human PCLS might also be suitable to characterize respiratory irritation and inflammation induced by chemicals. PCLS were exposed to 20 chemicals and IC_{50} were calculated. We investigated cytokine patterns (e.g. IL-1, TNF, IL-8) for the differentiation between respiratory and contact allergens. Indeed, IL-8 production is increased after stimulation with TMA whereas DNCB failed to induce the release of IL-8 to the same extent. This suggests that the combination of cytokine production with cytotoxic data may represent a promising *in vitro* model for the screening of potential allergens.

Potential epidermal cell and dendritic cell-based tests for assessing sensitization

S. Gibbs

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The aim of the European project Sens-it-iv is to develop and optimize a battery of novel *in vitro* assays, which will be ready for prevalidation. These assays should distinguish sensitizers from non-sensitizers; be at least as accurate as the current animal tests and mimic the human *in vivo* cellular processes which occur during sensitization. Four epidermal cell (EC) and dendritic cell (DC)-based assays are currently undergoing optimization and refinement:

i) Comparison of DC-like cell lines MUTZ-3, U937 and THP-1 using IL-8 and CD86 maturation biomarkers. The learning process of setting up an inter-laboratory ring study:
ii) EC based inter-laboratory study assessing the ability of NCTC 2544 keratinocyte cell line and biomarker intracellular IL-18 to distinguish sensitizers from non-sensitizers and respiratory sensitizers.
iii) A DC-based migration assay assessing the ability of MUTZ-LC to migrate towards CXCL12 upon exposure to sensitizers and CCL5 upon exposure to non-sensitizers.
iv) A 3D epidermal equivalent assay to rank sensitizer potency. This assay is based on determining the sensitizer concentration which results in 50% reduction in metabolic activity of the culture (MTT EC_{50} value) – the lower the EC_{50} value the greater the potency of the sensitizer.

These assays are currently being standardized, protocols are being harmonized and cells are being exposed to fully defined test panels of chemicals under guidance from ECVAM. The most promising assays will be available for further pre-validation studies.

T cell priming and amplification: exploiting key events in contact sensitization

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More than 4,000 chemicals cause allergic contact dermatitis, a T cell mediated inflammatory skin disease. One of the hallmarks of contact allergens is their reactivity with proteins, which causes the activation of skin inflammation and results in the generation of antigenic determinants for recognition by T cells. This immunogenicity of contact allergens is exploited within the EU funded project Sens-it-iv to develop an *in vitro* T cell priming assay as well as a T cell amplification assay. In the *in vitro* T cell priming assay naïve human T cells are activated with myeloid dendritic cells, either directly modified with putative contact allergens or fed with contact allergen modified human serum albumin. Contact allergens are identified by their ability to induce specific T cell proliferation or cytokine production. In the T cell amplification assay, rare naïve allergen-specific precursor T cells are expanded several hundred-fold into clonally activated T cell blasts by polyclonal stimulation. The second step of antigen-specific stimulation allows the identification of cultures containing specific T cells as well as the isolation of antigen-specific T cell clones. With this assay frequency and avidity of naïve T cells specific for different types of antigens including protein allergens are measured (e.g. Der p I from house dust mite). Chemically-modified self-proteins are now being tested.

These assays may be a valuable tool to avoid some of the shortcomings of the innate immune cell-based assays and could be useful as a second or third line test for substances that cannot be safely classified.

Session EB11: Introduction and status report on ESNATS – EU project

Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS); progress report after 18 months

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The aim of the ESNATS project is to develop a novel all-in-one toxicity test platform based on embryonic stem cells (ESCs), in particular human ESC (hESCs), to accelerate drug development, reduce related R&D costs and propose a powerful alternative to animal tests in the spirit of the Three R's principle.

ESNATS addresses current shortcomings in drug toxicity testing:

- Testing takes place late in the development cycle, implying use of high numbers of animals and generating significant costs;
- Animal-based test systems bear the risk of non-prediction due to inter-species variation;
- *In vitro* assays rely on primary cells or cell lines of malignant origin that are hard to standardise and limited in regard to quantity, homogeneity and genetic diversity;
- Existing assay systems based on primary animal cell lines do not reliably represent the physiological situation of cells in native tissue.

To overcome these shortcomings, ESNATS will develop a novel testing system taking advantage of the unique potential of ESCs, including:

- their capacity to self renew, constituting an unlimited source of standardised cells;
- their pluripotency, providing a source for cells of different phenotypes required for toxicity testing;
- the physiological relevance of ESC-derived somatic cells for toxicity endpoints, guaranteeing high test predictivity;
- at least for murine ESC (mESC), their easy genetic manipulation, allowing use of reporter gene expression as a powerful toxicity testing tool.

Highlights and progress of the consortium within the first 18 months will be presented in the meeting.

Embryonic stem cells as tools for neurotoxicology

K. H. Krause

Medical Faculty and University Hospitals, Geneva, Switzerland

Neurotoxicology testing is becoming increasingly important in the context of drug development, but also for safety assessment of environmental chemicals. The usefulness of rodent models is limited because of species differences, particularly pronounced in the central nervous system. In the context of the European ESNATS consortium, we therefore develop new neurotoxicology assays systems based on neuronal differentiation of mouse and human embryonic stem cells, ESC. ESC-derived neurons can be obtained either in classical two dimensional culture, or engineered in three dimension as a neuronal tissue. Both systems are complementary and useful for neurotoxicology testing. Read-outs for neuronal toxicity include electrophysiology, Ca2+ measurements, ROS measurements, but also changes in protein and mRNA expression. We are confident that the neurotoxicology platform developed within the context of the ESNATS consortium will contribute to improved neurotoxicology testing and will also allow a larger number of compounds to be tested.

Lunch Sessions

Session SL5: Animal use policies: the future of animal welfare legislation

The revision of EU directive 86/609/EEC on the protection of animals used for scientific purposes

S. Louhimies

EC, DG Environment, Brussels, Belgium

Around 12 million animals are used on a yearly basis in scientific procedures in the EU today. EU legislation, Directive 86/609/EEC, for the protection of animals used for scientific purposes, is being revised. The European Commission proposal under discussion by the European Parliament and the Council is based on four fundamental elements; steering away from animal experiments as the ultimate goal; accepting that doing away with animal testing is not yet feasible with current scientific knowledge; recognising animals as sentient beings, having intrinsic value in themselves, which must be respected, and, finally, confirming that the principle of the Three Rs is the key to more humane and better science and thus the way forward.

The measures include in the proposal build a legislative framework that provides for detailed scrutiny of work carried out on animals through systematic ethical evaluation as well as authorisation of persons, establishments and projects using animals. A number of measures will ensure comprehensive implementation of the principle of the Three Rs and the promotion of alternative methods. Finally, specific attention is paid to flexible implementation to ensure that existing, well functioning infrastructures can continue to work and EU research and industry remains competitive.

The need for a coherent and comprehensive strategy to phase out animal experiments

S. van Tichelen

Eurogroup for Animals, Brussels, Belgium

To avoid that animals suffer has been the overall aim for the animal welfare movement since it became existent. This includes in particular the area of animal experimentation. To achieve this aim, the 3Rs concept, and particularly the replacement of animal experiments with humane alternatives has become an accepted idea in that regard.

Notwithstanding the growing interest over the last twenty years and a variety of initiatives; from setting up of alternative centres to investment in validation, the overall numbers of animals used continue to rise.

Why we ask? There seems to be an increased demand for animal experiments as our society attempts for high levels of safety, longer life expectation through new medicines and an avalanche of new products resulting from clever technology such as nano-applications and genetic modification.

In the recent years this trend has virtually nullified other developments to phase out the use of animals.

A shift of paradigm is required. Our modern society must get away from using sentient beings as test machines and measuring instruments. In this context, the only way to achieve a signifi-

cant change is not only to regulate the use of animals in research more rigorously but also to invest in alternatives. An overall coherent strategy is needed which looks at all areas of animal use, how and when they should be replaced, including research and possible emerging risks which may result in increased demand for animal use.

As a first step countries should revise and make more rigid the legislation which controls and monitors animal use. Animal use which is not essential to achieve societal needs should no longer be allowed. In some countries/regions products such as cosmetics or tobacco may not be tested on animals. But many more types of products exist where animal use obviously is in contradiction to our society's ethical standards for animals.

In the European Union, the present amendment of Directive 86/609 is a good example of how citizens' concerns and expert advice have been considered in the drafting of a new legislative text. However, we have to fear that after going through the political process many provisions that are undoubtedly substantiated by agreed ethical principles, public opinion and scientific findings will be threatened because they would interfere with the interests of industry and scientists.

In addition to this, other type of EU-activities/legislation would directly counteract such measures to protect animals in research and testing. For example, a new legislation for the regulation of chemicals, pesticides and biocides has been set up at the same time as the proposal for an amended Directive was produced. That legislation will directly lead to more animal tests in the respective fields. Another example is the EU's research funding programmes which often directly generate new animal experiments even on species (e.g. non-human primates) and in fields (pure basic research) raising ethical concerns.

For these reasons regulatory requirements and research programmes – in particular those prescribed by international bodies such as OECD, EU, WHO or ISO – should be critically questioned on a regular basis. Finally, what has been formulated in the EU Treaty – to consider animal welfare in all of EU's legislation and policies – needs to put into practice.

Session SL6: Vaccines: acceptance of the 3R methods

No abstracts arrived

Poster Section

PO21/22: Skin and eye toxicity

ID ABS: 5

A modified short-time exposure (MSTE) test for cosmetics: an alternative to the Draize eye irritation test

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Shanghai is traditionally a strategic port, where cosmetics from the European Union, United States of America, Japan and other Asian countries are imported into China. Therefore, the Shanghai Entry-Exit Inspection and Quarantine Bureau of China periodically executes a sampling inspection of cosmetics for adequate quality control. In an attempt to address recent issues in animal welfare, we innovated an alternative for the Draize eye irritation test on rabbits. The short-time exposure (STE) test is known as a simple *in vitro* eye irritation test using a rabbit corneal cell-line (SIRC). We evaluated 19 chemicals and 23 cosmetics using the modified STE method (mSTE), and the results were compared with the Draize test. The sample-concentrations in the *in vitro* test were 0.05, 0.50 and 5.0%, while that in the *in vivo* test was 100%. The mSTE test of the 19 chemicals at concentrations of 0.05, 0.50 and 5.0% registered sensitivity rates of 37.5, 37.5 and 62.5% with specificity rates of 100, 81.8 and 72.7%, respectively. As for the 23 cosmetics at corresponding concentrations, the mSTE test indicated sensitivity rates of 61.5, 92.3 and 92.3% with specificity rates of 80.0, 80.0 and 50.0%, respectively. Although results of the 19 chemicals showed poor sensitivity with reliable specificity levels, those of the 23 cosmetics manifested reliable results in the sensitivity and specificity levels at the 0.50% concentration. These findings indicate that the mSTE test may be a reliable safety assessment assay for eye irritancy of cosmetics, albeit inadequate in assessing eye irritancy of chemicals.

ID ABS: 16

Total protein contents and release of LDH as an endpoint to evaluate potential irritation of surfactants

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There is increasing interest in developing simple in vitro methods to detect potential skin irritation caused by new surfactants. The need is especially acute in the cosmetic industry, following the European ban on the use of animals to assay new finished cosmetics or ingredients. Although a range of 3D methods has been validated for this propose, there is still a need to find other, cheaper alternatives.

The release of cytoplasmic enzymes is an indicator of cell membrane integrity and cell viability. The total protein content is an index of the relative number of cells. We measured these two parameters in keratinocytes NCTC 2544 exposed to both commercially available and new amino acid based surfactants.

We observed dose-response behaviour for all the surfactants tested. The increase in lactate dehydrogenase (LDH) release

correlated with a decrease in protein content. When these values are plotted, they show a crossing point at different concentrations, depending on the irritancy of the surfactant. Strongly irritant surfactants such as HTAB present this crossing point at lower concentrations than non-irritant surfactants such as Tegobetaine. This simple method is proposed as a screening strategy on the way to differentiate between irritant and non-irritant surfactants.

ID ABS: 35

Validation study for non-radioisotopic local lymph node assay based on BrdU incorporation (LLNA-BrdU)

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The local lymph node assay (LLNA) is a stand-alone test method to assess skin sensitization potential of chemicals based on the antigen-induced cell proliferative response in the draining lymph node of mice, and this method is currently widely used as an alternative test method for guinea pig prediction tests (GPMT/BT). However the method requires use of radioisotopes. The LLNA-BrdU method developed by CERI measures the uptake of 5-bromo-2'-deoxyuridine (BrdU) instead of 3H-thymidine and thus has the great advantage that it operates without radioisotopes. We have conducted the inter-laboratory catch-up validation study to evaluate the inter-laboratory reproducibility and its potential to substitute the standard LLNA method.

We conducted the inter-laboratory validation study with ten chemicals excluding the positive control substance (50% hexylcinnamicaldehyde). Among them, 3 common chemicals were examined in all 7 laboratories and 7 other chemicals were examined in 3 laboratories each. The chemicals were provided in an unprepared form, and working solutions were prepared in each laboratory according to instructions. Positive/negative decisions were made based on the criterion SI>2.

Variation of positive control responses were small and high intra-laboratory reproducibility was confirmed. Analyses for all test chemicals showed good dose-responsiveness and successful inter-laboratory reproducibility. Moreover the outputs of the LLNA-BrdU containing independent assay data with 31 chemicals in CERI were almost completely consistent with those reported in the standard LLNA. These results show the LLNA-BrdU method has high inter-laboratory reproducibility and reliability, and the method is a promising alternative to the standard LLNA method.

ID ABS: 84

Mechanism-based mathematical modelling for skin and respiratory allergens

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Skin sensitization and respiratory sensitization are complex multi-step biological processes with many similarities but some key differences. There is substantial evidence in both cases that the key step is covalent reaction of the sensitizer or its metabolite with protein nucleophiles. By focussing on this step, which is dependent on the nature of the sensitizing chemical, predictive models for both endpoints, based on chemical properties, can be derived. These involve 1) Identifying the nature of the reaction chemistry, i.e. assigning the chemical to be predicted to its reaction mechanistic applicability domain (This can often be done by expert knowledge; if not it can be determined by chemical experimentation) and 2) Obtaining reactivity data and hydrophobicity data for the chemical (Often this can be done *in silico*, but if not it can be done *in chemico*, e.g. by experimental measurement of rate constants with model nucleophiles). By comparison of the reactivity and hydrophobicity parameters with those of known sensitizers and non-sensitizers in the same domain, sensitization potency can be predicted using mechanism-based QSAR models or by read-across.

The approach offers the potential to replace the animal testing laboratory by the chemical laboratory, and can be applied to complement *in vitro* assays.

This project was sponsored by Defra through the Sustainable Arable Link Programme.

ID ABS: 94

Dermal xenobiotic metabolism: a comparison between human native skin and four *in vitro* test systems

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The skin is the first site of contact with maximum exposure of external stimuli and protects the body in several ways. Its barrier function determines the local and systemic bioavailability of dermally applied substances. In addition, the dermal xenobiotic metabolism contributes to potential toxicity of substances by converting penetrated compounds into harmless or toxic metabolites. Consequently, growing efforts are put into species and organ-specific safety assessment of dermally applied compounds: corrosion, irritation, genotoxicity, and sensitization. However, in contrast to the liver, little is known about xenobiotic metabolism of skin and appropriate *in vitro* systems.

To select an *in vitro* system which is most similar to the *in vivo* situation, human native skin was compared to several *in vitro* models of different physiological complexities: (1) the Phenion® Full Thickness Skin Model comprising dermis and epidermis, (2) an epidermal model, and (3 + 4) monolayer cultures of fibroblasts or keratinocytes. To exclude donor variability the four *in vitro* models were produced in house with cells of biopsies from the same donor. First, the basal gene expression of phase I and II enzymes of three different donors were investigated by quantitative RT-PCR. Second, protein expression (e.g. FMO3+5) and enzyme activity (EROD, BROD, PROD, GST, UGT) were compared between the *in vitro* systems.

The results demonstrate that *in vitro* skin models with rising physiological complexity mirror the native situation more realistically: of 14 analyzed genes only two display reduced expression in epidermal models but 12 and 9 in keratinocytes or fibroblast monolayer cultures respectively.

ID ABS: 104

Histopathology in the isolated chicken eye test; comparison of different stainings of the cornea

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The Isolated Chicken Eye Test (ICE), developed by TNO, is one of two validated alternative methods accepted by OECD for identification of severe irritants. The OECD ICE guideline encourages preservation of eyes for histopathology. Eyes are collected in formalin following routine assessment using slit-lamp microscopy of corneal swelling, opacity, and fluorescein retention by damaged epithelial cells over a 4 hr period (10 second exposure). For more than 5 years TNO has routinely conducted histopathology of the cornea in ICE assays. Histopathology of the cornea is believed to strengthen evidence of absence/presence of irritation and help clarify borderline effects (e.g. between R36/R41).The goal of this study was to compare 5 different stainings of the cornea, two general (H&E, PAS) and three connective tissue (Trichroom, AZAN, EVG) stainings, in preliminary experiments to select specific stainings that could be of use in assessing corneal depth-of-injury, which constitutes lesions of the epithelium (erosion, necrosis), stroma (disorder, pyknotic nuclei) and endothelium (necrosis). Additional evaluation of the collagen-rich basement, Bowman's and Descemet's membranes in the cornea may provide valuable information. Slides of control and treated corneas were evaluated by assessing general morphology quality, and basement membrane and Bowman's membrane visibility. Results showed that general morphology quality was good with H&E and PAS and moderate with Trichroom, AZAN, EVG. Bowman's membrane was clearly visible with all stainings, whereas the basement membrane was clearly visible with PAS only. This would indicate that PAS may be the optimal stain for evaluating histopathological effects of effects on the cornea in ICE assays.

ID ABS: 111

An *in vitro* strategy for phototoxicity testing based on complementary biological endpoints

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Some chemicals used in drugs, cosmetics or food sometimes produce adverse biological effects when exposed to sunlight. There is thus a need for methodologies providing relevant photo-biological data at the molecular and cellular levels for such compounds. For ethical and practical reasons, *in vitro* models have gained an increasing importance in safety screening. Here, we propose a strategy based on complementary tests for assessment of the phototoxic potential. First, a solar simulator that mimics environmental solar UV is used as light source. Then, complementary methods with increasing complexity are implemented in order to get a rational evaluation of the phototoxic risk: (I) supercoiled circular DNA for *in tubo* assessment of photoreactivity, (II) yeast *Saccharomyces cerevisiae* for evaluation of photocytotoxicity and photomutagenicity, (III) in addition to the validated 3T3 NRU test, use of normal cultured human skin cells where photogenotoxicity can be detected using the comet assay (IV) and finally reconstructed skin where cell death as well as DNA damage can be measured after either systemic or topical application of the studied chemical. Such a strategy allows the evaluation of compounds with very different physico-chemical properties, and it is thus particularly well adapted to rigorously ensure the safety of products exposed to sunlight.

ID ABS: 118

Human skin metabolism and its relevance to safety assessment of topically applied chemicals: learning from the COLIPA skin metabolism project

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The development of non-animal consumer safety assessment strategies for cosmetic ingredients will rely on demonstrating the human relevance of new *in vitro* models. This is especially important for genotoxicity, for which *in vivo* models have been banned by the EU Cosmetics Directive since March 2009, and traditional *in vitro* models produce many false positive outcomes. The skin is the primary target organ for cosmetic ingredients; therefore, we are characterising the metabolising capacity of human skin and 3D skin models in order to better understand the role of metabolism in bioactivation and detoxification of dermally applied chemicals.

We are using a proteomics approach to analyse in *ex vivo* human whole skin microsomal fractions. So far, we have detected more than 1500 proteins and have identified a variety of phase I and II enzymes. We now plan to compare relative levels of expression of these proteins in different human donors and in available *in vitro* skin models.

Functional assays indicate that basal phase I enzyme activities are not detectable in human skin microsomes or 3D epidermis models. In contrast, glutathione S-transferase, UDP glucuronosyltransferase and cyclooxygenase activities are present in human skin and in epidermis models. The work, utilising sensitive analytical techniques, continues to corroborate the general observation that the skin is more protective than activating when exposed to chemicals. The expression and function of metabolising enzymes in skin is also qualitatively and quantitatively different to the liver.

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

ID ABS: 133

Potential of predictive biomarkers for *in vitro* skin corrosion tests

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To perform successful *in vitro* skin corrosion tests validated human 3D test systems and adequate predictive biomarkers are necessary. In this study a human full-thickness skin equivalent developed by Fraunhofer-IGB and excised human skin were compared as *in vitro* models.

4 OECD substances according to guideline 431 were applied to both tissues to determine interleukin release patterns and protein synthesis profiles. 2-tert-Butylphenol and 1,2-Diaminopropane served as corrosive agents, 4-Amino-1,2,4-triazol as a skin irritant and Eugenol as a sensitizing agent. Medium was collected to perform IL-1α, IL-6 and IL-8 analysis by ELISA, supported by WST-1 viability-assay, histological and immunohistological investigations. To detect cytochrome P450 protein (CYP) and heat shock protein 60 (Hsp60) expression Western blots were performed.

Different levels of epidermal barrier damage could be shown for the 4 substances by histological staining. All substances caused viability reduction of 90% in the skin equivalent. All chemicals led to a significant upregulation of the measured interleukins. In the presence of the tested irritants, aberrant cytokine release profiles could be observed compared to the sensitizing agent. Induction of CYP isoform 3A4 was found for corrosive Butylphenol, Diaminopropane and Aminotriazol, not for sensitising Eugenol. An upregulation of stress marker Hsp60 could be observed for all test substances.

Therefore, CYP isozymes and cytokine release pattern could be relevant indicators to discriminate between corrosive, irritant and sensitizing potentials of chemicals.

ID ABS: 143

A combined test skin irritation evaluation: monolayer cell, 3-dimensional skin model tests and human patch test

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Many cosmetics, including foreign products, have to be tested before they are allowed onto the Chinese market. Hitherto, we have been conducting rabbit tests to evaluate skin irritation for safety vigilance. However, we have recently evaluated cosmetics for skin irritation using a monolayer cell (MC) test, where upon detection of irritation, test-samples were subsequently subjected to the rabbit test and human patch test (HP) for re-evaluation. Although the MC method drastically reduces animal use (35th Japan Toxicological Sciences Annual Meeting), it is not a reliable alternative to the dermal irritation test.

In this study, we examined a combined evaluation system comprising the MC test, human skin models (3D models) and the HP. Mouse (BALB/3T3) fetal fibroblasts incubated in 96-well plates were exposed to test-samples diluted with PBS(-) for 5 min to derive the LC_{50}. Test-samples with LC_{50} <10% were defined as suspicious irritants, which were then confirmed with 3D models. The preincubated 3D models were exposed to test samples for 15 min, and cell viability (CV) was assayed at 42 h post incubation; test-samples yielding CV ≥50% were defined as non-irritants, otherwise were irritants. As a final confirmation, the 24 h HP was required. Test-samples yielding LC_{50} ≥10% should not induce irritation in the HP. Test-samples with LC_{50} <10% and those yielding CV ≥50% would indicate no irritation in the HP. This combined evaluation system manifested a concordance rate of ca. 93% with the HP.

In evaluating skin irritation of cosmetics without animal use, the present battery evaluation may serve as a replacement assay in accordance with the 3Rs.

ID ABS: 149

Viability of the red blood cell assay: blood storage and reliability of measurements

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The red blood cell assay is widely used as a screening method to discern whether surfactants are potential eye irritants, while photohaemolysis is reported to discriminate potential photoirritant compounds. Both assays are performed with an erythrocyte suspension obtained from whole blood, and two major endpoints are determined: lysis (HC_{50}) and haemoglobin denaturation and/or oxidation. However, the reliability of the results depends on how long the erythrocyte suspension has been stored. We therefore studied the stability of various erythrocyte suspensions obtained from healthy volunteers (male and female) over a period of 7 days. Spontaneous haemolysis and haemoglobin denaturation induced by SDS (ratio A575/A540) was monitored for one week. Similarly, blood stability in the absence and in the presence of UV-light was also studied using chlorpromazine as a photo-haemolytic compound. There was a slight increase in spontaneous haemolysis (3.6%), but a decrease in the HC_{50} values for SDS and haemoglobin absorption at 575, which was stronger on day 7. In the case of photohaemolysis, we observed a tendency to increase the HC_{50} and the oxidation of haemoglobin until day 4. On day 7, methaemoglobin decreased, probably due to the auto-oxidation of haemoglobin to other oxidative forms. In conclusion, the erythrocyte suspension should be used within four days after extraction to obtain reliable results and not to overestimate or underestimate the potential toxicity of the compound tested.

ID ABS: 155

Use of HPLC/UPLC instead of photometry for evaluation of MTT in *in vitro* RhT assays for irritation: assessment of coloured materials

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In Reconstructed human Tissue (RhT) assays, a test material's irritation potential is typically determined by measuring cell viability in treated tissues using the colorimetric MTT reduction assay. A known limitation is possible interference with absorbance measurement of reduced MTT (formazan) for intrinsically coloured materials. Aims of this work were: 1) develop an analytical approach using HPLC/UPLC to detect formazan separately from intrinsically coloured test material; 2) demonstrate for non-coloured chemicals and dyes without colour interference that HPLC/UPLC results are comparable with standard photometry; 3) apply HPLC/UPLC analysis only for intrinsically coloured dyes with colour interference.

For this work, MatTek EpiOcularTM assay was used. Tissues were treated with test material (plus negative and positive controls), stained with MTT, and formazan was extracted in isopropanol. Extract solutions were used for photometry measurements at 570nm (standard methodology) and for HPLC/UPLC analysis. A selection of chemicals/dyes without intrinsic colour interference and 2 dyes interfering with MTT due to intrinsic colour were tested. Results demonstrate that when photometry can be used, good correlation exists between photometry and HPLC/UPLC. When photometry is not possible (colour interference) cell viability can still be measured by HPLC/UPLC only.

In conclusion, HPLC/UPLC use in an *in vitro* eye irritation RhT assay can extend its applicability to intrinsically coloured materials that may interfere with MTT assay using standard photometry. Also, based on these data, it is proposed that use of HPLC/UPLC should be applicable to any *in vitro* assay using viability measurement via the MTT assay, e.g. RhT assays for skin irritation.

ID ABS: 160

A new *in vitro* method for identifying skin sensitizers and predicting LLNA EC3 values

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The purpose of this study was to develop a novel *in vitro* human cell-based model to detect chemicals and finished products that are skin sensitizing agents. The present study describes the use of human (HaCaT) cells in culture and a 3D human skin model to identify skin sensitizers and predict LLNA EC3 values by utilizing concentration response, exposure time, and expression of genes controlled by the antioxidant response element (ARE). The model was developed using a set of 14 chemicals previously classified as non-, very weak-, weak-, moderate-, strong-, and extreme-sensitizers. Test chemicals were applied at concentrations that ranged from 0.01 μM to 1 mM. Viability was monitored with MTT or histology. Total RNA was extracted and changes in the expression of quinone reductase (NQO1), interleukin 8 (IL-8), and aldoketo reductase (AKR) were measured by RT-PCR. The strong sensitizer 1-chloro-2,4 dinitrobenzene produced significant increases in IL-8 and AKR at an exposure of 10 μM. In comparison, the non-sensitizer benzoic acid had no effect at an exposure concentration of 1 mM. An algorithm based on an analysis of the magnitude of gene expression, concentration response, the number of genes responding, and time provides a single value of response, the *in vitro* toxicity index (IVTI). Comparison of the IVTI to known LLNA EC3 values by exponential regression analysis resulted in an R value of 0.92 in both cell models. This *in vitro* system provides a useful tool for identifying sensitizers and predicting LLNA EC3 values without the use of animals.

ID ABS: 162

Assuring safety without animal testing: assessing hazard for skin allergy risk assessment

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Assuring consumer safety without the generation of new animal data is currently a considerable challenge but one that we believe is ultimately achievable. Skin allergy (sensitisation) is an important consumer safety endpoint for home and personal care products and an endpoint where animal data (e.g. mouse local lymph node assay data) are often needed to inform risk assessments. The biological complexity of this endpoint is such that data from multiple tests, assessing distinct pathways in the underlying biology, will likely be required in order to provide robust data for risk assessment.

Previously, in collaboration with Entelos, Unilever developed an *in silico* model of skin sensitisation based on a biological understanding of the events and mechanisms involved. The purpose of building this model was to determine the importance of the underlying biological pathways to the sensitisation response and thereby focus *in vitro* assay development. Analysis confirmed that exposure, chemical reactivity, antigen presentation and inflammatory pathways are all important drivers of the sensitisation response (i.e. allergen specific T-cell proliferation).

These findings were used to recommend investigation of candidate assays assessing the underlying biological pathways of epidermal inflammation, Langerhans cell activation, epidermal exposure and chemical reactivity. Up to 40 chemicals have been investigated in these assays and various statistical techniques used to analyse the data. Here we present our evaluation of these assays and outline the beginnings of a probabilistic approach to integrate such data in a mechanistic and biologically relevant way that is suited to consumer safety risk assessment.

ID ABS: 186

Development of a new reconstituted human corneal model to assess the ocular irritating test

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Alternative methods to the Draize eye irritation test, such as the BCOP, HET-CAM, ICE, and IRE, are used to evaluate the ocular irritation potential of cosmetic, livelihood articles or industrial chemicals.

In order to improve the sensitivity and specificity of the alternative eye irritation test, we developed a novel three-dimensional human corneal model that uses normal human corneal epithelial cells. Our corneal tissue model consists of normal human corneal epithelial cells on inert cell culture inserts at the air liquid interface, differentiating to form a stratified, squamous epithelium similar to that found in the human cornea and exhibiting *in vivo* like morphological and histological characteristics.

In this study, two laboratories tested 20 reference chemicals using the same study protocol. A good intra/inter-laboratory reproducibility and correlation with *in vivo* and other *in vitro* corneal model results were obtained. The results were compared to previously published *in vivo* eye irritation as well as existing data obtained in the other three-dimensional corneal model test.

ID ABS: 188

Continued developments in the Colipa eye irritation task force strategy and programme for development of *in vitro* methods

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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* endpoints more predictive of the *in vivo* human response to chemical injury through understanding mechanisms of eye injury/recovery. This would result in new/improved *in vitro* methods that would proceed to formal validation. A key project on method development/optimisation has focused on Reconstructed human Tissue (RhT) assays using MatTek EpiOcular and SkinEthic HCE human corneal models. We have, working with the test method developers, completed pre-validation datasets enabling these methods to enter a prospective validation study in collaboration with ECVAM. The focus of our research programme is availability of models that address depth of injury/recovery as a mechanistic basis for eye irritation. Ongoing work is focused on continued development/optimisation of multilayer ocular models including isolated eyes, isolated corneas and bioengineered corneal constructs and incorporation of evaluation parameters that measure depth of injury as this relates to extent of recovery. We are also actively working in collaboration with external organisations such as ECVAM, academia and regulatory authorities to achieve validated alternatives. To ensure that our work remains state-of-the-art, a workshop involving invited international experts was conducted in September 2008 to benchmark the focus and content of the programme.

This poster provides a detailed overview of the current Colipa programme strategy and content including an update of the RhT assays validation status. Further detailed information on the RhT assays industry pre-validation programme can be located in individual posters for each test method.

ID ABS: 190

Fundamental experiments for risk assessment of nanoparticulate carrier systems using validated *in vitro* test procedures

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

Thus, to test the dermal safety of SLN and CMS nanotransporters the EPISKIN® skin irritation test was performed. The results predict no irritant potential according EU classification R38. Interestingly, the acute irritant potential of the positive control sodium dodecylsulphate was reduced when loaded onto CMS nanotransporters.

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

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

ID ABS: 207

Validation of LabCyte EPI-MODEL24, an *in vitro* assay for detecting skin irritants

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In April 2007, the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed (1) the use of the EPISKIN *in vitro* skin irritation assay as a stand-alone replacement for the *in vivo* Draize skin test for distinguishing between irritating and non-irritating substances, according to the UN Globally Harmonised System of Classification. Based on the ECVAM Scientific Advisory Committee (ESAC)-endorsed performance standards(2) for EPISKIN we evaluated the reliability (intra- and inter-lab reproducibility and transferability) and relevance of LabCyte EPI-MODEL24, an *in vitro* assay developed in Japan for detecting skin irritants. In Phase I of the validation study, we evaluated protocol transferability and assay reliability by testing ethanol, glycerol, and napthalene acetic acid, along with a positive control (5% SLS), in 7 labs. The protocol was easily transferable and all labs obtained the same call for the 3 chemicals, supporting further validation of this assay. In Phase II, the 9 irritants (the other skin irritant could not be purchased in Japan) and the 10 non-irritants in the EPISKIN performance standards2 were tested among the same 7 labs. The assay demonstrated acceptable reliability of positive control (100 %) and accuracy (74% overall accuracy, 67% overall sensitivity, 80% overall specificity) for use as a stand-alone assay for distinguishing between skin irritants and non-irritants.

Supported by the Japanese Society for Alternative to Animal Experiments.

(1)ECVAM statement on the validity of *in vitro* tests for skin irritation (2007) (http://ecvam.jrc.it/index.htm)
(2)ECVAM SIVS: Performance standards for applying human skin models to *in vitro* skin irritation testing (2007)

ID ABS: 216

Investigation of the test method for eye irritation potential using the reconstructed human corneal model

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An *in vitro* eye irritation testing alternative to animal testing, such as the rabbit Draize test, is required from an animal welfare standpoint. In order to develop an alternative *in vitro* method to the Draize test, we studied the tissue culture method of three-dimensional (3D) corneal models with normal human corneal epithelial cells and explored an alternative method to the eye irritation test using this model.

Human normal corneal epithelial cells were grown with 3T3-J2 feeder cells. These cells were seeded in culture inserts and cultured for 13 days. Similarly to human cornea epithelial tissue, the stratification of the cells and squamous epithelial layer at the surface was observed during the 3D-culturing process of cornea epithelial cells. Both Claudin-1, a component of tight junctions in the superficial layer, and E-cadherin, a component of the desmosome in the pterygoid layer, were strongly expressed in the stratified squamous epithelial layer. Additionally, it was confirmed that Mucin-1 was expressed in the stratified squamous epithelium layer. These results suggested that this model was correlated with the tissue structure of normal human corneal epithelium. Since this result was correlated with the degree of *in vivo* eye irritation, i.e. the Draize score, this 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 test.

ID ABS: 219

Assuring safety without animal testing: non-animal models for skin allergy risk assessment

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Assuring consumer safety without the generation of new animal data is currently a considerable challenge but one that we believe is ultimately achievable. Skin allergy (sensitisation) is an important consumer safety endpoint for home and personal care products and an endpoint where animal data (e.g. mouse local lymph node assay data) are often needed to inform risk assessments.

A number of key events in the biology of skin sensitisation are known to occur and their importance has been confirmed in our *in silico* modelling work; epidermal exposure to the ingredient, covalent modification of proteins and cytokine-mediated activation of Langerhans' cells to migrate to the lymph node and initiate a T-cell response. For some ingredients the additional step of metabolic activation may also be required.

Here we report our recent work in each of these areas. In brief, the *in vitro* approaches being investigated include: *ex vivo* human skin for assessment of ingredient dose delivered to the epidermis; the use of protein nucleophiles to assess the reactivity profile of an ingredient; *in vitro* models to study the activation of immune system cells (e.g. Langerhans' cells) and inflammatory response of skin cells (e.g. keratinocytes) following ingredient exposure; and approaches to assess the potential for skin metabolism of an ingredient.

These non-animal approaches are currently being investigated with a view to defining experimental systems that could be used to generate hazard characterisation data capable of informing future consumer safety risk assessment decisions in skin allergy.

ID ABS: 222

Skin and eye hazard tested *in vitro* in a group of chemicals with reference human skin irritation data

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Various *in vitro* protocols, involving tissue and organotypic models, have been assessed with the aim to evaluate skin and eye irritation hazard, as special emphasis is currently given to this subject in Europe under the REACH chemical strategy and the Cosmetics Directive. The key difficulty in determining the validity of alternative *in vitro* methods is that in vivo animal or human data is both scarce and often of limited utility for hazard prediction. Consequently, recently obtained human 4 h patch test data generated according to a standardized protocol were extended by a number of *in vitro* data related to skin and eye irritation potential. A group of selected chemicals employed in previous EU validation studies of *in vitro* methods for skin irritancy classification and a limited number of cosmetic formulations were subjected to further *in vitro* testing. Additional *in vitro* methods including Hen's Egg Test – Chorioallantoic Membrane (HET-CAM), Neutral Red Release Assay and Epi-Ocular tissue model (MatTek, USA) were performed. Human and animal skin/eye irritation hazard data were compared to the results of the *in vitro* methods. The *in vitro* models for testing of skin/eye hazard seem to be useful for the prediction of human hazard, particularly for consideration of initial concentration for confirmatory human patch tests or clinical trials to prove absence of local irritative effects and to confirm safety of consumer products, e.g. cosmetics. The advantages and limitations of individual *in vitro* methods are discussed with the view of their inclusion in tiered testing strategies.

ID ABS: 224

Alteration of skin permeation properties – mimicking different types of skin damage

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Penetration properties are altered by the environment and change during the penetration process with the physical environment while passing from the stratum corneum through the epidermis into the dermis. The barrier integrity of the exposed skin is influenced by experimental conditions like solvents but also by the receptor fluids used. Additionally, aspects of slightly damaged skin have to be taken into account, because the barrier integrity of skin exposed to occupational situations may not be ideal. Even a slight damage is assumed to increase the penetration of chemicals through the dermal route.

To evaluate the influence of the organic composition of the receptor fluid on the permeability of a substance we investigated the dermal penetration of Dye BR12. We used dermatomized skin from pig ears and applied BR12 using three receptor fluids differing in their ethanol content (0%, 20% and 50%). The total amount penetrating the skin with PBS buffer and PBS buffer containing 20% ethanol was comparable, but a higher percentage was found in the in the receptor fluid. In contrast, using 50% ethanol in the receptor fluid increased the amount penetrating by a factor 8-10, both in the receptor fluid and within the viable skin tissue.

In the second part we monitored the skin barrier function by measuring the transdermal conductivity after different ranges of skin damage. The damage was achieved either mechanically or chemically. The strongest effect is observed, as expected, by incubation with organic solvents, which remove lipids from the stratum corneum.

ID ABS: 225

Skin sensitization *in vitro* assays – a tiered strategy to replace animal testing

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The process of sensitization includes at least three steps: 1) penetration into the viable cell layers of skin tissue, 2) binding of the hapten to endogenous proteins, 3) activation of the skin Langerhans cells, inducing their maturation and migration to the lymph nodes.

To be able to react with proteins the compound has to have an electrophilic character or must have the ability to induce oxidation of the amino acid moieties within the proteins. In this study we present the results of our established peptide reactivity assay. With this cheap and fast method the electrophilic potential of different substances is determined by their reaction with a peptide containing cysteine and lysine residues. The free SH-group is monitored in a fluorescence assay in a time- and concentration-dependent manner. Afterwards the activation of dendritic cells is mimicked in a cell culture assay by the use of the two cell lines THP-1 and U396. The same set of substances is tested for the ability to increase surface expression of CD54 and CD86 after incubation with the different compounds.

Additionally we tried to include also the aspect of different penetration properties of the substances. For this purpose we use fresh dermatomized skin tissue from pig ears. The skin discs are treated with the same substances and the maturation and migration of the skin Langerhans cells is monitored by FACS analyses (CD1a, CD83, CD86).

All results are compared and discussed with results achieved with the LLNA in mice.

ID ABS: 249

Comparative evaluation of cosmetic formulations with different alternative methods for eye irritation

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Under the REACH regulation (440/2008 EC) all chemical substances produced in or imported into the EU in quantities greater than 1 tonnage per annum, will have to be assessed for their eye irritation potential. For chemicals in quantities between 1 and 10 tonnes per annum, only *in vitro* testing is permitted for the assessment of eye irritation. For cosmetic ingredients animal experiments will be forbidden for all acute toxicity endpoints, including eye irritation, from March 2009, as stipulated in the 7th Amending Directive 2003/15/EC to Cosmetic Directive 76/768/EEC.

A number of *in vitro* tests are accepted within the EU as screens for the prediction of severe eye irritation. At present, no alternative test has been fully validated and accepted as a stand-alone replacement for regulatory use. In this work, the eye irritating potential of 5 cosmetic formulations were determined by 5 different alternative methods for eye irritation (Hens-Egg-Chorio-Allantoic-Membrane (HET-CAM), Bovine-Corneal-Opacity-and-Permeability-Test (BCOP), Isolated-Rabbit-Eye (IRE), an *in vitro* method using a human cornea model (EpiOcular™ from MatTek), and the Red-Blood-Cell-Test (RBC)). The cosmetic formulations were chosen to cover the whole range of irritating effects: non, mild, moderate, and severe/very severe. The results of this study indicate that the eye irritation models, HET-CAM, BCOP, IRE, and EpiOcular™ are able to discriminate between the above mentioned irritating effects. This study shows that existing alternative methods are able to discriminate between non, mild, moderate, and severe/very severe irritants and may substitute the *in vivo* Draize Rabbit Eye Irritation test.

ID ABS: 255

An inter-laboratory study of short time exposure (STE) test for predicting eye irritation potential of cosmetic ingredients and formulations

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The Short Time Exposure (STE) test is an *in vitro* eye irritation test using SIRC (rabbit corneal cell line) cells. In this test, the cells are evaluated after a 5-minute exposure to the test material, and cell viability (MTT test) is used as the endpoint to evaluate the eye irritation potential. Two test concentrations (5 and 0.05%) are used to predict Draize test data obtained with 100 and 10% concentrations, respectively. The aim of this study was to confirm the predictive capacity of the STE test to assess the eye irritation potential, not only of ingredients, but also formulations. Therefore, an inter-laboratory study was conducted at three different laboratories on 51 cosmetic ingredients and 20 formulations using the same protocol and cell lots. The transferability and reproducibility of the STE test were also evaluated. The obtained results showed good correspondence with the Draize data and GHS classification (accuracy: approximately 90%), good transferability and reproducibility. These data

suggest that the STE test shows good predictive capacity, easy transferability, and good reproducibility, making this method a useful *in vitro* eye irritation test. In addition, the STE test is easy to perform and can evaluate the eye irritation potential both of ingredients and formulations.

ID ABS: 257

Evaluation of eye irritation potential of cosmetic raw materials using the *in vitro* short time exposure (STE) method

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Testing of any cosmetic in animal eye irritation tests has been banned in the EU. Although some alternative methods of eye irritation using various cell lines are being developed around the world, none of them are yet accepted as an OECD test guideline. The Short Time Exposure (STE) method, an alternative eye irritation method, involves exposing SIRC (rabbit corneal cell line) cells for 5 min to test material. A good correlation has been confirmed between the irritation rank classified by the prediction model based on the cell viability at two concentrations (5% and 0.05%) and the eye irritation score in the Draize test. In the present study, we evaluated the eye irritation potential of 70 cosmetic raw materials using the STE method. Then, we examined the association of STE irritation rankings with the Global Harmonization System (GHS) classification. A good correlation was confirmed between the STE irritation rank assayed by the 5% concentration and GHS ranking of non-irritant or irritant; accuracy was above 80%. Moreover, the STE ranks of 1, 2 and 3 classified by the prediction model highly correlated with the GHS ranks of non-irritant, category 1, and category 2, respectively (accuracy was above 80%). Accordingly, it was demonstrated that the STE test possessed a good predictive performance of eye irritation and it might be a promising alternative method for the eye irritation test.

ID ABS: 258

Prediction of ocular irritation potential of surfactants-based formulations at different concentrations using the EpiOcular model

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Evaluating ocular irritation is essential to the safety assessment of facial and eye-area cosmetics. The EpiOcular tissue construct model has been used extensively to predict the traditional eye irritation potential endpoints of those products used around the eye area. This study investigated whether the concentration tested in the EpiOcular tissue construct model affected its ability to distinguish the degree of sensory discomfort potential to the eye area. Two surfactants-based facial cleanser formulations (Fd and Fm) were tested using the EpiOcular assay, and the results were compared with data obtained from clinical and consumer studies. At a concentration of 3%, Fm was shown to have a significantly shorter ET_{50} (duration of exposure causing a 50% decrease in tissue viability) than Fd, whereas no difference between the ET_{50} was found when both were tested at 10%. The eye irritation potential was evaluated by direct ocular instillation of a 1% solution. Fm was shown to cause a greater frequency and magnitude of sensory eye irritation than Fd, though neither formula induced objective tissue damage. Ocular discomfort caused by Fm was also noted in a consumer use study where panelists used the two cleansers for 12 weeks. Markedly increased frequency and magnitude of sensory eye irritation from using Fm in consumer panelists were noted, while Fd did not cause any sensory eye irritation. These results indicate that use of relatively low test article concentrations for eye area products may allow the EpiOcular assay to better distinguish the degree of sensory discomfort of surfactants-based formulations.

ID ABS: 259

Comparison of the EpiOcular assay with human clinical use studies and post-market consumer data

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Several methods may be used in preclinical evaluation of eye irritation potential of cosmetic products. Ten eye area cosmetic products were chosen for review of their *in vitro* and *in vivo* eye irritation studies. In the preclinical assessment, formulations were applied neat to the EpiOcular tissue construct (MatTek) for up to 4 hours or 20 hours (depending on the expected degree of irritancy) and the exposure time required to reduce the tissue viability to 50% (ET_{50}) determined. The human clinical safety-in-use studies were performed under the supervision of an ophthalmologist to evaluate eye irritation potential under normal use. Post-market consumer feedback was compiled over eighteen months for each product. All comments received were unconfirmed adverse reactions and not all were specific to eye irritation. The products had ET_{50} values ranging from >240 minutes to >1440 minutes and were all considered "non-irritating." All ophthalmologic examinations remained within normal limits during each study. A correlation was found between ET_{50} responses and eye-area irritation in the human clinical testing, indicating that the data from an EpiOcular may assay lend support to the results of an *in vivo* assessment of the product. The post-market data further support this observation. All unconfirmed consumer reactions were minor in nature, such as mild itching, and were consistent with minor events observed in the human clinical studies. The reactions commensurate with normal individual and/or seasonal phenomena and do not indicate significant product-induced irritation. Thus, in the products reviewed, the studies described accurately predicted low eye irritation potential.

ID ABS: 260

Colipa's industry pre-validation program using reconstructed human tissue-based methods for predicting eye irritation for chemicals: MatTek epiocular

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Colipa, (European Cosmetics Association) is actively working to bring *in vitro* eye irritation tests to formal validation with ECVAM. This poster details the Colipa program on technology transfer and reproducibility of MatTek's EpiOcular assay as one of the two human reconstructed tissue assays. This EpiOcular protocol differs from previous time-to-toxicity protocols in that it uses a single exposure period for each chemical and a prediction model based on a cut-off in relative survival (<= 60% = irritant (I) (R36 and R41); >60% = non-classified (NC)). Test substance exposure time is 30 minutes with a 2 hour post-exposure incubation for liquids and 90 minutes with an 18-hour post-exposure incubation for solids. After the post-exposure, tissue viability is determined by tetrazolium dye reduction (MTT). Combinations of 20 coded chemicals were tested in 7 laboratories. A standardized protocol and laboratory documentation were used by all laboratories. MatTek provided initial training and then each laboratory operated independently during the technology transfer study. Twenty liquids (11 NC/9 I) by ECVAM plus 5 solids (3 NC/2 I) were selected so that both exposure regimens could be assessed. Concurrent positive (methyl acetate) and negative (water) controls were tested in each trial. Chemical decoding occurred only after study completion. In all, 298 independent trials were performed and demonstrated 99.7% agreement in prediction (NC/I) across the laboratories. Coefficients of variation for the % survival of tissues across laboratories was generally modest (<16%) except where tissue survival values were low.

ID ABS: 264

An *in vitro* tier evaluation for the identification of cosmetic ingredients which are not ocular irritants

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Monolayer cell culture of SIRC cells and three-dimensional dermal models were separately shown to have high inter-laboratory reproducibility as alternative methods for eye irritancy testing in the previous Japanese validation study. The aim of this study was to evaluate a tier system combining both models for the safety assessment of ingredients of cosmetics and medicated cosmetics.

The tier evaluation system using monolayer culture of SIRC cells and a three-dimensional dermal model was designed based on the Japanese validation studies reported by Ohno et al. Forty-eight (10 positive, 38 negative) of 59 ingredients for which corneal injury data at 10% concentration were available were used for evaluation of the tier system. Seventeen were accurately classified as negative by SIRC cytotoxicity test. There was one false-negative. When 19 substances that tested positive and 11 with poor solubility in the culture medium of the SIRC cytotoxicity test were tested in the three-dimensional dermal model, 14 substances were negative. Finally, 31 of 38 ingredients were classified accurately as non eye irritants with the tier system. There were 7 false-positives and one false-negative, which was phenethyl alcohol.

The number of *in vivo* results was 108 (data were available at several concentrations for some of the 59 ingredients). When the three-dimensional dermal model was used at similar concentrations to those used *in vivo*, the accuracy was relatively high and there was no false-negative.

The results indicate that the tier system may be suitable for the safety assessment of eye irritancy of ingredients of cosmetics and medicated cosmetics.

ID ABS: 287

Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system

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Skin protein reactivity is a well established key step in the development of skin sensitization. Understanding the relationship between a chemical's ability to react with or modify skin protein and skin sensitization has led to the development of the Direct Peptide Reactivity Assay (DPRA) in our laboratory. A current constraint of the DPRA is that it cannot readily measure the reactivity of pro-hapten sensitizers. Pro-haptens are not directly reactive and must be bioactivated *in vivo* to form an electrophilic intermediate(s). Results from this work demonstrate the utility of using horseradish peroxidase and hydrogen peroxide (HRP/P) for assessing the skin sensitization potential of pro-hapten chemical sensitizers. Significant increases in peptide depletion for all pro-haptens examined were observed following co-incubation with HRP/P. Conversely, the percent peptide depletion for all pre-haptens was equally high with and without HRP/P, demonstrating an auto-oxidation pathway. The optimal HRP/P concentrations, incubation time and optimal peptide:chemical ratio were determined using a liquid chromatography-mass spectrometry detection method. Dithiothreitol was incorporated to reduce thiol dimers in the cysteine peptide nucleophile. This work shows the potential to incorporate an enzyme into an *in chemico* skin sensitization assay for the detection of all types of sensitizers.

This work is funded in part by The European Cosmetics Association, Brussels, Belgium.

ID ABS: 288

Direct peptide reactivity assay (DPRA) for screening skin sensitization potential of chemicals

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One characteristic of a chemical allergen is its ability to react with proteins prior to the induction of skin sensitization. The majority of chemical allergens is electrophilic and reacts with nucleophilic amino acids like cysteine or lysine. To determine if reactivity correlates with sensitization potential, chemicals representing varying allergenic potencies were evaluated for their ability to react with a cysteine or lysine peptide. Following incubation of test chemical with each peptide, the reactions were analyzed to measure peptide depletion by HPLC. Previous results for 82 chemicals have demonstrated a good correlation between reactivity and allergenic potency. The test set of chemicals has been expanded to 135 (33 strong, 36 moderate, 29 weak and 37 non-sensitizers) spanning a broad range of chemistries and reaction mechanisms for modifying proteins. The reactivity data were analyzed against existing LLNA data using classification tree methodology to rank peptide reactivity as minimal, low, moderate and high. By classifying minimal reactivity as non-sensitizers and low, moderate and high reactivity as sensitizers, the accuracy between the DPRA and LLNA is 86%. Of the chemicals that are misclassified, some can be explained by looking at their structures while others were related to incompatibilities with the assay. Aside from its accuracy, the DPRA shows excellent reproducibility as demonstrated by tracking run to run variability of several chemicals.

This work is funded in part by The European Cosmetics Association, Brussels, Belgium

ID ABS: 290

Alternative measurements with crucial refinements in the murine local lymph node assay (LLNA)

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In the standard LLNA, OECD protocol 429, the read-out is based on lymph node cell proliferation after intravenous injection of ^{3}H-thymidine.

We have investigated two examples of alternatives to the use of intravenous ^{3}H-thymidine injection in the LLNA: 1) The measurement of the cell counts in the local lymph nodes of individual animals and 2) the use of labeling with ^{3}H-thymidine *in vitro* instead of the *in vivo* labeling.

The advantage/refinement is that the animals are not injected with ^{3}H-thymidine but proliferation is retained as endpoint. The present work is based on LLNA studies performed with the following test materials: Industrial enzymes and positive and negative control substances.

Conclusion: When applying the stimulation index cut-off of 1.4 for cellularity to estimate a positive response (as recommended in existing literature), this cell count based read-out is found to be comparable to the traditional radioactive read-out. Further, the *in vitro* labeling versus the standard *in vivo* labeling with ^{3}H-thymidine shows similar stimulation indexes of the tested materials. The investigated alternative measurements seem promising and provide major animal welfare benefits.

ID ABS: 296

ECVAM activities on eye irritation

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To reduce and/or replace the Draize rabbit eye test, testing strategies combining the strengths of particular *in vitro* assays were proposed during an ECVAM expert meeting in 2005. The testing scheme proposes a Bottom-Up approach, beginning with test methods that accurately identify non-irritants, or a Top-Down approach, beginning with test methods that accurately identify severe irritants, for the progression of further *in vitro* testing based on the expected irritancy of the tested substance. Furthermore as its core activity, ECVAM participated in the retrospective validation and has peer reviewed the scientific

validity of four organotypic assays and undertook the retrospective validation of four cytotoxicity- and cell based- assays. The BCOP and ICE organotypic assays were endorsed as scientifically valid by ESAC for identifying severe irritants, and OECD Test Guidelines are under adoption. The NRR, FL and CM cytotoxicity-/cell function-based assays were recommended by the ECVAM Validation Management Group for the identification of non-irritants or severe eye irritants in the Bottom-Up and Top-Down approaches. These assays are currently under ESAC peer review. Finally, a joint ECVAM-COLIPA prospective validation study was initiated in 2008 to evaluate the ability of two Reconstructed human Tissue (RhT) models to discriminate non-irritants from eye irritants, based on the test strategies proposed. The ultimate goal is to combine the validated *in vitro* assays for eye irritation, based on their performances and applicability domains, to define the most suitable testing strategy to classify substances according to their irritation potential and ultimately replace the Draize rabbit eye test.

ID ABS: 300

Prevalidation of a hemi-cornea model for corneal safety and permeability testing

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A hemi-cornea model exclusively based on human corneal cell lines has been developed to provide a tool for corneal safety- and transcorneal drug permeability testing in order to replace animal experimentation. Serum-free culture conditions have been optimized for the maintenance of functional and structural characteristics of the epithelial and the stromal part of the cornea model. Under appropriate serum-free conditions the epithelium of the hemi-cornea model displays an effective barrier function. The stromal keratocytes represent the undifferentiated phenotype, which can be induced to differentiate into contractile myofibroblasts.

The current prevalidation phase aims to demonstrate the applicability of hemi-cornea models in eye irritation testing and transcorneal drug permeation studies. This includes

- inter-laboratory method transfer for the hemi-cornea contruction
- assessment of the reproducibility of the hemi-cornea construction (intra- and inter laboratory variability) in all participating laboratories.
- definition of a preliminary prediction model for eye irritation and transcorneal permeation
- transfer of the test protocols

The quality of the hemi-cornea model production is assessed from histology and the cell viability of the Negative Control (PBS) and after exposure to 0.3% Triton X-100 as the Positive Control. In addition, the barrier function of the construct is determined from the transepithelial electrical resistance (TEER) and the permeation of a fluorescent hydrophilic marker.

ID ABS: 309

A potential two tiered cell based assay to distinguish sensitizers from non-sensitizers and to classify sensitizers according to their potency

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Introduction: The use of animal tests aiming to predict the sensitization potential of chemicals will be banned by the European Union in 2013. Current promising assays are able to distinguish sensitizers from non-sensitizers. However they do not classify sensitizers according to their potency. This study aims to use two *in vitro* assays, developed by our laboratory, in a tiered manner, in an attempt to distinguish sensitizers from non-sensitizers and also to classify sensitizers according to their potency.

Method: Tier 1: distinguishes sensitizers from non-sensitizers using MUTZ-3 progenitor cells and CXCL8 secretion (ELISA) as readout. In this tier, 4 sensitizers and 3 non-sensitizers were tested. Tier 2 determines sensitizer potency. Epidermal equivalents were topically exposed to 11 sensitizers in a dose response manner and EC_{50} values were calculated based on the decrease in epidermal equivalent metabolic activity (MTT assay EC_{50}: chemical concentration which induces 50% reduction in cell metabolic activity).

Results: CXCL8 secretion by MUTZ-3 cells was only induced after sensitizer exposure. Epidermal equivalent EC_{50} values decreased in proportion to increasing sensitizer potency and correlated closely to the LLNA EC3 values.

Discussion: Our preliminary results indicate that combined assessment of CXCL8 secretion by MUTZ-3 and epidermal equivalent decrease in metabolic activity (EC_{50}) may not only distinguish sensitizers from non-sensitizers but may also enable sensitizer potency to be determined. This study warrants further investigation using an extended chemical panel and further correlation with human and LLNA data.

ID ABS: 354

Experiences with the HET-CAM and the rabbit eye irritation test in the routine testing of a broad variety of chemicals and formulations

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New chemicals are usually tested for their potential to cause eye irritation as an important part of the toxicology program, e.g. for occupational safety. For the routine testing of a broad variety of chemicals and formulations we used the Hen's Egg Test - chorioallantoic membrane (HET-CAM) method. In the course of a tiered testing strategy and due to the lack of regulatory acceptance we also performed rabbit eye irritation tests according to OECD Test Guideline 405.

Of 145 tested substances according to the EU classification system 76% were non to mild irritants, 13% were irritating and 11% were severe irritants *in vivo*. The severe cases were based on the irreversibility of effects and not due to sufficiently high irritation scores during 72 hours post application.

Retrospective analysis revealed that the HET-CAM test's overall accuracy was 65% and the overall rate of false negatives (FN) and false positives (FP) was 50% or 33%, respectively. The HET-CAM was sufficiently specific (few FP) for water soluble substances, but failed to identify nearly all severe irritants within this group. In contrast, it was highly sensitive (no FN) for non- and oil-soluble substances, but the specificity for this group was rather low.

Therefore the HET-CAM method appears not useful in our present testing strategy. But due to the excellent sensitivity for non and oil soluble substances it might be applicable to further reduce the number of *in vivo* tests in a modified test-battery, provided that regulatory acceptance is given.

ID ABS: 367

Prevalidation of the *in vitro* skin irritation test method based on human reconstructed epidermis EST-1000

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Skin irritation is a reversible damage of the skin occurring as a consequence of the application of a test chemical. Skin irritation has typically been assessed involving animal experiments. Owing to animal welfare concerns, substitute *in vitro* methods based on reconstructed human epidermis were developed. Some of these methods have been validated to meet the criteria of the according test guideline.

In the course of the adoption of the United Nations Globally Harmonised System for the Classification and Labelling of Substances (GHS) the former valid *in vivo* cut-off score defined in the European classification system for irritant substances was shifted from 2.0 to 2.3. As a consequence three former (R38) irritants had become (no cat.) non-irritants under GHS, and the list of reference substances was not balanced anymore between irritant and non irritant chemicals. To achieve a balanced status of the number of irritant and non-irritant substances the list of reference substances has been changed in the draft proposal for the new OECD guideline released on 20 March, 2009.

We present the data of a prevalidation, testing the new GHS reference substances for skin irritation on EST-1000 epidermis models. The study was conducted according to the new performance standards for skin irritation test methods based on human reconstructed epidermis released by the ECVAM on February 17, 2009.

ID ABS: 377

Inter-laboratory reproducibility for a human cell line activation test (h-CLAT) in the Colipa ring trials

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There is no established OECD non-animal skin sensitization test method. Evaluation of changes in cell surface marker expression of DC-surrogate cell lines represents one non-animal approach. The human Cell Line Activation Test (h-CLAT) examines the level of CD86 and CD54 expression on the surface of THP-1 cells, a human monocytic leukemia cell line, following 24 h of chemical exposure. Ring trials (RTs) of alternative test methods for skin sensitization were conducted under the European Cosmetics Association (Colipa). Among these methods, h-CLAT has been evaluated by five independent laboratories. The results of the first and second ring trials demonstrated that the protocol was transferable and basically a good predictor. However there were some false negative data. To improve the performance of the test, the protocol and prediction model were revised. The 3rd RT evaluated the improved prediction model and good results were obtained. From these RTs data the feasibility of utilizing cell lines as surrogate DC in development of *in vitro* skin sensitization methods shows promise. The data also support moving the h-CLAT on into a formal pre-validation process.

ID ABS: 382

In vitro eye irritation assessment of colored and colorant-like substances using the SkinEthic™ HCE ocular model needs appropriate controls

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The *in vitro* SkinEthic™ reconstructed Human Corneal Epithelium (HCE) is part of an ongoing ECVAM formal *in vitro* eye irritation validation study aiming a possible incorporation of the test method in a tiered test strategy to replace the Draize eye test. The method, based on tissue viability assessment using the MTT test, was designed to allow the discrimination between irritant and non irritant substances. The reduced MTT is quantified by a standard colorimetric method. During formazan extraction, any unspecific color remaining in the tissue may be extracted and may result in a possible final viability overestimation.

The purpose of this study was to introduce specific controls allowing the use of the HCE test method for the irritancy prediction of colored substances. The protocol consisted mainly in a short 10 minutes topical treatment or a long 1 h +16 h post-treatment incubation period. After rinsing, colored substances retained in the epithelium may leave residual staining. Unspecific color was quantified by using treated tissue controls following the standard protocol course but not exposed to MTT. These controls enabled the quantification of Non Specific Optical Density and the correction of final OD (true OD due to mitochondrial activity). Histological analysis was conducted in order to document strong coloring substances. 9 irritants and 10 NC dyes were evaluated by this strategy.

We show that the HCE assay is a suitable method for *in vitro* eye irritation prediction of colored chemicals. The applicability domain of this assay can therefore be extended to these substance families.

ID ABS: 390

The Colipa strategy for developing and evaluating animal alternatives for skin sensitization testing

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Allergic contact dermatitis is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). Currently, the sensitizing potential of chemicals is usually identified on the basis of animal studies, such as the local lymph node assay (LLNA) or guinea pig tests. There are, however, increasing public and political concerns regarding the use of animal testing for the screening of new chemicals. Consequently, the development of *in vitro*, *in chemico* or *in silico* models for predicting the sensitizing potential of new chemicals is receiving widespread interest.

The Colipa Skin Tolerance task force currently collaborates with several academic research groups to expand our understanding of the molecular and cellular events occurring during the acquisition of skin sensitization. At present fundamental and applied research is being funded in multiple key areas, such as modeling of skin bioavailability, hapten chemistry, peptide binding, skin metabolism, dendritic cell activation and T cell proliferation. Knowledge gained from this research is being used to support the development and evaluation of novel alternative approaches for the identification and characterization of skin sensitizing chemicals. At present one *in chemico* (direct peptide reactivity assay (DPRA)) and two in vitro test methods (cell based assays (MUSST) and (hCLAT)) have been evaluated for their potential to predict skin sensitization potential within Colipa interlaboratory ring trials and were submitted to ECVAM for pre-validation.

ID ABS: 395

A Bayesian weight of evidence approach to assess eye irritation – test case

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Existing *in vitro* eye irritation assays have domains of applicability in terms of the range of irritancy and classes of chemicals they can predict. It is generally agreed that no one *in vitro* assay will fully replace the Draize test and that combinations of assays will be needed for this purpose. As such, a framework to objectively combine information from different tests is needed for transparent and consistent decision making. This need will increase as validation activities, e.g. prospective validation studies and retrospective evaluations, are completed and more validated *in vitro* assays with specified domains of applicability become available. One approach is to apply a Bayesian Weight of Evidence framework represented as a Bayesian Network. Bayesian Network seeks to resolve conflicting evidence, reason consistently given different data sets and/or incomplete data and results in generation of a probability statement about activity of a chemical based on a specific assay combination. A tiered testing strategy (Bottom-Up/Top-Down Approach) to predict eye irritation categories was proposed in a 2005 ECVAM workshop. As a test case we implemented the Bottom-Up/Top-Down Approach as the Bayesian Network, using published data on a selected set of chemicals for two *in vitro* assays – the Bovine Corneal Opacity Test (BCOP) and the SkinEthic™ Human Corneal Epithelium (HCE) assay. An analysis of the Bayesian WoE for BCOP and HRT assays will be presented. It will include Weight of Evidence quantification of individual tests and effectiveness analysis of this combination of assays in the Bottom-Up/Top-Down Approach.

ID ABS: 403

Evaluating the micronucleus induction potential for the genotoxicity assay using the Keraskin™ human skin model

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The micronucleus test is part of a battery of genotoxicity screening programs. The *in vitro* micronucleus assay is a mutagenic test system for the detection of chemicals which induce the formation of numerical or structural chromosomal damage. The reduction and replacement of *in vivo* toxicity testing require the development of *in vitro* models to predict the genotoxic potential. Reconstructed human skin models present various structural and functional advantages as compared to mouse skin for human risk assessment. Keraskin™, a reconstructed human tissue model, reflects metabolic complexities of *in vivo* and human specific responses and is used in safety or efficacy screening tests. In this study, five genotoxins and five non-genotoxins were applied to the Keraskin™ model and genotoxic potential was evaluated using Giemsa staining. A good reproducibility and correlation with *in vivo* and other *in vitro* model results were obtained. Our Keraskin™ model has a higher predictive potential of micronucleus assay and utility as part of an *in vitro* genotoxicity assay.

ID ABS: 404

The EpiSkin skin irritation validated test method: global performances merging evaluation sets according to the new GSH-EU classification

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The reconstructed human epidermis EpiSkin model test method was validated by ECVAM (ESAC statement, 2007) as a stand alone test for the prediction of acute skin irritation. The validated test method is mainly based on tissue viability assessment (MTT) following a 15 min exposure time and a 42 h post-treatment incubation period.

The implementation of the Global Harmonization System (GHS) in the European Union Classification (EU) has led to a new GHS-EU classification. The *in vivo* score cut-off value shifted from 2 to 2.3 for distinguishing non-irritant (no category) from irritant substances (category 2). Therefore, substances presenting *in vivo* scores comprised in this interval (GHS category 3) are considered as non-irritants in the GHS-EU classification. As a result, this reclassification led to more unbalanced working sets in terms of *in vivo* irritants versus non-irritants.

Taking into account this GHS-EU update, global performances of the EpiSkin skin irritation test method were recalculated by merging working sets (including the ECVAM validation set) evaluated in our laboratory. Analyses were performed on an overall set of 103 chemicals composed of 1 quarter irritant and 3 quarters non-irritant. Predictive capacities were defined by using the new GHS-EU classification and showed a sensitivity increase together with a slight specificity decrease as compared to the EU classification.

The overall performance of the EpiSkin test method was in accordance with the criteria defined by ECVAM. However, these results evidenced the impact of *in vivo* classification rules on *in vitro* methods performances.

ID ABS: 406

In vitro skin irritation assessment of colored test substances by using the validated SkinEthic RHE "42 bis" test method

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The SkinEthic Reconstructed Human Epidermis (RHE) "42 bis" skin irritation test method was validated by ECVAM as a stand alone replacement test for the prediction of acute skin irritation discriminating irritant and non-irritant substances (ESAC statement November 2008). The RHE "42 bis" test method is based on the quantification of tissue viability (MTT reduction assay)

after a 42 min exposure of the test substance on tissues followed by a 42 h post-incubation period.

The purpose of the study was to introduce specific controls allowing the use of the RHE "42 bis" test method for the irritancy prediction of colored substances. After rinsing, colored substances could be retained in the epidermis, mainly in the stratum corneum. During formazan extraction (reduced MTT), any unspecific color remaining in the tissue may be extracted as residual staining, and induce a possible final viability overestimation. Unspecific color was quantified using treated tissue controls following the standard protocol course but were incubated in medium instead of MTT. These specific controls enabled the quantification of the resulting non-specific color-related optical density (OD) and the correction of final true OD due to mitochondrial activity of a test substance. The applicability domain of the SkinEthic RHE "42 bis" skin irritation test method could therefore be considered compatible with colored test substances defined either as irritant or non-irritant.

ID ABS: 407

In vitro prediction of the validated SkinEthic RHE "42 bis" skin irritation test method for the EU-GHS classification

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The SkinEthic Reconstructed Human Epidermis (RHE) "42 bis" test method was recognized as a stand alone test to assess acute skin irritation by ECVAM by discriminating two categories, irritant and non-irritant substances, according to the European Classification System (ESAC Statement, November 2008). An adaptation of the Global Harmonization System (GHS) by the European Union (EU) has led to a new EU-GHS Classification. According to the new rules for classification, the cut-off score to distinguish between no category to category 2 substances was shifted to 2.3 from 2.0 (former EU system). Consequently, test substances with *in vivo* scores between 2 and 2.3 are now considered as non-irritants.

The aim of the study was to determine *in vitro* prediction of the SkinEthic RHE test method. For this purpose, the global performances of the test method were recalculated on a large set of test substances representative of different physico-chemical properties (including the catch-up validation set). The predictive capacity values (specificity, sensitivity and accuracy) were at least comparable to the required predictive value criteria defined by ECVAM.

ID ABS: 408

EpiSkin: a promising alternative tools to human skin for skin permeation study

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In human risk assessment, dermal absorption needs to be considered to predict the exposure and the risk of cosmetic ingredients. To date the OECD Guideline 428 recommends the use of human skin or alternatively pig skin for *in vitro* studies. However, the use of such methods is limited by the number of skin explants available as well as tricky and time-consuming sample preparation. Reconstructed skin models could be appropriate alternative methods for the assessment of skin permeation and penetration *in vitro*. The aim of the study was to investigate whether the commercially-available reconstructed human epidermis (RHE) models EpiSkin are suitable for *in vitro* skin absorption testing. We observed that the ranking of the permeation through reconstructed epidermis models reflected permeation through human skin. The results indicated a good correlation between the penetrated doses in the receptor fluid obtained with human skin and RHE models despite a lower barrier function of the reconstructed models. In conclusion, this study demonstrated the promising capabilities of RHE models to evaluate the *in vitro* skin permeation. To allow more precise quantification of absorption in human skin using RHE models, an expanded chemical panel and an increase in the number of experiments are necessary for the establishment of a robust and well-recognized alternative method.

ID ABS: 411

IL-1alpha measured in standardized and controlled conditions can be a useful adjunct to the EpiSkin skin irritation test method viability assay

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The reconstructed human epidermis EpiSkin model was validated by the ECVAM Scientific Advisory Committee (ESAC) in 2007 as a full replacement test for the prediction of acute skin irritation. The validated test is mainly based on viability assessment (MTT test) following a 15 min exposure time and 42 h post-treatment incubation period. During validation, IL-1-alpha release in this model was demonstrated as being a potentially useful parameter for the detection of irritants (sensitivity increase). Nevertheless, some questions remained regarding standardization and variability of the marker.

In the present work, we propose an approach integrating standards and controlled references, enabling the use of IL-1-alpha in well-defined conditions.

Standardization of the data was obtained by using International Unit (IU) values following a proposed 3 step procedure: 1-check R&D Systems kit calibration (converting factor) with the reference IL-1alpha standard in the laboratory working conditions and convert pg/ml to IU/ml, 2- Limit operator-dependent effects on basal interleukin release by subtracting negative control IL-1alpha values from treated tissues values. 3- Add 2 reference control-chemicals for IL-1alpha in the tested set. Taking into account these controlled conditions, 18 chemicals were evaluated by using the combined approach [viability + IL-1alpha]. Intra and inter-batch variability was measured. In conclusion, IL-1alpha, evaluated in standardized and controlled conditions, is a helpful adjunct to viability assays, thus strengthening the detection of irritants when using the validated EpiSkin model test method.

ID ABS: 422

Xenobiotic metabolism CYP450 activities in the EpiSkin™ reconstructed human epidermis

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Skin is not a single passive physical barrier but also a biological structure involved in a wide range of metabolic activities. In the context of alternative methods to animal experimentation, the reconstructed human epidermis EpiSkin™ is a powerful tool for studying, *in vitro* skin toxicity of chemicals and their metabolism. This dynamic requires characterization of the models in terms of metabolic capabilities. Even if previous studies showed that EpiSkin™ expressed numerous cytochrome P450 (CYP) isoforms, this is not proof that mRNA are translated into proteins and that the corresponding enzymes are active. A Western blot methodology, applied to EpiSkin™ microsomes, allowed us to determine which were the main CYP translated into proteins and active in xenobiotic metabolism in the epidermal model (CYP1A1/1B1/2C18/2D6/2E1/2J2/3A4/3A5/3A7). In addition, CYP functionality was revealed by measuring the CYP1A1/1B1, 2B6/2C18/2E1 and 3A isoforms' catalytic activities using fluorogenic substrates. Results showed that 1A1/1B1 and 3A isoforms are the main CYP activities in the reconstructed epidermis. CYP2B6/2C18/2E1 activities were also detected but at a level too low to be quantified. We observed, as well, that there was no real correlation between the CYP mRNA expression, translation into protein and activity levels. Thus, catalytic activity studies seem to us necessary steps to prove the real metabolic capabilities of any tissue. In conclusion, these studies confirmed that EpiSkin™ is a metabolically active skin model for studying *in vitro* xenobiotic phase I metabolism and toxicity and selecting inducers or inhibitors for these enzymes.

ID ABS: 424

Cell surface marker of corneal epithelium stem cells and culture

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Purpose: We examined the manifestation of the tissue stem cell marker (p75NTR, CD271) which the actor in the corneal epithelial stem cell of the human corneal limbus. Also, we examined the primary culture method of new corneal epithelium cells. In addition, p75NTR is one of the epithelium stem cell markers and mesenchymal stem cell marker.

Method: We purchased a human cornea tissue from an American eye bank for using of research. The human cornea tissue was fixed by SUPER FIX (Japan patent NO 3723204, KURABO, JAPAN), made the paraffin specimen and immunostained by using p75NTR and p63 antibody. The cornea tissue including the corneal limbus was treated using enzyme and cultured at serum-free medium on coating dish. We examined the primary culture method that we did not use feeder cells about discrete cells from a cornea tissue including the corneal limbus.

Results: p75NTR-positive cells were observed around epithelial cell base of the corneal limbus. The p75NTR-positive cells were with positive p63 which expressed at a corneal epithelial stem cell. The culture cells from a cornea tissue including the corneal limbus were observed circular cells and were able to subculture three times over.

Discussion: Including the traditional report of the speaker, the tissue stem cell in the ophthalmology domains such as cornea, the crystalline lens, the iris was observed p75NTR-positive cells. Furthermore, this method was able to culture epithelium cells without using feeder cells.

ID ABS: 432

Oxidative deaminase activity in the EpiSkin™ reconstructed human epidermis

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Beside its physical barrier function, skin contains numerous metabolizing enzymes involved in skin metabolism and detoxification. New legislations, i.e. the 7th European Amendment to the Cosmetic Directive, have forced the cosmetic industry to develop reconstructed human skin models as alternative tools to animal experimentation. Characterizing these models in terms of metabolic capabilities became necessary to use them in safety studies and understand local toxicity mechanisms. Adenosine deaminase (ADA) and adenylate deaminase (AMPDA) are enzymes involved in the irreversible oxidative deamination of adenosine and adenosine derivatives (Ciuffreda, P. et al., 2007). These enzymes, which are widely present in normal human epidermis, play a critical role in skin homeostasis and maybe in the proliferation and maturation of certain mammalian cell types (Hiroko Koizumi, M. D. et al., 2009). We characterized the oxidative deaminase activity in EpiSkin™ using 2',3'-O-isopropylidene adenosine and corresponding ethyl ester as enzyme substrates. Inosine derivative formation was detected by UV-HPLC and demonstrated that ADA and/or AMPDA are present and active in the EpiSkin™ model.

ID ABS: 435

Characterisation of *N*-acetyl and glutathione *S*-transferase activities in skin and reconstructed human skin models

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Skin is mainly considered a physical barrier to the environment. However, it contains numerous metabolizing enzymes which give to it a potential role in terms of metabolism and detoxification. The 7th European Amendment to the Cosmetic Directive forbids the use of animal testing to evaluate the efficacy and safety of new cosmetics. Thus, the cosmetic industry has developed reconstructed human skin models as tools for alternative methods to animal experimentation. This requires that the mod-

els are characterized and compared with a normal human skin (NHS) in terms of metabolic capabilities. We characterized the *N*-acetyl transferase (NAT) and glutathione S-transferase (GST) activities particularly involved in xenobiotic detoxification at the skin level. Previous studies showed that NHS and reconstructed human epidermis such as EpiSkin™ and SkinEthic-RHE™ expressed several NAT and GST isoforms. Thus, NAT and GST activities of these models and NHS were quantified using *p*-aminobenzoïc acid (PABA) and chlorodinitrobenzene (CDNB) as substrates, respectively. Apparent Vmax, Km and clearance were measured for each tissue. Results showed that for both activities, even if the Vmax and Km between tissues were different, probably due to the substrate bioavailability, the clearances were equivalent. Besides, a high variability between samples was observed notably for NAT activity of NHS. Tissue spoiling or genetic polymorphism could explain this result. This variability was not observed with skin models which were reconstructed from a keratinocyte pool of several donors. In conclusion, these findings confirm that NAT and GST activities are present and active in skin and reconstructed models.

ID ABS: 439

Effects of collection, transportation, and BCOP methodology on bovine corneal histology evaluation

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

ID ABS: 442

Colipa's pre-validation program using reconstructed human tissue-based methods for predicting eye irritation: SkinEthic human corneal epithelium assay

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Colipa (European Cosmetics Association) is co-sponsoring the formal validation of *in vitro* eye irritation assays together with ECVAM. This poster details the technology transfer and reproducibility of the SkinEthic Human Corneal Epithelium (HCE) test method as one of the two Reconstructed human Tissue (RhT) assays submitted. The optimized SkinEthic HCE protocol, including two exposure time regimens (10 min and 1 h + 16 h post-exposure incubation) was established for a set of more than 100 chemicals. The prediction model was based on a 50% viability cut-off by measuring tetrazolium dye reduction (MTT), with 2 prediction classes (irritants and non-irritants). Intra- and inter-laboratory reproducibility was assessed in 8 participating laboratories. Reproducibility was demonstrated for both exposure times using at least a subset of 20 coded chemicals. Across the laboratories and the multicentric performed studies, 95% of chemicals were identically classified as well as concurrent positive and negative controls. The Direct Peptide Binding Assay (DPRA) chemical reactivity criteria enabled the choice of the most appropriate exposure treatment time regimen of the SkinEthic HCE protocol using 90 chemicals. The biological *in vitro* eye irritation SkinEthic HCE test method was able to discriminate irritants from non-irritants with an overall accuracy of 79% and balanced sensitivity and specificity performances.

ID ABS: 451

Results of a two laboratory reproducibility study using a harmonized bovine corneal opacity and permeability (BCOP) protocol

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The Bovine Corneal Opacity and Permeability assay (BCOP), an internationally recognized alternative to the Draize eye irritation test, uses excised bovine corneas to predict ocular irritation. Originally developed by Gautheron (1992) and utilizing the irritation class prediction established by Sina (1994), BCOP has been used independently at MB Research and at the Institute for *in vitro* Sciences (IIVS) for over fifteen years for product development, worker safety, and safety claims substantiation. The assay is currently under regulatory review by EPA, ECVAM and ICCVAM, and has recently been endorsed for prediction and labeling of severe/corrosive eye irritants. Since MB Research and IIVS have extensive experience performing the BCOP assay utilizing a variety of specific protocols to discriminate among mild and moderate, as well as severe/corrosive eye irritants, they agreed to develop and evaluate the reproducibility of a standard harmonized protocol for regulatory labeling. Nine blind-coded chemicals, primarily comprised of surfactant dilutions, as well as imidazole and pyridine, were tested in three independent GLP-compliant trials using exactly the same protocol. Intra-laboratory and inter-laboratory reproducibility evaluations showed that both laboratories typically obtained the same irritation class predictions. The resulting *in vitro* scores were compared to Draize MMAS results (ECETOC, 1998). Some of the surfactant dilutions (sodium dodecyl sulfate, cetyl pyridinium bromide) were found to be under-predicted using the standard BCOP protocol for liquid test chemicals.

ID ABS: 457

Chemical metabolizing activity of an *in vitro* human epidermal (EpiDerm™) model, and genotoxicity as determined by *in vitro* skin micronucleus assays

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Human dermal and airway epithelia contain xenobiotic metabolizing enzymes (XME) that could cause biotransformation of cosmetic ingredients, hair-dyes and other chemicals into toxic/mutagenic metabolites. The present work evaluated functional expression of XMEs in highly differentiated *in vitro* models of human epidermal (EpiDerm™) and airway (EpiAirway™) epithelia. RT-PCR and quantitative real-time PCR array experiments were conducted to analyze expression of 168 Phase I and Phase II XMEs in the models. To evaluate the functional activity of XMEs, an in vitro skin micronucleus assay was also performed with genotoxic chemicals known to require metabolic activation. Phase I enzymes found to be expressed in the models include cytochrome P450 (CYP) isoforms, alcohol dehydrogenases, aldehyde dehydrogenases, monoamine oxidases, flavin-containing monooxygenases and others. Further expression of some enzymes could be induced by 3-methylcholanthrene (3MC). Phase II enzymes found to be expressed included glutathione S-transferases, glucuronosyl transferases, sulfotransferases, N-acetyl transferases, epoxidases, esterases, and others. In vitro skin micronucleus assays conducted on EpiDerm™ tissues topically treated with genotoxins confirmed metabolic activation of four chemicals that are known to require metabolic activation in order to produce genotoxicity. These results show that the EpiDerm™ and EpiAirway™ *in vitro* human skin and airway epithelial models possess functional drug metabolizing activity that can result in biotransformation of chemicals and generation of genotoxicity as determined by an *in vitro* skin micronucleus assay. These models and assays should prove useful for *in vitro* genotoxicity testing of cosmetic formulations as well as *in vitro* testing of chemicals for the REACH program.

ID ABS: 468

Development of an EpiDerm *in vitro* skin irritation test (SIT) for the globally harmonized system (GHS) of classification and labeling of chemicals

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Purpose: Recent legislation and a ban on animal testing for cosmetics have heightened the need for validated *in vitro* skin irritation tests (SIT)s. The EpiDerm model has been validated for *in vitro* skin corrosion testing worldwide, and for *in vitro* SIT in the EU in studies sponsored by the European Center for the Validation of Alternative Methods (ECVAM). The EU SIT system distinguishes 2 classifications – skin irritants (R38) and non-irritants (no label). However, a UN treaty endorsed by the US, EU, China, Japan, Australia and others has outlined a GHS of Classification and Labeling of Chemicals. The GHS classifies skin irritancy of chemicals into three categories: non-irritant, slight irritant or irritant. Therefore, additional efforts are underway to validate an EpiDerm SIT for GHS.

Methods: 15 test chemicals with known *in vivo* Draize skin irritation scores were applied to EpiDerm to identify *in vitro* skin irritation biomarkers and establish a preliminary EpiDerm-GHS-SIT prediction model. Biomarker endpoints evaluated include EpiDerm viability (MTT assay) and inflammatory mediator release by ELISA and/or Multiplex (Bio-Rad BioPlex) assays.

Results: The MTT viability response was the most predictive and least variable biomarker, providing 80% concordance with the *in vivo* Draize classification (i.e. 80% sensitivity and specificity for assigning GHS classifications). Among the mediators investigated, significant levels of IL-1α, IL-1ra, IL-8, IL-18, GROα and PGE_2 were produced by EpiDerm tissues. These biomarkers did not improve the classification. This preliminary prediction model will be further tested and refined to form the basis for formal multi-laboratory EpiDerm-GHS-SIT validation studies.

ID ABS: 471

EpiOcular tissue model protocols for 1) REACH ocular irritation screening and 2) ultra-mild eye care cosmetics

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Purpose: *In vitro* tests for assessing ocular irritancy of consumer/household product chemical ingredients are urgently needed to comply with EU legislation such as the 7th Amendment of the Cosmetics Directive and the REACH Directive. Eye care cosmetics (ECC) also need to be non-irritating (i.e. "ultra-mild") in order to be successful in the marketplace. This poster summarizes two different protocols that have been developed for use with the *in vitro* EpiOcular tissue model in order to accommodate both purposes

Methods: For REACH irritation testing, a single exposure period is used: 30 min with a 2 h post-exposure incubation (liquids) or 90 min with 18 h post-exposure incubation (solids). A single cut-off in relative survival is used for classification: more than 60% = irritant (I) (R36 and R41); >60% = non-classified (NC). Tissue viability is determined by MTT assay. For ultra-mild testing, exposure times between 8 and 24 h are used, and tissue viability (ET-50) is also determined by MTT assay.

Results: For irritation screening of chemicals, 99.7% agreement in prediction (NC/I) was obtained from 298 independent trials across seven laboratories (Harbell et al., *The Toxicologist 108(1),* 2009). For ultra-mild testing of 10 mascaras, a range of ET-50s was obtained from 8.7 h to >24 h. Other formulations with low levels of surfactants known to be irritating at higher concentrations could also be discriminated by their ET-50 values. Thus, the EpiOcular model appears to function well for both chemical testing for REACH purposes, as well as ultra-mild screening of ECCs and other materials.

ID ABS: 472

The use of HET-CAM for assessing the potential of skin irritation of surfactants

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Introduction: Although commercial reconstituted skin kits for determining skin irritation and corrosion are already available, their short validity makes it difficult to buy them for countries outside Europe, since the process of receiving via customs is very complicated and risks the loss of material. Since the redness observed in the Draize rabbit skin irritation test is an inflammatory reaction shown as a vascular alteration, we studied the possibility of correlating animal findings with HET-CAM observations. The aim of this study was to determine the dilution needed for use in the HET-CAM assay in order to obtain the same response grade (non irritant) as in the animal test.

Material and Methods: Seven surfactants were tested both *in vivo* and *in vitro*. Historic rabbit test data were obtained from previous tests. The Draize test was carried out using 6 rabbits per surfactant, by applying 0.5 ml of different surfactant concentrations. The same or lower concentrations of these surfactants were tested in the HET-CAM, using 4 SPF eggs per sample.

Results: Anionic surfactants were classified as non irritant when diluted 1:320. Non-ionic and cationic surfactants did not present any correlation when dilutions were compared to *in vivo* results (non-ionic 1:200 and 1:1280; cationic 1:20, 1:40 and 1:160).

Conclusion: HET-CAM presented the same non-irritant classification as the rabbit test only for anionic surfactants, when diluted 1:320. This means that if HET-CAM presents a positive reaction when diluted less than 1:320 of the concentration of use, the product can be considered a potential skin irritant.

ID ABS: 474

Early detection of toxic damage at the corneal epithelium surface using multiple endpoint analysis on HCE

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Introduction and aim: The Multiple Endpoint Approach (MEA) on human reconstructed corneal epithelium (HCE) constitutes a valuable approach to determine the mechanisms of eye irritation. The aim of this study was to optimize the MEA in the field of ophthalmo-toxicology. Using a 0.001% to 0.1% range of BAK concentrations, we investigated various parameters likely to be modified by the toxin.

Material and methods: Using the HCE model from SkinEthic we modified the MEA protocol by modifying the classical MTT procedure, by measuring the TEER at defined concentrations and by including the study of mRNA expression of occludin, a structural component of tight junctions. We also investigated on frozen sections and entire epithelia the expression and spatial distribution of cellular markers involved in cell death, proliferation and inter-cellular junctions.

Results: The modified MTT procedure shows a higher sensitivity in detecting low toxic effects. Low doses of BAK induced apoptosis restricted to the superficial cell layers, and proliferation mainly located in the basal layers, while high doses of BAK induced apoptosis in all cellular layers, hindered cell proliferation and modified occludin expression patterns. The occludin gene expression tended to increase with the low range of concentrations, while it decreased with the high range of concentrations. This study is an example of a MEA that can be used in toxicological testing. Such an approach may help to detect the infra-clinical signs of cellular toxicity that are possibly responsible for ocular inflammation.

ID ABS: 475

Reproducing 3D conjunctiva: a challenging new *in vitro* model

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Introduction: Due to the large surface area of the conjunctiva, particularly compared to the cornea, for example, the ability of a compound to penetrate the conjunctival barrier is an important consideration in the evaluation of its potential toxicity. The conjunctival route is important for hydrophilic compounds with large MW, and it is less influenced by lipophilicity. *In vivo* conjunctival uptake via its blood vessels may lead to systemic absorption.

Material and methods: An American Type Culture Collection (ATCC) conjunctival cell line was cultured in low and high-calcium medium for 6 to 10 days to form both a monolayer culture and a stratified culture on coated inserts.

Results: The new conjunctival cell line retains many of the characteristics of primary conjunctival cell cultures including: morphology on plastic; growth rate; types of expressed keratins (including, for example, keratin 4); expression of adhesion molecules (e.g. ICAM-1); expression of mucin (including MUC5ac); basal cytokine production (including IL-12, IL-12a); presence of goblet cells (a specialized, mucin-secreting cell of the conjunctiva); ultrastructural features (including tight junctions, desmosomes, apical microvilli) and transepithelial barrier function (as evaluated by measurements of transepithelial electrical resistance (TEER).

ID ABS: 477

Use of the reconstructed EpiVaginal tissue model to screen irritation potential of feminine hygiene products

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The vaginal mucosa is commonly exposed to chemicals and therapeutic agents that may result in irritation/inflammation and that can make women susceptible to infections such as HIV-1 and HSV-2. Hence, chemical/formulation or therapeutic agent induced vaginal irritation is a concern for toxicologists. Traditionally, testing of such materials has been performed using the rabbit vaginal irritation (RVI) assay. In the current study, we investigated whether the organotypic, highly differentiated EpiVaginal tissue could be used as a non-animal alternative. The EpiVaginal tissue was exposed to a single application of six chemicals at three concentrations and the effects on tissue viability (MTT assay), barrier disruption (measured by transepithelial electrical resistance, TEER, and sodium fluorescein, NaFl, leakage), and inflammatory cytokine release (Interleukin, IL-1α, IL-1β, IL-6, and IL-8) were examined. When compared to untreated controls, two irritating test articles, benzalkonium chloride and nonoxynol 9, reduced tissue viability to <40% and TEER to <60% and increased NaFl leakage by 11-24% and IL-1α and IL-1β release by >100%. Four other non-irritating materials had minimal effects on these parameters. Assay reproducibility was confirmed by testing the chemicals using three different production lots and by using tissues derived from cells of three different donors; coefficients of variation were <12%. In conclusion, decreases in MTT and TEER, and increases in NaFl and IL-1α and IL-1β release appear to be useful endpoints for preclinical toxicity screening of chemicals, formulations, and medical devices and should be investigated further as an *in vitro* alternative to the current RVI assay.

ID ABS: 490

International evaluation of bovine corneal opacity and permeability assay as an alternative method to eye irritant test

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The bovine corneal opacity and permeability (BCOP) assay is well established as one of the alternative methods to the Draize test. BCOP has not yet been accepted as a regulatory validated method in Korea. To validate our BCOP assay internationally, we

compared our BCOP results with ICCVAM results. We performed the BCOP procedure according to an ICCVAM detailed protocol in which all experimental details were described and each chemical was used in at least three independent experiments. The test materials were liquids (five), solids (five) and surfactants (two), representing a range of different physical chemical characteristics. Liquid and surfactant materials were incubated for 10 min and solids materials for 30 min. The BCOP score was graded according to four irritation scale. Although our BCOP scores of some materials were lower than ICCVAM BCOP scores, the BCOP irritation scale was the same as ICCVAM's. Histophysiological study revealed the relative ranking of opacity and permeability. Irritant materials caused edema and vacuolization in stroma and epithelial cell layers were partly lost depending on the dose.

ID ABS: 507

The prevalidation of alternative methods of new reconstructed human corneal equivalents for the assessment of eye irritation

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Several alternative *in vitro* methods for identifying eye irritants have been developed recently. One of the most promising is a reconstituted human corneal model. In this study, we demonstrated the new reconstituted human skin model, human corneal epithelial (HCE) model (MCTT Co, Korea), showed a good performance in terms of intra- and semi inter-laboratory variability and predictive capacity to assess acute eye irritation. During Phase 1 (protocol optimization) and Phase 2 (protocol transfer) of the pre-validation study, reproducibility and predictive capacity of the method were optimized. In order to improve the performance of the HCE model, the existing similar protocol was adopted with appropriate refinement. This refinement consisted of changing the chemical treated exposure time and volume of corneal epithelium and incubation time after treatment. The main endpoint measured in the HCE model was cell viability (MTT or WST). In samples which gave cell viability above the threshold of 50%, cytokine (IL-1α and IL-8) release and LDH leakage were also measured. Sensitivity, specificity and accuracy of the method using the new HCE model were measured. Linear equations were used to convert the results into the corresponding EU eye irritation categories and GHS data. We concluded that this new three-dimensional model, developed from human corneal epithelium, could be promising for the prediction of eye irritation induced by chemicals.

ID ABS: 509

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

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

Methods: Normal human epidermal keratinocytes (KC) and dermal fibroblasts (FB) were cultured to produce the highly differentiated full-thickness skin model. Small wounds of several mm diameter were induced in the epithelial model by means of a battery operated cauterizer or a dermal biopsy punch. The wounded EpiDerm-FT cultures were fixed at various time points and H&E stained paraffin sections were prepared to evaluate the wound and the wound healing process.

Results: Immediately after burn wounding, necrotic epithelium and denatured collagen dermal matrix were evident. Within one day, the denatured collagen matrix began to degrade and epithelial KC were observed migrating inward from the wound edges. Over a time course of seven days, migrating KC repopulated the wounded area to form a fully covered epithelium. Dermal fibroblasts were also observed to be proliferating within the wound area and generating new dermal matrix material. Biopsy punches were used to produce wounds that removed only the epidermis. These wounds also healed within a timeframe of 3-7 days. Increased FB proliferation in dermal areas directly adjacent to migrating KC was observed. These results demonstrate that EpiDerm-FT is a useful animal alternative skin model for investigating dermal-epidermal interactions during wound healing and to evaluate the role of specific growth factors or new therapeutics in the dermal wound healing process.

ID ABS: 513

Validation of the reconstructed human epidermis (RHE) skin irritation assay for full replacement of the Draize test

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Purpose: The Directive 67/548/ECC or OECD TG 404 to fully replace the *in vivo* Draize skin irritation test was reinforced with the 7th Amendment of the Cosmetic Directive and the REACH regulation. Two reconstructed human epidermis models, EpiDerm and EpiSkin, were scientifically validated with reliability, as for EpiSkin, to discriminate skin irritants (R38) from non-irritants (no label) as defined by EU risk phrases. The aim of this study was to assess whether the *in vitro* Reconstructed Human Epidermis (RHE) model, commercialized by SkinEthic Laboratories, could be an alternative to the Draize rabbit test.

Methods: According to the standard documents to evaluate accuracy and reliability (ECVAM SIVS, 2007), a blind multicentric validation study using the RHE model was performed in three independent laboratories: L'Oréal, Coty and Oroxcell. Twenty reference compounds (cpds) with *in vivo* irritancy score were selected in this validation study for submission to ECVAM. RHE tissues were incubated with each of the test compounds for 42 min, rinsed and then post-incubated for a 42 h period to assess cell viability by MTT assay. IL-1α release was measured as a second end point. Irritant cpds were those giving <50% cell viability.

Results: Predictivity of skin irritancy potential for the 20 cpds was found high using the MTT assay: 90% sensitivity and 80% specificity. Accuracy was 85% and was not improved by IL-1α release assay.

Conclusions: The RHE model using the MTT assay provides similar results to those found with the EpiSkin model validated by ECVAM using both MTT and IL1α assays.

ID ABS: 515

Inter-laboratory 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 *in vitro* eye irritation test that assesses cytotoxicity in SIRC (rabbit corneal cell line) cells following a 5 min dose treatment. To assess reliability (including transferability and inter-lab reproducibility) and relevance of the STE test, a validation study with 5 laboratories was conducted (supported by the Japanese Society for Alternatives to Animal Experiments). At first, three standard chemicals (sodium lauryl sulfate, calcium thioglycolate, and Tween 80) were evaluated to confirm transferability. Three experiments for each chemical were evaluated using 5%, 0.5%, and 0.05% test material in vehicle (saline). The mean cell viability (CV) value of each substance was similar to the developer's background data. Also, the rank classifications based on the prediction STE model for these substances were 3, 2, and 1, respectively, in all 5 laboratories and developer. From these data, a good transferability of the STE test was obtained. For the next step, between-lab reproducibility and relevance were evaluated using 25 blinded chemicals. STE irritation rank based on the CV of the 5% solution showed good correlation with GHS rankings (NI; I: Cat. 1 and 2). Moreover, STE rank (1, 2 or 3) classified by the prediction model using the CV of the 5% and 0.05% solutions showed good correlation (above 80%) with GHS rank (NI, Cat. 2, Cat. 1) in all laboratories. Also inter-lab reproducibility for 25 chemicals was good. Based on these findings, the STE test could be easily standardized as an alternative eye irritation test.

ID ABS: 552

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 expression to native human skin. This model has shown to be useful as an alternative testing model in the skin irritation and corrosion test. Although there is a strong urge to restrict the animal experiments for toxicity tests, an alternative *in vitro* phototoxicity tests using KeraSkin™ has not yet been established. Therefore, this study was conducted to validate the *in vitro* phototoxicity test method using KeraSkin™. Nine phototoxic or non-phototoxic chemicals were topically treated on KeraSkin™ and after 24 h incubation the KeraSkin™ was exposed to 6 J/cm^2 of UVA. Test chemicals were removed, and cell viability was quantified by MTT assay after incubation for another 24 h. Predictions of phototoxic potentials were highly reproduced, and the established prediction standard was effective, showing consistency with previously reported *in vivo* test results. In conclusion, *in vitro* alternative phototoxicity test method using KeraSkin™ was successfully established. Surely KeraSkin™ can be used as a good alternative test method for assessment of phototoxicity of chemicals.

ID ABS: 583

International acceptance of *in vitro* alternative ocular safety testing methods: the isolated chicken eye (ICE) test method (Draft OECD TG 438)

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The ICE test method is an *in vitro* method that provides short-term maintenance of the chicken eye. Potential ocular damage is assessed by corneal swelling, opacity, and fluorescein retention. ICCVAM recommended that ICE could be used to classify positive substances as ocular corrosives and severe irritants. While not a complete replacement for the *in vivo* rabbit eye test, ICE can be used in a tiered-testing strategy for regulatory classification and labeling within a specific applicability domain. These recommendations were accepted by U.S. Federal agencies, and positive results from ICE may now be used in the U.S. instead of the rabbit eye test for certain regulatory hazard classification decisions. To have the greatest impact on reducing animal use, ICCVAM, with input from stakeholders in the U.S., EU, and Japan, drafted an OECD Test Guideline (TG) that is based on the ICCVAM ICE test method protocol. This protocol was developed following an international peer review evaluation with contributions from ECVAM and JaCVAM. The draft TG 438 was recently accepted by the OECD WNT. Once formally adopted by the OECD Council, TG 438 will be accepted by all 30 OECD member countries in accordance with OECD Mutual Acceptance of Data. The use of ICE will reduce the use of rabbits for eye safety testing and eliminate the *in vivo* testing in animals of most substances likely to cause severe pain and discomfort. ILS staff supported by NIEHS contract N01-ES-35504. The views above do not necessarily represent the official position of any government agency.

ID ABS: 584

International acceptance of *in vitro* alternative ocular safety test methods: bovine corneal opacity and permeability (BCOP) assay (draft OECD TG 437)

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The BCOP is an *in vitro* method that provides short-term maintenance of normal physiological and biochemical function of the bovine cornea. Potential ocular damage is assessed by changes in opacity and permeability to fluorescein. ICCVAM recommended that BCOP could be used to classify positive substances as ocular corrosives and severe irritants. While not a complete replacement for the *in vivo* rabbit eye test, BCOP can be used in a tiered-testing strategy for regulatory classification and labeling within a specific applicability domain. These recommendations were accepted by U.S. Federal agencies, and positive results from BCOP can be used in the U.S. instead of the rabbit eye test for making certain regulatory hazard classification decisions. To have the greatest impact on reducing animal use, ICCVAM, with stakeholders in the U.S., EU, and Japan, drafted an OECD Test Guideline (TG) that is based on the ICCVAM BCOP test method protocol. This protocol was developed following an international peer review evaluation with contributions from ECVAM and JaCVAM. Draft TG 437 was recently accepted by the OECD WNT. Once formally adopted by the OECD Council, all 30 OECD member countries in accordance with OECD Mutual Acceptance of Data will accept TG 437. Use of BCOP will reduce rabbit use for eye safety testing and eliminate such testing in animals of most substances likely to cause severe pain and discomfort. ILS staff supported by NIEHS contract N01-ES-35504.

The views expressed above do not necessarily represent the official position of any government agency.

ID ABS: 585

Evaluation of potential false negative corrosive chemicals in proposed *in vitro* dermal irritation assays

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EpiDerm™ and EPISKIN™ have been proposed as replacements for the *in vivo* rabbit skin irritation test and for inclusion in an *in vitro* testing strategy to replace *in vivo* rabbit testing for regulatory hazard classification and labeling of skin corrosivity and irritation. If these methods are to be considered complete replacements for animal tests, they must provide equal or greater protection. Therefore, an *in vitro* testing strategy must be capable of identifying approximately 12% to 21% false negative corrosive substances, which are currently identified using the *in vivo* rabbit skin irritation/corrosivity test. NICEATM and ICCVAM designed a multi-phased study to assess the performance of the EpiDerm™ irritation assay when testing false negative corrosive substances to establish criteria for identifying corrosives or to identify exclusion rules for substances that cannot be accurately evaluated in an *in vitro* testing strategy. Phase 1 evaluated the effectiveness of an MTT correction step in reducing false negatives in the corrosivity assay and whether the EpiDerm™ irritation assay can distinguish dermal corrosives from irritants. Data from Phase 2 were used to inform suggestions on modifying decision criteria for the EpiDerm™ irritation protocol that would also identify corrosives. Information generated from this study, which was developed by NICEATM and ICCVAM, with input from the ECVAM Validation Study Management Team, will be critical to regulatory authorities in their consideration of *in vitro* skin irritation test methods. ILS staff supported by NIEHS contract N01-ES-35504. The views expressed above do not necessarily represent the official positions of any Federal agency.

ID ABS: 633

Extended storage of corneas for use in BCOP assay

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The draft OECD guideline for the Bovine Corneal Opacity and Permeability (BCOP) assay requires that laboratories performing this assay use excised bovine corneas within five hours of slaughter. The timely availability of excised bovine corneas for laboratory testing is determined by the proximity of abattoirs and the time of animal slaughter. The duration of the BCOP procedure frequently results in the requirement for opacity and permeability measurements to be performed during unsocial working hours, particularly for solid test materials. We have performed comparative BCOP assays for a range of solid and liquid test materials using freshly excised corneas within five hours of slaughter and following storage of excised corneas overnight at room temperature. Imidazole and ethanol positive controls were included in these comparative tests. The *in vitro* irritancy scores obtained using stored corneas were comparable with fresh corneas for both solid and liquid test materials. Our results indicate that it is acceptable to extend the current testing limitation of "within five hours" to accommodate overnight storage of excised corneas and permit testing within ca. 24 hours of animal slaughter.

PO23: Systemic toxicity and target organs

ID ABS: 25

Preservation of hepatocellular functionality in cultures of primary rat hepatocytes upon exposure to 4-Me2N-BAVAH, a hydroxamate-based HDAC inhibitor

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5-(4 dimethylaminobenzoyl)-aminovaleric acid hydroxamide (4-Me2N-BAVAH), an amide-based structural analogue of trichostatin A (TSA), was previously shown to induce cell cycle arrest in mitogen-stimulated rat hepatocytes. Since the use of primary hepatocyte cultures in pharmaco-toxicological research is limited by the occurrence of phenotypic alterations, including loss of xenobiotic biotransformation capacity, it was investigated whether the anti-proliferative properties of 4-Me2N-BAVAH might promote the maintenance of a differentiated hepatic phenotype in culture. To that end, genome-wide gene expression analysis was performed. Several genes coding for xenobiotic biotransformation enzymes, including cytochrome P450 (CYP) enzymes, were found to be positively regulated throughout culture time upon exposure to 4-Me2N-BAVAH. For CYP1A1, CYP2B1 and CYP3A2, these observations were confirmed by qRT-PCR and immunoblot analysis. In addition, significantly higher 7 ethoxyresorufin O deethylase (EROD) and 7 pentoxyresorufin O dealkylase (PROD) activity levels were measured. These effects were accompanied by an increased expression of CCAAT/enhancer binding protein alpha (C/EBPα) and hepatic nuclear factor (HNF)4α, but not of HNF1α. Finally, 4-Me2N-BAVAH was found to induce histone H3 acetylation at the proximal promoter of the albumin, Cyp1a1 and Cyp2b1 gene, suggesting that chromatin remodelling is directly involved in the transcriptional regulation of these genes. In conclusion, histone deacetylase (HDAC) inhibitors (HDACi) prove to be efficient agents for better maintaining a differentiated hepatic phenotype in rat hepatocyte cultures.

ID ABS: 51

Effects of furan on thymus of male rats: from weaning to puberty

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Furan occurs naturally in various kinds of foods that undergo heat treatment, such as canned and jarred foods. Human may be exposed to furan via consumption of food products and via other ways such as breathing. Furan may have potential risks in human, especially in children. Therefore, in the present study, the effects of furan on thymus of weaning male rats are evaluated. There were five groups of 8 male 3-4 week old rats each, i.e. control group, oil control group, furan treated groups. In this study, furan was given by gavage to the rats in the treatment groups at doses of 2 mg/kg/day, 4 mg/kg/day and 8 mg/kg/day for 90 days. At the end of the experiment, the thymus was removed and examined morphologically, histopathologically and immunohistochemically. In immunohistochemical investigations, apoptotic cells were determined and evaluated for immunolocalization of fibronectin and Transforming Growth Factor-beta.

Absolute and relative weights of thymus decreased significantly in rats treated with 4 mg/kg/day and 8 mg/kg/day doses of furan. In the histopathological examination, increased connective tissue and congestion were observed. Increase in apoptotic cell numbers in thymus was detected. In conclusion, this study suggests that furan affects the thymus in growing rats.

ID ABS: 103

Refinement of the fasting period for a standard gastrointestinal (charcoal meal) study

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The charcoal meal test is a model used to provide information on side-effects such as constipation and diarrhoea commonly occurring in man. These effects are unlikely to be life-threatening, but may affect patient compliance, quality of life and contribute to adverse interactions with other drugs.

Rodents are routinely fasted overnight (~18-24 hours), to ensure an empty stomach (reducing any variation in results due to food). Animals receive a dose of test compound, followed by a charcoal meal orally. 15 minutes later, the amount of charcoal remaining in the stomach (an index of gastric emptying, GE), and the distance any charcoal has moved down the intestines (% intestinal transit, IT) are measured. Prolonged fasting can cause stress, aggression (requiring single-housing), bodyweight loss, body temperature reductions and blood glucose reductions. We investigated whether a fasting period was required, and if so, could a reduced fasting period be used without impact on study outcome, but with benefits to the welfare of the animals.

Rats/mice were fasted for 0, 3, 6 or 18 hours before dosing. Animals were dosed orally with water (as a control) or atropine (a compound known to inhibit gastric function, used as a reference), 1 hour prior to a charcoal meal, then GE and IT assessed.

Fasting rodents for 6 hours gives similar results in GI function to overnight fasting, whilst having less effect on bodyweight and clinical signs (e.g. aggression), thus enabling group-housing for mice. This outcome may be more widely applicable in other studies where fasting overnight is also used.

ID ABS: 135

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

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Developing alternative methods to animal testing is crucial to the cosmetic industry owing to ethical reasons, the REACH regulation and the 7th Amendment of the European Cosmetic Directive. This directive foresees a marketing ban from March 2009 of cosmetics containing ingredients or combinations of ingredients tested in animals for, among other toxicity endpoints, acute toxicity.

Over the last years, considerable efforts have been made to develop *in vitro* methods that would be sufficiently robust to re-

place *in vivo* animal testing. It has been recognized that one way to anticipate acute toxicity was by considering metabolic pathways. Therefore, the aim of the present work was to evaluate the ability of the Solidus biochips to predict metabolism-mediated toxicity. Such a technology, integrating a cell-based (Datachip) and enzymatic systems (Metachip), provides mechanistically-based information; which is supportive of the European ongoing goal and the ToxCast program.

50 chemicals representative of the industry portfolio have been used for this purpose. Results obtained demonstrated the ability of the chip technology to i) detect both Phase I and Phase II enzyme effects on cytotoxicity and ii) predict animal toxicity with sensitivity and specificity values over 85% by using an LD_{50} threshold of 500 mg/kg. This is an exploratory work, and further investigations, such as assessing the relevance of such an approach in an Integrated Testing Strategy (ITS) workflow, are still in progress.

ID ABS: 136

Towards a new preditive method in toxicology? Case study with a multiparameter, cell-based *in vitro* model

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Current legislation constraints (7th Amendment to the Cosmetics Directive and REACH) and increasing societal concerns for animal welfare have made the industry enter a new phase in its innovation and R&D process. The challenge is to move from a descriptive to a predictive toxicology, implying the development and use of non-animal alternatives. Such a challenge is huge, especially in the area of systemic toxicity, as developing an alternative requires the prediction of numerous and complex biological processes. The aim of the present work was to evaluate a multiparameter, cell-based *in vitro* system for predicting rat acute systemic toxicity. For this purpose, a set of 76 non-proprietary chemicals pertaining to different chemical categories were tested using the Ceetox panel® and algorithm developed for the estimation of the LD_{50} value. Predictive performances of the technology for LD_{50} thresholds ranging from 100 mg/kg to 2,000 mg/kg were calculated. Results obtained demonstrated the ability of such an approach to shed light on potential subcellular targets and also to identify which of the categories defined by the Globally Harmonized System (GSH) were correctly predicted. We showed that chemicals falling into the "non-toxic category" (LD_{50} >2,000 mg/kg) were identified with a sensitivity and a specificity of 91% and 78%. At this stage, the Ceetox approach could be used not to predict the extent and nature of all possible *in vivo* toxic effects but rather to estimate the risk of failure if a new chemical entity was to be evaluated with conventional long term *in vivo* studies.

ID ABS: 158

Culture, characterization, and *in vitro* toxicity of arsenic in mouse primary uroepithelial cells

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The purpose of this study was to develop a novel *in vitro* mouse uroepithelial cell culture to improve cancer risk assessment for arsenic, including use of genomic data for understanding mode of action, and cellular exposures for potential quantitative and innovative dose-response assessment. Female mice 56-60 days old (C57BL/6J) were used to acquire primary bladder epithelial cells. Bladders were surgically excised and rinsed with keratinocyte-SFM medium (37°C). Epithelial cells were isolated by making a longitudinal incision to expose the interior and then gently scraping to release cells. Bladder cells from 50 mice were pooled, centrifuged, and washed with keratinocyte-SFM to remove debris. Total cell yield was approximately 0.5 x 106 cells per bladder and viability was >80%. RT-PCR in the cell pellet confirmed a high expression of Keratin-10, a marker of epithelial cells. Cells were seeded at approximately 0.4 x 106 per well into collagen-coated, 24-well inserts. After acclimation of cells through growth and maintenance, cells were treated for 24 hr with 0, 0.1, and 0.5 μM arsenite, plus combination arsenite (As) and dimethylarsinic acid (DMA) exposures at (1.6 μM As + 0.7 μM DMA), (3 μM As + 3 μM DMA), and (5 μM As + 10 μM DMA). Cell viability analysis showed a concentration-dependant decrease in ATP relative to controls from approximately 80% at (1.6 μM As + 0.7 μM DMA) to less than 40% at (5 μM As + 10 μM DMA). This cell model may be useful in assessing arsenic toxicity and improving risk assessment.

ID ABS: 191

Effects of furan, naturally occuring food contaminant, on endocrine glands in male rats at puberty

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Furan (C_4H_4O) occurs naturally in a number of foods that undergo heat treatment, such as canned or jarred foods, and is a volatile and colourless liquid. It is also used in some processes of chemical manufacturing industry. Furan can be formed by thermal degredation of carbohydrates, ascorbic acid or unsaturated fatty acids. This chemical is carcinogenic in mice and rats. In this study, furan was administered orally to 3-4 week old male rats for 90 days by dissolving it in corn oil at doses of 2, 4 and 8 mg/kg/day. In total there were 5 groups including control group, vehicle control (corn oil) group and treatment groups; each group consisted 8 animals. At the end of the experiment, endocrine glands, such as pancreas, adrenal gland, thyroid and testes, were investigated morphologically and histopathologically. While furan had no adverse effects on thyroid glands, we found some changes in adrenal glands, testes and pancreas.

ID ABS: 209

Estimation of acute oral toxicity using the NOAEL values from the 28-day repeated dose toxicity studies

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The 7th Amendment to the Cosmetics Directive (76/768/EEC) introduced in 2009 a testing and marketing ban of cosmetic products with ingredients tested on animals for several human health effects. Acute systemic toxicity is one of the endpoints of concern. Although considerable effort is dedicated to the development and validation of non-animal alternatives for acute toxicity, it is unlikely that validated and regulatory accepted methods and/or strategies will be available soon.

Recently, the European Centre for Validation of Alternative Methods (ECVAM) proposed an approach to identify compounds with LD_{50} >2000 mg/kg ("not classified" according to EU classification) using information from 28 days repeated dose toxicity studies (Bulgheroni et al., 2009). Taking into account the high (87%) prevalence of substances with LD_{50} > 2000 mg/kg in the New Chemicals Database, it was possible to set a threshold of NOAEL >200 mg/kg that allowed the correct identification of 63% of not classified compounds, while only for less then 1% of harmful compounds the acute toxicity would be underestimated. Since *in vivo* data from repeated dose toxicity studies can be used for hazard assessment of cosmetic ingredients until 2013, the proposed approach could have an immediate impact for the testing of these products in Europe.

Reference

Bulgheroni, A. et al. (2009). *Regul. Toxicol. Pharmacol. 53*, 16-19.

ID ABS: 210

Preliminary results from the ECVAM follow-up validation study on the predictive capacity of the 3T3 neutral red uptake assay

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To address the urgent demands for validated alternatives to animal testing laid down by the 7th Amendment of the Cosmetics Directive (76/768/EEC) and the new chemicals regulation REACH, the European Centre for the Validation of Alternative Methods (ECVAM) initiated several projects to replace or reduce animal testing for systemic acute oral toxicity. Taking into account the high (~87%) prevalence of substances with acute oral LD_{50} >2000 mg/kg in the New Chemicals Database (Bulgheroni et al., 2009), ECVAM commissioned in 2008 a follow-up of the international validation study of the 3T3 Neutral Red Uptake (3T3 NRU) cytotoxicity assay (Anon, 2006) with the objective of demonstrating the capacity of the assay to discriminate between toxic/hazardous (LD_{50} <2000 mg/kg) and not classified (LD_{50} >mg/kg) substances. 57 coded industrial chemicals (including 60% cosmetic ingredients) have been tested in three independent laboratories using slightly different protocols. The Health and Safety Laboratory (UK) performed the test using the validated manual procedure. In parallel, an automated version of the validated protocol was established on the robotic platform at the Institute for Health and Consumer Protection (E. C. Joint Research Centre, Italy). Finally, the Institute for *In Vitro* Sciences (US) assessed an abbreviated version of the protocol, which is less costly and more industry-friendly. The preliminary results of the validation study will be summarized.

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ID ABS: 211

Investigation of kidney toxicity in benzyl benzoate treated male rats

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Benzyl benzoate (C6H5CO2CH2C6H5), which is widely used in cough syrup, cinnamon oil, pharmaceutical industries and chewing gums is a brown component of Peru and Tolu balsams. Also, it is used for topical treatment of scrabies and it is reported that extract from sweet gum resin, containing benzyl benzoate, inhibits the function of Angiotensin-II.

In this 90 days sub-chronic toxicological study, male rats were treated with two doses of benzyl benzoate (25 mg/kg bw/day and 100 mg/kg bw/day) in accordance with the ADI dose (0-5 mg/kg bw). The kidney tissue was removed and investigated for growth factor (transforming growth factor beta), extracellular matrix component (fibronectin) and for kidney microenvironment components (Matrix metalloprotease-2 and tissue inhibitor of metalloproteinases-2) by immunohistochemistry. Additionally, for calculating glomerular volume of kidney, the average diameter of glomeruli and thickness of renal cortex were measured. There were differences in measurements between control and treatment groups. Region dependent changes in immunolocalization of fibronectin, matrix metalloprotease-2 and tissue inhibitor of metalloproteinases-2 were determined.

ID ABS: 318

Biological reactivity and cytotoxicity evaluation of pharmaceutical materials

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Samples of polymeric materials, plastics, and medical devices were evaluated to determine their potential biocompatibility in contact with physiological systems. The biological reactivity test was performed by agar diffusion using the mammalian fibroblast cell cultures (ATCC cell line CCL 1, NCTC clone 929), with the cell suspension having a density of about 3 x 10^5 cells/mL, and the end-point of lysis or cell death shown by the discharge of neutral red as zone around the specimens. The neutral red uptake cytotoxicity assay, based on the exposure of the NCTC 929 cells to the samples, was performed on a 96 well microtiter plate maintained at 37ºC in a CO_2 incubator for 24 h. The rupture of the cells and the neutral red released were evaluated by the addition of extractant solution and the absorbance was read at 540 nm. The samples were also evaluated by the *in vivo* test in mice, following extraction and systemic injection, observing the mortality. The assay was carried out also by intracutaneous injection in rabbits. The responses observed as reactivity zone, edema, necrosis, erythema or mortality, were used for the correlation between the proposed methods, demonstrating respectively, non-toxic effects for the samples. Moreover, the proposed methods were also applied for the evaluation of natural products and degradation studies of drugs, demonstrating the capability of the *in vitro* cytotoxicity test. The agreement found between the *in vitro* and *in vivo* responses contributes to the establishment of valid alternatives for the toxicity and reactivity evaluation of pharmaceuticals.

ID ABS: 369

Effects of prenatal dihexyl and dicyclohexyl phthalate exposure on female reproductive tract development of Wistar rats

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This study was performed to determine the effects of maternal DHP and DCHP exposure on female reproductive tract of Wistar rats at various doses and on different developmental stages. Dams were dosed daily by gavage at gestation days (GD) 6-19. The groups were as follows: Control, vehicle control (corn oil), 20, 100, 500 mg/kg/day DHP and 20, 100 and 500 mg/kg/day DCHP. The effects of DCHP and DHP on female reproductive tract were examined at prepubertal (post-natal day (PND) 21), pubertal (PND32) and adult (PND90) stages. The serum samples were analyzed for hormones (estradiol, FSH and LH). Ovaries and uterus were investigated histopathologically. Estradiol levels of prepubertal females increased in DHP treated groups compared to control groups. In the 100 mg/mg/day DHP, 20 and 100 mg/kg/day DCHP treated groups, the levels of estradiol increased compared to control groups. In the 100 mg/kg/day DHP group, LH levels of pubertal female rats increased compared to controls. In the 500 mg/kg/day DHP and 20 mg/kg/day DCHP groups, the estradiol levels of adult female rats increased compared to control groups. LH levels of adult female rats increased in 20 and 500 mg/kg/day DHP treated groups compared to control groups. Histopathological analysis showed that there were polyovular follicles, irregular shaped oocytes and picnotic granulosa cells in antral antrum in the ovaries of prepubertal, pubertal and adult females in DHP and DCHP treated groups. The findings of this study indicate that prenatal exposure to DHP and DCHP may cause adverse effects on the development of the female reproductive system.

ID ABS: 396

Development of an *ex vivo* human gut mucosal challenge model to replace animal testing

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Collaboration between the Rowett Institute of Nutrition and Health and Biopta Ltd. was initiated to develop a functional *ex vivo* human colon tissue explant culture model. The intention is to develop a human gut test system that replaces animal tests of functional biological and pharmacological responses of colon tissue and that permits investigation of colon pathologies. Preliminary studies have established procedures for removal of the entire intact colonic epithelium and explant culture using serum based media formulations; maintenance of mucosal microarchitecture over a 4 hour culture period; extraction of total RNA of sufficient quality and yield for application of novel GenomeLab System (Beckman) gene expression profiling studies. Application of a custom designed colon cancer gene GenomeLab multiplex assay has been applied to cultured colon explants to monitor gene expression. Diseased explants have been cultured to further develop non-animal alternatives for *in vitro* pharmacology and study of colon pathologies, such as irritable bowel disease (IBD) and cancer. The model provides a tissue alternative that closely approximates *in vivo* responses in human tissue and offers sufficient throughput to be used for the screening of potential new drugs. It is considered that during the process of developing the model opportunities will be presented to gain insights into basic colon biology and physiology. The principles involved may also have further applications in the development of *ex vivo* models of similar tissue systems elsewhere in the body.

ID ABS: 433

The effects of Aloe vera extract on male reproductive tract of Swiss albino mice

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This study was performed to determine the effects of Aloe vera extract (AVE), which is a commercially available product marketed for human use, on the male reproductive tract of Swiss albino mice. Male mice aged 4 weeks separated into four groups randomly received AVE by gavage daily for 28 days. The groups were as follows (n=5): control, x0.5 dose group received half the recommended AVE dose, x1 dose group received the recommended AVG dose, and x2 dose group received twice the recommended AVE dose. LH levels of mice in the treatment groups decreased compared to control. Testosterone levels decreased in x2 dose group compared to control. Sperm head count in treatment groups did not change compared to control, sperm shape abnormalities in x1 and x2 dose group increased compared to control. Histopathological analysis showed that there were atrophic tubules, germ cell debris, picnotic cells and Sertoli cell vacuolization in testes of x1 and x2 dose groups. Moreover, there were atrophic tubules, prostatic intraepithelial neoplasm and mononuclear cell infiltration in prostate glands of the treatment groups. There were no changes in malondialdehyde (MDA) and reduced glutathione (GSH) levels of testes among groups. The findings of this study indicate that exposure of AVE may cause adverse effects on the male reproductive tract of mice by affecting the secretion of reproductive hormones. The results of the study also suggest that the mechanism of the AVE induced damage on male reproductive tract is independent of oxidative stress.

ID ABS: 441

The use of a long shelf-life *in vitro* cell model of the human airway epithelium (MucilAir™) for inhalation toxicity assessment

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

MucilAir™ is morphologically and functionally differentiated, and it can be maintained at a homeostatic state for more than one year. The typical ultra-structures of the human airway epithelium, such as the tight junctions, the cilia, the basal cells, and the mucous cells can be observed. The epithelium is electrically tight (TEER ~ 450 ohms.cm^2). The ion channels are fully functional and respond normally to their specific inhibitors and activators. Moreover, the epithelial cells react to pro-inflammatory mediators in a physiological manner. Remarkably, the epithelium has a strong capacity of regeneration after mechanical or chemical injuries. Epithelia from several different pathologies can be reconstructed (e.g. Asthma, COPD, CF, smoker, etc.).

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

1)Acute, Long-Term and Chronic Toxicity testing

2)Inflammatory effect assessment

3)Progress in the detection of airway sensitization

ID ABS: 454

Criticism of the Limulus amebocyte lysate (LAL) test as a final alternative method to the *in vivo* rabbit pyrogen test (RPT) to detect endotoxins

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This work is part of the project "Replacement Strategies for research and classes with animals" and intends to debate the acceptance of the Limulus amebocyte lysate (LAL) test as a definitive, although still important, alternative method to the *in vivo* rabbit pyrogen test (RPT) in detecting endotoxins. There is evidence that the extraction of hemolymph from *Limulus polyphemus* (horseshoe crabs) causes the death of about 30,000 crabs per year in the United States (Hoffmann et al., 2005). That identifies the biomedical industry as one of the main causes of death of that arthropod (Walls and Berkson, 2000; Hurton and Berkson, 2006). Added to that is our ignorance of the degree to which the phylogenetic scale of consciousness goes (Searle, 1998). Therefore, the inexistence of consciousness of damaging stimuli in those animals is unknown. That is why the LAL should also be replaced, and five tests are already validated for the detection of pyrogens instead of the RPT, which are based on measurement of proinflammatory cytokines (interleukin [IL]-1β, IL-6, tumor necrosis factor-α) (Presgrave, 2003; Barth et al., 2007). Four of those five tests use human blood. There are several ethical advantages in using human blood, since human donors must be able to understand the research and give their consent to harmless donation. Since *in vitro* tests with human blood are potential final replacement methods with no ethical consequences, more studies for catch-up validation of those testes in other products should be conducted and encouraged to be later incorporated to pharmacopoeias.

ID ABS: 548

Ultrastructure alterations in the skeletal muscle after the injection of an analgesic non-steroidal antiinflammatory drug (NSAID)

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An experimental study with the aim to investigate by electronic microscopy the ultrastructural changes of the muscle gastrocnemio after the intramuscular injection of a non-steroidal analgesic anti-inflammatory drug. There were selected 3 experimental groups with 8 male mice, NIH RR, 25-27 g, and sodium diclofenac was given the first one intramuscular, to the rest of the groups there was given in similar conditions distilled water (control vehicle) and acetic acid 6 % (as positive control), respectively. By means of surgical ablation 2 mm^2 of the muscle gastrocnemio of the rodents at 48 and 96 hours was taken after the intramuscular administration. The samples surrendered to routine processing of thin cut for transmission electronic microscopy. They were fixed with Karnovsky solution in buffer phosphate of Millonig pH 7.4 and 320 mOsm and post-fixed with OsO_4 under the same conditions of pH and osmolarity. Then they were dehydrated in increasing concentrations of ethanol and incorporated in epoxy resin. The thin cuts, 60-90 nm, were obtained by an ultramicrotome Porter-Blum MT2-B. The grids were examined in a transmission electronic microscope (JEM-1011 80 kV). The ultrastructural analysis showed that the sodium diclofenac treatment produced damage in the contractile elements of the muscle fiber, muscle atrophies and destruction of the capillary, similar to the one of glacial acetic acid a 6%. Such results are compatible with the response of the skeletal muscle to the damage.

ID ABS: 549

Levels creatine kinase (CK) after the intramuscular injection of a non-steroidal antiinflammatory analgetics (NSAID) and antibacterial drugs

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An experimental study was carried out in male mice, 25-27 g, per group of the National Institutes of Health, proceeding from the Bioterio of the INH RR, food and water were given ad libitum, temperature of 24°C, humidity of 61.3%, period of light/dark of 12 hour with the aim to determine the levels serum CK after the intramuscular injection of diclofenac sodium, ketoprofeno, penicillin G benzatinica and ceftriaxona.The serum enzyme activities were determined 24, 48, 72 and 96 hours after the injection, were measured using a commercially available Kit CK – NAC UV unitest and AA made by Laboratories Wiener lab and a BT3000 Plus Spectrophotometer. The observation that the levels of CK serum increase after the intramuscular administration of the different solutions of the drugs studied, would indicate their capacity to damage muscular tissue. The levels of CK serum increased in 85% in the group treated with a solution of diclofenac sodium $p < 0.05$ which indicates that this drug probably causes severe muscular injury upon intramuscular administration; on the other hand the antibacterial drugs increased the levels of CK serum at 24 hour $p < 0.05$ returning to normal values at 96 hr after the intramuscular administration $p > 0.05$, indicating that the muscular injury is a product of the trauma caused by the injection of the drugs. This study will allow formulating changes in the evaluation of intramuscularly applied drugs in order to rationalize the requirements needed by the regulatory authorities.

PO24: Genetics and carcinogenicity

ID ABS: 108

Cell transformation assays – past – present – future

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Carcinogenesis has been shown to be a multistep process, which involves sequential genetic alterations in a single target cell, which cause subtle alterations in growth control and culminate in cells that are able to form malignant tumors. There is accumulating evidence that at least some of the rodent cell transformation assays exhibit sensitivities and specificities for predicting chemical carcinogenicity, which are comparable to, or better than, those shown by several of the established genotoxicity tests.

Rodent cell transformation assays involve either finite lifespan cells such as the SHE (Syrian Hamster Embryo) cell assay, or immortalised fibroblast cell lines such as the Balb/c 3T3 or C3H/10 T1/2. Morphological transformation of cell colonies and focus formation on a monolayer are the most commonly used endpoints, regardless of the type of system used.

Promising results were obtained related to the in the inter- and intralaboratory comparison and the predictivity of the SHE and the Balb/3T3 system at an international validation study organised by ECVAM, and also a retrospective analysis of published data indicates a high sensitivity, specificity and overall concordance of the Balb/c 3T3 and the SHE assay protocol.

An ideal transformation assay would be one which utilises human cells and avoids the subjectivity in scoring of the transformed phenotype by using image analysis or biochemical and molecular biological markers.

ID ABS: 204

In vivo Comet assay: update on the on-going international validation study

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The *in vivo* rodent alkaline Comet assay is frequently used worldwide for detecting genotoxic chemicals, and is expected to be recommended in the revised ICH-S2 guidance document as the second *in vivo* genotoxicity test to use when testing pharmaceuticals. The assay, however, has not been formally evaluated for its reliability and relevance. Thus, JaCVAM (the Japanese Center for the Validation of Alternative Methods) has been coordinating an international validation study to evaluate the *in vivo* rodent alkaline Comet assay as a potential predictor of genotoxic carcinogens and as an alternative to the currently recommended *in vivo* rat unscheduled DNA synthesis (UDS) assay. The validation study consists of several phases, with initial phases to optimize the protocol and to ensure transferability of the protocol between laboratories. The current version of the protocol involves 3 treatments (0, 24, and 45 h) before collecting liver and glandular stomach tissue samples at 48 h after the first treatment. This protocol allows for the detection of DNA damage using the Comet assay and of micronuclei in blood erythrocytes using the standard micronucleus assay in the same animals. Phase IV of the validation study consists of multiple laboratories in several countries testing compounds representing the range of genotoxic mechanisms and physical-chemical properties.

ID ABS: 401

Bayesian weight-of-evidence for multiple tests: assessing rodent carcinogenicity potential from *in vitro* genotoxicity test batteries test case

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There is a need to develop objective methods for interpreting results from *in vitro* test batteries for making an inference about an *in vivo* endpoint and optimizing their overall predictivity. We develop a formal Weight of Evidence framework for a test battery based on Bayesian inference. This approach allows for a science-based, fully transparent consensus building when heterogeneous or conflicting data is provided and formally generates the probability that a chemical is, or is not, active based on a specific battery outcome. To illustrate the approach, we interpret results from 3 genotoxicity *in vitro* tests: the Ames Test (A), the Mouse Lymphoma Assay (MLA) and Chromosomal Aberrations Test (CA) to assess carcinogenicity potential. We develop a quantitative measure of evidence that allows for comparing utility of different tests evaluating the consequences of adding new results to a battery. Reduction in the certainty of the battery outcome due to conditional dependence between tests is demonstrated.

ID ABS: 423

International validation study of the *in vitro* alkaline Comet assay

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The Comet assay has been widely used for detecting initial DNA damage in individual cells. The *in vitro* Comet assay, especially, could be an alternative for other genotoxicity tests, because it can simply identify the genotoxic hazard of chemicals. The performance of the assay is, however, dependant on the technique applied, and the results vary greatly. We have now organized an international corroborative study for the *in vitro* Comet assay, supported by JaCVAM, and established a robust protocol of the *in vitro* Comet assay and validated its capacity. We treated TK6 human lymphoblastoid cells with a chemical for 4 h with or without S9, and then conducted the Comet assay according to the JaCVAM's *in vivo* Comet assay protocol. As cytotoxic parameters, trypan blue dye exclusion (TBDE), non-detectable cell nuclei (NDCN; hedgehog), and cell growth after the treatment were measured. The recommended top concentration is one with 80% TBDE, 20% NDCN, or no cell growth. Until now, 5 laboratories examined 11 genotoxic or non-genotoxic chemicals. Overall, the *in vitro* Comet assay without S9 could appropriately yield positive response for genotoxic chemicals (EMS, 9-Aminoacridine, Camptothecin, Etoposide) except for a cross-linking agent (MMC), and negative for non-genotoxic chemicals (Cyclohexamide, Triton-X, Mannitol). However, S9 worked poorly for genotoxic chemicals requiring metabolic activation in the Comet assay (2-Aminoanthracene, Cyclophosphamide, DEN), although their cytotoxicity was highly induced in the S9 condition. Optimization of the protocol for the S9 condition is needed.

ID ABS: 428

Bhas 42 cell transformation assay to detect carcinogens using 96-well plates and a robotic system for high-throughput screening

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High-throughput screening technology using multiwells and a robotic system are widely adopted to discover a new drug and detect toxicity. Here, we report an application of this technology to the Bhas 42 cell (v-Ha-ras transfected BALB/3T3 cells) transformation assay to detect possible carcinogens. The system consists of a control unit by PC and a clean bench unit including plate transfer apparatus, liquid dispensers and aspirators, and provides a series of the operation of transformation assay (plating cells, treating with chemicals, changing medium, fixing and staining cells). When we treated the cells with 3-methylcholanthrene (0.1-1 μg/ml) as an initiator and 12-O-tetradecanoylphorbol-13-acetate (0.03-0.3 μg/ml) as a promoter, transformation frequencies by this system were similar to those found by the manual method. The present study demonstrates the feasibility of high-throughput screening for *in vitro* carcinogenicity testing.

This research was conducted on a New Energy and Industrial Technology Development Organization (NEDO) project addressing "Chemical Substance Management (METI)".

PO25: Reproduction, development and fertility

ID ABS: 36

Evaluation of the embryotoxicity of alloys containing indium with whole-embryo culture and embryonic stem cell test

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The results of animal experiments have indicated the embryotoxicity of indium compounds (Nakajima et al., 2000). We examined the embryotoxicity risk of the alloy of Ag-20%. A developmental toxicity test was conducted using the rat whole-embryo culture method (WEC) and embryonic stem cell test (EST). The test results showed that there was no significant difference in yolk-sac diameter, crown-rump length, head length, number of somites or protein concentrations upon exposure compared to the control group. Therefore, there were no apparent abnormalities in the embryos. However, an examination of the embryotoxicity level of each extract using ES-D3 cells demonstrated the possibility of embryotoxicity for silver-20-%indium alloys. These results differ from the report of the animal experiment on the indium compound, and demonstrate that the alloys containing indium examined here carry a very low embryotoxicity risk.

ID ABS: 93

A first interlaboratory trial of the Hen's Egg Micronucleus (HET-MN) assay as a potential replacement for the *in vivo* Micronucleus Test (MNT)

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Existing *in vitro* genotoxicity tests exhibit very low specificity. Therefore, *in vivo* assays must often be performed to clarify the biological relevance of the *in vitro* experiments. However, due to the EU 7th Amendment to the Cosmetic Directive *in vivo* tests for cosmetic ingredients are banned since March 2009. In addition, due to the new chemical legislation REACH the number of *in vivo* mutagenicity assays will increase by an estimated factor of 3 (Van der Jagt et al., 2004).

Here we investigate whether the Hen's Egg Micronucleus (HET-MN) has the potential to serve as a replacement method for the *in vivo* MNT. The developing egg has been studied for a long time as an alternative to animal tests (Wolf et al., 1997, 2002, 2003, 2008). Since it is not protected by a maternal organism, the egg must already be competent in metabolising xenobiotics, like (pre)mutagens.

In order to investigate the predictability of the test system, the mutagenic potential of ten compounds was tested in two independent laboratories: non-mutagens: isopropylmyristate, ampicillin, L ephedrin; mutagens: p-chloroanilin, malathion, cyclophosphamide, dimethylbenzanthracene; presumably false-positive: isophorone, dichlorophenol; aneugen: carbendazim.

The mutagenic, non-mutagenic compounds and the aneugen were correctly predicted by the HET-MN. The presumably false-positive compounds were evaluated as non-mutagens in both laboratories.

The results are in line with *in vivo* MNT results. Their relevance should be supported by testing additional compounds. In summary, the HET-MN is a promising candidate for a prevalidation and might aid in the replacement of *in vivo* testing.

ID ABS: 203

Adequate conditions for performance of Comet assay using 3-dimensional human epidermal model

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To evaluate a risk assessment of genotoxicity when human skin is affected by chemicals, we tried to establish adequate conditions for the Comet assay using a 3-dimensional human epidermal model (LabCyte EPI-MODEL, Japan).

Mitomycin C, methylmethanesulfonate (MMS) and 4-NQO (4-Nitroquinoline 1-Oxide) were utilized as test chemicals. Each test chemical solution was applied directly to the surface of the models and incubated for 4 h, then washed off and the models were incubated for a further 20 hours. Maximal dosage was calculated according to the cytotoxicity. As to the Comet assay, each test chemical solution refering to the cytotoxicity was applied and incubated for 4 h.

1) Cells were detached using Liberase solution (Roche) or Trypsin solution (GIBCO), and an adequate cell suspension was obtained.

2) The three cytotoxicity tests used for the studies were:

a) Trypan blue dye exclusion test (TBDE) directly after the treatment

b) Counting non-detectable cell nuclei (NDCN; hedgehog)

c) Relative cell growth for 24 h after the treatment

More single cells could be efficiently retrieved using Trypsin solution, especially with treatment for 25 min, than using Liberase solution. Under relative cell growth for 24 h after the treatment, Comet signals mediated by Mitomycin C (150 μM) and 4-NQO were shown. However, MMS had no clear effect.

We established a practical, rapid and easy Comet assay protocol using a 3-dimensional human epidermal model.

ID ABS: 335

Development and characterization of a totipotent stem cell line in goat

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Reproductive cloning facilitates conservation and multiplication of rare and high quality animals. However this technique yields very few viable embryos and on transfer to the recipient leads to disorders like epigenetic modifications and dystocia. Blastomere cloning is an alternative, but the cell availability is limited. Totipotent stem cells (TSC) derived from the pre-compact morula (8-16 cell) are a possible solution. These TSC could be used in reproductive cloning, to multiply a specific animal to identical multiples. Therefore a study was undertaken to develop TSC from pre-compact morula in goat and to characterize them with stem cell markers. Embryos were developed by IVMFC of 559 goat oocytes of slaughter house origin. The presumptive zygotes were cultured in mSOF; 61 oocytes cleaved and 20 embryos developed to pre-compact morula. These morulae were made zona free by pronase treatment. Blastomere clumps were then cultured in CR11 media with LIF on a mitomycin-C inactivated fetal fibroblast monolayer. On the 30th day of culture a few cells were isolated, fixed on slides and used for characterization using Alkaline Phosphatase, Stage Specific Embryonic Antigen-1. Pluripotent stem cells (PSC) from an early fetus acted as control. 80% of the blastomeres divided and formed TSC colonies. As the colonies grew, cells were detached from the original colonies and formed fresh colonies. However, marker intensity in TSC was much lower compared to the control. It may be concluded that TSC can be developed in culture, and a specific TSC marker must be developed as PSC markers do not work properly on these cells

ID ABS: 370

Development of a predictive assay of developmental toxicity with fish medaka (*Oryzias latipes*) early life stages

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To identify teratogenic chemicals for mammals (including humans), we developed an assay with medaka (*Oryzias latipes*) early life stages (from blastula to eleutheroembryo stage), before they reach the larval stage protected by EU regulation (Dir 86/609/EEC).

The test sensitivity was evaluated with 26 reference chemicals known to be teratogenic and 22 known to be non-teratogenic for mammals. Medaka embryos were exposed for 9 days (26°C) with a semi-static exposure regime (test solution renewal 3 times/week). At day 9, for each chemical, LC_{50}, EC_{50} (concentration causing abnormalities in 50% of the exposed organisms) and the teratogenic index (TI = LC_{50}/EC_{50}) were calculated. If TI >2.4, the substance was found to be embryotoxic for the medaka and is highly suspected of being teratogenic for mammals.

With this set of 48 reference chemicals, the sensitivity of the test reached 69% (identification of more than 2/3 of the mammal teratogenic chemicals) with a 100% specificity (no false teratogenic chemical identification).

We are working at increasing the assay sensitivity by adding behavioral parameters (spontaneous and triggered activity) and by identifying protein biomarkers thanks to differential DIGE-based proteomics studies. We conclude that this alternative method has the potential to be included in a coherent testing strategy for the identification of human teratogenic chemicals.

ID ABS: 384

Development of novel short-term tests for reproductive and developmental toxicity

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The aim of this project is to develop novel, short-term tests for reproductive and developmental toxicity using murine embryonic stem cells (ES cells) and whole embryo culture (WEC).
(1) ES cell study: In DNA microarray analysis of ES cells during differentiation into cardiomyocytes, genes which were substantially up-regulated during the differentiation process were isolated as candidate marker genes. 13 genes showed marked differences in expression in the teratogen in comparison to the

non-teratogen-treated groups, suggesting that these genes may be useful markers for predicting embryotoxicity. We have developed stable transgenic ES cells to detect the chemical-dependent changes in the candidate genes easily and conveniently.
(2) WEC study: We have tried to develop a novel WEC method with metabolic activation of chemicals using rat S9-mix. Effects of ten chemicals on cultured embryos were examined with or without S9-mix. The S9-mix addition enhanced the test chemical-dependent adverse effects on the cultured embryos. These data suggest that the novel WEC method with metabolic system might be a better test method than the existing ones to detect embryo abnormality caused by both the test chemical and its metabolites. We have also developed new equipment to reduce serum volume in the WEC method.

This project is supported by a research grant from the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

ID ABS: 385

The effect of chemical compound on cultured rat embryos in S-9mix

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We are attempting an improvement to optimize the embryo culture method as an alternative developmental toxicity test. In the present study, we investigated the effect of a chemical compound on cultured embryos in S-9mix.

Rat embryos at 11.5 days of embryonic age were cultured for 48 hours, and 10 μg/ml of imipramine was administered from 2 h after culture until the completion of culture for comparison of fetal abnormality between the S-9mix group and no-S-9mix group. The rat S-9mix concentration in the culture medium was 3% in all tests.

For the effect of imipramine on cultured fetuses, deformity rates were 67% in the S-9mix group and 54% in the no-S-9mix group after 48 hours of culture. Types of deformity included cleft lip, short tail and abnormal maxillary formation in the no-S-9mix group, while deformity in the S-9mix group included many cases of fetal atrophy, hematoma and edema; each group had different types of abnormalities. The difference in types of abnormality seemed to be due to a difference in types of abnormality induced in the cultured embryos between imipramine and its metabolite desipramine.

These findings suggested that an embryo culture method combining a metabolic system may be a test method more likely to be able to confirm abnormality due to a chemical as well as its metabolites than the existing embryo culture methods.

This work was supported by the grant P06040 from the New Energy and Industrial Technology Development Organization (NEDO).

ID ABS: 394

Cellular mechanism underlying embryo-maternal relationship in intraspecific and interspecific pregnancy

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Mouse and vole embryos were transferred into pseudopregnant CD-1 and immunodeficient (scid) female mice. Cellular changes involved in the formation of decidua in the pregnant mouse uterus up to day 8 of pregnancy were examined by histological, electron microscopic, and histochemical techniques. On day 6 of pregnancy, the vole embryos were laid in the interstitium of the antimesometrial side of the uterus, as in intraspecific pregnancy. Compared with the intraspecific pregnant mouse, blood vessels were numerous in the decidua around the vole embryos in interspecific pregnancy. Both distribution and dilation of the blood vessels were increased on day 8. A part of cells in the inner cell mass had no nuclei, suggesting damaged vole embryos on day 8. At the implantation site, the uterine decidua was invaded by extravillous trophoblast (EVT) cells, whose function it is to destroy the walls of the uterine spiral in order to provide an adequate blood flow to the fetus. Moreover, the decidua was infiltrated by a population of natural killer (NK) cells and macrophages. These cells were particularly numerous in the decidua basalis at the implantation site, where they came into close contact with invading EVT cells. These results suggest that interaction between NK, macrophage and EVT provides the controlling embryo-maternal relationship in intraspecific and interspecific pregnancy.

ID ABS: 414

Effects of pyrazinamide and disulfiram on reproductive health in Wistar rats

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Drugs work through active pharmaceutical ingredients that are absorbed in the mammalian body. This has raised concerns about potential impacts on people, animals and the environment, e.g. contribution to antibiotic resistance, effects of highly potent drugs on environmental organisms. In spite of broad utilization of pyrazinamide in tuberculosis and AIDS treatment schemes its effects on reproductive function and posterity remain insufficiently investigated and present results are discrepant.

Effects of pyrazinamide (500 mg/kg b.w., 1000 mg/kg b.w., 2000 mg/kg b.w.) and disulfiram (30 mg/kg) on liver and testis biochemical parameters and reproductive toxicity indices were studied in experiments on Wistar rats.

It was shown that pyrazinamide caused a dose dependent increase of p-nitrophenol hydroxylase activity (from 1.4 to 2.3 times) and NADPH-dependent lipid peroxidation (to 86%) in the liver microsomal fraction. Use of the CYP 450 2E1 inhibitor disulfiram prevented p-nitrophenol hydroxylase activation, the increase of total CYP 450 2E1 and the rate of NADPH-dependent formation of TBA-products. Pyrazinamide administration produced a decrease in the spermatozoid number in epididimys in dependence of dose. Decrease of number of spermatogonia and compensatory increase of 12-th meiosis stages with synchronous dystrophic changes of spermatogenic epithelia were also registered under these conditions. Disulfiram introduction with pyrazinamide produced three times decreased meiotic activity of spermatocytes, but did not influence degenerative changes of spermatogenic cells. Therefore pyrazinamide caused a dose dependent increase of preimplantational embryo death and postnatal fetal death in different terms, disulfiram introduction lowered the negative effect of pyrazinamide on parameters of preimplantational embryo death and postnatal fetal death with a simultaneous increase of postnatal vitality.

ID ABS: 415

The zebrafish embryo: an alternative model to screen for neurodevelopmental toxicity and teratogenicity of chemicals and drugs

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The development of novel alternatives for neurodevelopmental toxicity and teratogenicity in order to reduce animal experiments is gaining great interest. The purpose of our research project is to investigate zebrafish embryos and larvae as a simple and fast high-throughput system to predict potential morphological and neural disorders during early development.

Teratogenic and neurotoxic compounds have been selected, nd they are evaluated using in-house standard protocols. The teratogenic assay includes the time-related evaluation of morphological endpoints like heart beat, tail detachment, formation of somites, otoliths, eyes, spinal cord, etc. at embryonic and larval stages up to 144 h post fertilization (hpf). Spontaneous tail coiling (24-26 hpf) and swimming (120 and 144 hpf), selected as measures for neurobehavioral disorders, are analyzed with the appropriate software packages as part of the neurotoxic assay for egg and larval stages respectively. These procedures are currently applied for a number of compounds (retinoic acid, caffeine, valproic acid, ethanol, chlorpyrifos, acrylamide, lead, mercury, etc), and results allow ranking of chemicals according to their potency.

The validity of the zebrafish test for the screening of chemicals will be discussed and evaluated through comparison with available mammalian *in vivo* and *in vitro* data. Further studies will include the determination of teratogenic and neurotoxic potential of an extended panel of chemicals (up to 20), which then should be used to develop a prediction model for mammalian teratogenic and neurodevelopmental effects.

ID ABS: 429

Toxicity of directly observed therapy short course (DOTS) to the male reproductive system

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Metabolism of xenobiotics and its effects on the male's reproductive system is one of the least investigated problems of toxicology. In spite of broad utilization of directly observed therapy short course (DOTS) in tuberculosis treatment its effects on reproductive function and posterity remain insufficiently investigated. Effects of oral administration of pyrazinamide, ethambutol, isoniazid and rifampicine in the DOTS regime on reproductive toxicity indices were studied in Wistar male rats, since the basic mechanisms underlying male reproductive function in the rat are well researched and reasonably representative of those in man. It was shown that using DOTS during the period of spermatogenesis caused a decrease in sperm counts to 35%, as well as decreased fertility of males and intact females, which were coupled with these males accordingly. Vitality of offspring of experimental males and intact females coupled against the background of DOTS was lower, i.e. 4.6%. Similar investigations after 45 days recovery time showed the restoration of spermatozoid production and fertility indices. It was indicated that offspring mortality of males coupled in late periods after treatment was higher than in controls (48.53% and 9.38% correspondingly). These findings support the supposition that simultaneous administration of antituberculosis drugs in the DOTS regime can provoke adverse effects on male reproductive function. Alterations in reproductive endpoints may be the result of direct or indirect toxicity to the male reproductive system. We think it is necessary to carry out a further profound *in vivo* investigation to effectively overcome the unfavourable influence on male reproduction.

ID ABS: 440

Critical factors impacting intralaboratory transferability of the mouse Embryonic Stem Cell Test

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

ID ABS: 449

In vitro tests for endocrine active compounds: performance of MELN assay and steps to validation

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Scientific and societal concerns arose as reproductive dysfunctions in wildlife animals showed a link to elevated levels of persistent pollutants in the environment. Moreover, increased prevalence of human health problems, such as birth defects, can-

cers and decreased fertility, have been attributed to exposure to endocrine active compounds, chemicals suspected of interfering with the normal function of hormones. This topic became a high priority issue for organizations involved in validation of alternative methods, as some reproduction toxicity tests require large numbers of animals. European, Japanese and US Centres for the Validation of Alternative Methods (ECVAM, JaCVAM & ICCVAM) initiated studies to evaluate the usefulness and limitations of *in vitro* test methods which identify estrogen or androgen-like chemicals. Currently 2 estrogen receptor (ER-) and 2 androgen receptor (AR-) gene reporter assays, next to receptor binding assays, are prevalidated within the EU 6thFP project ReProTect, coordinated by ECVAM. VITO is the leading lab for the MELN assay, an ER-transactivation assay, and results for the 1st & 2nd module of the ECVAM validation scheme will be presented. MELN test procedures for estrogenicity by agonistic or antagonistic mode, combined with cytotoxicity evaluation were optimised, and criteria for test performance and acceptance were defined. These procedures were successfully transferred to Bayer Schering Pharma, partner within ReProTect. Good performance and reproducibility for within and in between lab variability were shown. Potency of estrogenic chemicals, derived from calculated effect levels, were in agreement with indicative potency (*in vivo* data), while presumed negative compounds showed no effect.

ID ABS: 617

Mouse embryonic stem cells for testing the role of neuropathy target esterase in cell embryogenesis

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In vivo studies have demonstrated that Neuropathy Target Esterase (NTE) is a protein with a vital and still unknown role during embryogenesis. We studied the gene and enzymatic expression of NTE in D3 embryonic stem cells. D3 cells in monolayer increase NTE gene expression immediately after initiating differentiation, reaching a maximum during the first 24 h, which corresponds with a maximum of NTE enzymatic activity expression around 6 h later. Around 48 h after starting the differentiation both enzymatic and genetic NTE expression drop to basal levels, and they are maintained during at least 22 hours. D3 cells cultured in monolayer expressed a NTE enzymatic activity of 23 nmol phenol/min/mg protein, which corresponds to 1 nmol phenol /min/10^6 cells. Our results suggest that: 1) D3 embryonic stem cells might be an appropriate alternative model for investigating the role of NTE in embryogenesis; 2) the modulation (either through inhibition or over expression) of NTE expressed in this model might be an appropriate approach for reaching this goal.

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

PO26: Disease models

ID ABS: 60

Characterization of pathomechanisms relevant to Alzheimer's disease in a human neuronal model system

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Alzheimer's disease (AD) is a devastating disorder characterized by the progressive decline of cognitive function caused by the degeneration of neurons. The two pathological hallmarks of AD are extracellular "senile plaques", consisting of aggregates of the amyloid β peptide (Aβ), which is enzymatically released from amyloid precursor protein (APP), and intracellular "neurofibrillary tangles", whose main component is the hyperphosphorylated form of the tau protein. In order to model human pathology, dozens of transgenic mouse models have been generated by introduction of human genetic material into mice.

As an alternative approach we have used human neurons directly, differentiated *in vitro* from the conditionally immortalized neuronal cell line LUHMES. Initially we characterized in detail how these cells assume basal neuronal characteristics upon switching off the immortalizing v-myc oncogene. Using immunocytochemistry/-blotting, qPCR, scanning electron microscopy and ELISA, we demonstrated that during five days of differentiation, axonal growth and the formation of an elaborate neurite network take place, and that important pre- and post-synaptic marker proteins are upregulated, localizing to neurite processes. Importantly, the levels of most AD relevant proteins increase and Aβ is secreted into the medium. Pharmacological studies revealed that Aβ formation is efficiently blocked by various inhibitors of the APP-cleaving enzymes at IC_{50} values similar to those found in primary neurons. Furthermore, we showed that treatment with the phosphatase inhibitor okadaic acid leads to hyperphosphorylation of tau and neurite degeneration. Thus, typical AD pathologies known from transgenic mouse models can be studied directly on a human neuronal background with the LUHMES cell system.

ID ABS: 129

Validating animal models: increased human benefit at the price of more animals used?

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Ethical acceptability of animal-based research is widely viewed as dependent on its harm-benefit balance; nevertheless the benefit is rarely systematically assessed. Model validity is one factor determining benefit which can be assessed scientifically. Presently, this is mostly only done informally through a theoretical discussion of in particular the construct validity of models. This is a limitation, particularly in the case of preclinical models used to inform first-in-human trials in pharmacological research. Here predictive validity is crucial to avoid exposing human research subjects to unnecessary trials. A new medicinal compound entering human clinical trials is estimated to have only 8% chance of reaching the market – which is partly explained by low predictive validity of preclinical models. Validating preclinical models in a similar way as presently required for alternative methods in toxicology testing could improve predictive validity. Of the five steps established by ECVAM for alternative methods, animal models presently informally undergo the first two, test development and pre-validation. For full validation, the models should be tested in inter-laboratory blind trials with subsequent independent assessment. This would potentially increase the chance of translating results based on animal research into successful clinical results. However, proceeding to a system of full validation is time consuming and costly and requires the use of animals specifically for the validation. Thus there is a price to be paid in terms of increased animal use – at least in the short run. It is argued that the potential benefits may outweigh the costs.

ID ABS: 183

Development of a 3D *in vitro* model of the inflamed colonic mucosa

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In *in vivo* studies of inflammatory bowel diseases (IBD) and their treatment, intestinal inflammation in rats or mice is often induced by highly irritant hapten2,4,6-trinitrobenzenesulphonic acid (TNBS) or by dextran sodium sulphate (DSS) treatment. Animals develop severe colitis with high weight loss and rectal bleeding, which leads to elevated death rates. As an alternative to this ethically and scientifically questionable *in vivo* approach, we developed an organotypic cell culture model reflecting pathophysiological changes of intestinal mucosa in the state of inflammation.

A triple co-culture model was designed, consisting of human blood monocyte-derived macrophages and dendritic cells, which were embedded within a collagen layer on transwell filter inserts, with intestinal epithelial Caco-2 cells grown atop. After Caco-2 confluency, the system was stimulated with interleukin-1β. Inflammation was quantified via real-time PCR measurements and enzyme immunoassay. Transepithelial electrical resistance (TEER) was monitored, and structural changes in the tight junctions were investigated by immunostaining and confocal microscopy.

In the co-culture, Caco-2 cells formed intact monolayers with tight junctional structures and TEER values comparable to monocultured cells. Stimulation of inflammation by addition of interleukin-1β disturbed tight junctions as shown by a 20–25%

reduction of TEER, a reorganization of tight junctional protein ZO-1 and significantly increased levels of interleukin-8 and TNF-α expression.

Results indicate a successful inflamed 3D model that reflects pathophysiological changes in the inflamed intestine and can now be used as an alternative screening tool for drugs and formulations directed against IBD.

This work was supported by the Sixth Framework Programme Integrated Project MEDITRANS, Contract No.026668.

ID ABS: 197

Kaempherol decreases nitric oxide level in renal ischemia/reperfusion injury

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Nitric oxide (NO), produced by inducible nitric oxide synthase seems to play an important role during renal ischemia/reperfusion (I/R) injury. Bioflavonoids such as kaempherol have antioxidant properties and NO scavenging activity. This study was design to investigate the possible protective effect of kaemperol on I/R induced renal injury. Wistar albino rats were unilaterally nephrectomized, and two weeks later they were subjected to 45 min of left renal pedicle occlusion followed by 3 h of reperfusion. Rats were administered either L-N6-(1-iminoethyl) lysine (3 mg/kg, i.p., 30 min prior to I/R) or kaempherol (7 mg/kg, i.p., 1 h prior to I/R). Serum BUN levels (112%) and creatinine levels (355%) were elevated in the I/R group as compared to the control group. Similarly, I/R caused a significant increase in plasma cGMP level (75%), which was accompanied with a significant increase in nitrite/nitrate level (135%) of plasma. Pretreatment of rats with L-NIL or kaempherol significantly attenuated both renal dysfunction and elevation in plasma cGMP levels and also restored the increased plasma nitrite/nitrate level. Both L-NIL and kaempherol diminished the positive immunohistochemical staining of the 3-NT and iNOS.

The present study shows for the first time that kaempherol, with its potent NO scavenging properties, seems to be a highly promising agent in protecting kidney against nitrosative damage due to I/R.

ID ABS: 323

The use of fibroblast populated collagen lattice as an *in vitro* model for post burn hypertrophic scar wound healing

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Hypertrophic scars are a common problem in the clinic and in surgery daily practice. Despite their relevance, there is a lack of knowledge about their mechanism of formation and alternatives for prevention and treatment. In 1979, Bell et al. showed that culturing fibroblasts in hydrated collagen gels (fibroblast populated collagen lattice, FPCL) led to gel reorganization. This study investigated the differences in matrix reorganization with normal and hypertrophic scar fibroblasts. Fibroblast cultures were initiated from full thickness normal human skin (NHF–n=10) and from hypertrophic scars (HSHF–n=10). Subcultures were seeded in a collagen lattice and then assayed for contractile activity. After, 24 h and 48 h, dishes were photographed and analyzed using image analyzer software. Results were statistically analyzed using generalized estimating equations. There was a significant increase of lattice contraction in the HSHF group when compared to the NHF group. From zero to 24 h, HSHF exhibited a 33% reduction in contraction while NHF contraction was 26%. After 24 h, there was no statistically significant increase in lattice contraction ($p<0.0001$).

In the present study we observed that the maximum contraction occurred during the first 24 h. Despite FPCLs were still visually contracting; the GEE statistical analysis showed no difference in FPCL areas between 24 h and 48 h in both HSHF and NHF lattices. The data obtained in these experiments indicate that this model could be used in hypertrophic scar wound healing studies.

ID ABS: 331

Improvement of a primary human *in vitro* skin cancer model using a full human cancer-associated dermal microenvironment

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Squamous cell carcinomas (SCCs) of the skin represent a large clinical problem, which is particularly dramatic in immune-suppressed individuals. Since medical intervention is crucial against successive and recurrent SCCs, refined therapies are indispensable. Therefore, well-targeted therapeutics should be screened in representative human skin carcinoma models. Our goal is to generate and validate an *in vitro* human skin cancer equivalent that mimics human SCCs to reduce and refine the current exploitation of animal models. For this purpose, *in vitro* skin models were engineered directly from primary SCC-biopsies of immune-suppressed individuals. These models reproduce important hallmarks of SCC, including invasion, hyperproliferation, aberrant differentiation and expression of putative SCC markers as determined by morphological and immunohistochemical analysis. In order to further mimic SCC behavior *in vitro*, the dermal microenvironment was modulated by introducing cancer-associated fibroblasts (CAFs) into the dermal compartment and by using human CAF-derived dermal matrix instead of rat-tail derived collagen. These CAFs influenced invasion and epidermal morphogenesis differently than normal human fibroblasts. Furthermore, CAFs presented with aberrant morphology, loss of contact-inhibition, increased contractility and motility, and increased production of extracellular matrix components. Altogether, these observations emphasize the crucial role of the dermal microenvironment in tumor development. By using primary human carcinomas and carcinoma-associated fibroblasts, the natural heterogeneity of SCCs is reflected by this *in vitro* skin cancer model. Ultimately, this work aims to contribute to the replacement of animal models for therapeutic, diagnostic and screening purposes towards human skin cancer.

ID ABS: 383

Photodynamic treatment of localized infections with a new cationic Zn(II) phthalocyanine derivative

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The growing resistance of pathogenic microorganisms against antimicrobial agents has generated research for alternative treatments for localized infections (Saskia et al., 2005). Photodynamic therapy (PDT), currently applied as treatment for cancer and for certain benign conditions (Oleinick et al., 2002), is a promising candidate. Moreover superficial wound infections are potentially suitable for treatment by PDT because of their ready accessibility for topical delivery of the photosensitizer and light.

In this study we report the activity of compound RLP068, a new photodynamic agent synthesized in our laboratories (Roncucci et al., 1999) tested on a fungal and bacterial infection animal model in comparison to classic antibiotic therapy.

The higher ability of light-activated RLP068 to reduce the rate of progression of the infection confirms this treatment as an alternative to conventional antibiotic therapy for localized lesions.

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ID ABS: 438

Contribution of animal models to the understanding of the metabolic syndrome: a critical analysis

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The metabolic syndrome (MetS) is a major challenge to medicine internationally. We present an overview of the use of animals in this research area.

Using the MeSH search in Pubmed, we identified 137 papers reporting 67 different animal models and, in addition, 38 papers report comparative reviews of models. Experimental studies with these models addressed etiology (e.g. genetics, fetal programming and diet), pathophysiology (e.g. insulin resistance, adipose endocrine response and low-grade inflammation), treatment (lifestyle, drugs) and associated diseases. Most of the models used rodents but large animal models including pigs, dogs, and certain non-human primates were also used. Reviews compared 43 mouse strains on the basis of MetS-related parameters and suggested CAST/EiJ, CBA/J, and MSM/Ms as particularly suitable. A similar approach for rats suggested male obese ZDF, obese Koletsky, obese SHHF/Mcc-facp, and obese ZSF1 rats. The Rat Genome Database indicates nine potentially useful strains but does not allow a detailed comparison.

The polygenic models that phenotypically resemble the human situation more closely still dominate this research field. The general weakness of all the animal models used is that the MetS is not defined in the different model organisms, since physiological parameters in trait clusters of the MetS differ considerably among species. The lack of a consensual definition of the MetS in humans contributes to this confusion.

Animal studies undoubtedly helped to understand basic pathophysiological mechanisms of MetS, but the translation of their results into effective preventive or intervention therapies, especially in case of the most commonly used rodent models are highly complicated.

ID ABS: 458

Tissue engineered *in vitro* human airway models (EpiAirway™) of asthma and COPD

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Asthma and chronic obstructive pulmonary disease (COPD) are two leading chronic respiratory diseases in industrialized countries. However, reliable *in vitro* human models are not widely available to researchers attempting to understand pathogenesis and develop therapeutic interventions for these diseases. Here we report on a program to create and maintain a human cell bank derived from airway epithelium of diseased individuals, and production of tissue engineered *in vitro* models of asthma and COPD from the cells. Tracheobronchial tissues are obtained from non-transplantable organs donated from normal, asthmatic or COPD individuals. Epithelial and mesenchymal cells are isolated, cryopreserved and maintained in a cell bank. As needed, cell are recovered and utilized to produce *in vitro* tissue engineered models. Tissue engineered models are cultured at the air-liquid interface and consist of well-differentiated human tracheal/bronchial epithelium (EpiAirway™) as well as human airway epithelium co-cultured with donor-matched human airway mesenchymal cells (EpiAirway-FT™). The models have pseudostratified epithelia with mucociliary phenotype similar to *in vivo* tracheobronchial epithelium. They can be cultured for several months, allowing for long-term experiments. These *in vitro* human models of asthma and COPD provide important unique attributes which animal models cannot provide, including the ability to address human individual variability and genetic factors and a means to determine mechanisms of human virus elicitation of asthma and COPD exacerbations. They will provide researchers with important new tools for investigating the role of airway epithelium and mesenchymal cells in asthma and COPD pathogenesis and for development and testing of new therapeutic treatments for these diseases.

ID ABS: 503

In vivo imaging system (IVIS) as a tool to monitor pathogen infections in live animals

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Group A Streptococcus (GAS) is a common human pathogen responsible for a large variety of non invasive infections, such as pharyngitis, impetigo or cellulitis, which, less frequently, can result in life-threatening and invasive conditions, such as bacteremia, pneumonia and necrotizing fasciitis.

Recently, there has been a resurgence of severe invasive *S. pyogenes* infections and emergence of antibiotic resistant strains, which makes desirable the introduction of an effective GAS vaccine able to confer broad coverage protection. In order to better study GAS infection mechanisms, we exploited the *In vivo* Imaging System to monitor infection progression in animals.

S. pyogenes strain DSM2071 was successfully transformed with a plasmid containing a gram-positive Photorhabdus luminescens lux operon, which allows random integration of lux genes into the bacterial chromosome. Mutants containing the exogenous DNA integrated downstream of a functional promoter sequence in the bacterial chromosome were highly bioluminescent, and when they were used to infect intraperitoneally either naïve mice or mice immunized with adjuvant only, a bioluminescent signal could be detected in the abdominal area, and infection progression could be monitored in real time for days. On the contrary, when bioluminescent bacteria were injected in mice previously immunized with a GAS antigen able to elicit an immune response conferring protection from GAS infection in an established animal lethal model, no bioluminescent signal could be detected.

In conclusion, IVIS technology appeared to be suitable to monitor bacterial infections "*in vivo*" and could possibly represent an appropriate tool to reduce the overall number of animals used for bacterial pathogenesis and vaccine studies.

ID ABS: 590

Effect of metformin in combination with malaysian tualang honey on glycaemia, body weight and oxidative stress in streptozotocin-induced diabetic rats

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Oxidative stress plays an important role in the etiology and pathogenesis of chronic complications of diabetes mellitus. The aim of this study was to investigate whether honey, metformin or a combination of glibenclamide and honey could ameliorate oxidative stress in kidneys of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced by STZ (60 mg/kg; ip). Rats were randomly divided into six groups. Diabetic rats received distilled water (0.5 ml/day), honey (1.0 g/kg/day), glibenclamide (0.6 mg/kg/day) or a combination of glibenclamide (0.6mg/kg/day) and honey (1.0 g/kg/day) orally for four weeks. Non-diabetic rats also received distilled water (0.5 ml/day) and honey (1.0 g/kg/day). The fasting plasma glucose (FPG), lipid peroxidation (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were significantly increased in diabetic rats. Body weight, reduced glutathione (GSH), total antioxidant status (TAS), catalase (CAT) and glutathione reductase (GR) activities were significantly reduced in diabetic rats. Honey significantly increased body weight, GSH, TAS, CAT and GR activities in diabetic rats. It also significantly reduced FBG, MDA levels and SOD activity. Although metformin decreased FBG and TBARS levels, it did not have significant effects on CAT, GPx and GR except SOD activity. However, metformin in combination with honey significantly increased body weight, TAS, CAT and GR activities while FBG, TBARS levels and SOD activity significantly decreased. These results suggest that tualang honey offers good therapeutic benefits by decreasing blood glucose and ameliorating oxidative stress in diabetes. The results further indicate that metformin, when administered together with honey, produces additive effects on antioxidant enzymes. These effects could be attributed to honey.

ID ABS: 775

Cell migration through the blood brain barrier (BBB) in feline immunodeficiency virus infection is significantly influenced by the pre-existence of virus and TNF-α within the CNS: studies using an *in vitro* feline BBB model

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Feline Immunodeficiency Virus (FIV) infection of cats is a model of HIV-1 associated neuropathology. Virus enters the brain early in infection, associated with leukocyte trafficking across the blood-brain barrier. Cytokines, including TNF-α, enhance adhesion molecules expression at the BBB. The present study evaluated cell-free FIVGL8 and FIV-infected lymphocyte (Mya-1) migration across an *in vitro* model of the feline blood-brain barrier and the extent to which this is influenced by TNF-α and virus in serum or CNS. Cell-free FIV migrated across the BBB model in statistically insignificant quantities, which were not significantly increased in the presence of TNF-α, and BBB tight junctions were not disrupted. In contrast, cell-associated FIV readily crossed the BBB in a similar magnitude to uninfected, activated cells, with neither cell population altering BBB integrity. With scenarios to mimic serum and/or CNS TNF-α concentrations, a statistically significant increase in transmigration of both cell populations was observed, and accompanied by a moderate disruption of barrier integrity. Further enhancement of migration occurred with infected cells and TNF-α within the brain, and this induced the most significant disruption of BBB tight junctions suggesting that, *in vivo*, small quantities of virus in the brain with the potential to trigger TNF-α production may attract greater viral entry into the CNS. The mechanisms of lymphocyte and virus transmigration were investigated using real-time PCR to quantify ICAM-1, VCAM and TNF-α expression on feline brain endothelial cells (FBEC), feline astrocytes and Mya-1 cells exposed to FIVGL8, TNF-α or a combination of FIV and TNF-α. VCAM expression was enhanced in FBEC cultures exposed to TNF-α alone or in combination with FIV, but not with FIV alone. In contrast, ICAM-1 and TNF-α expression on FBEC cultures was not altered in response to FIV or TNF-α. While Mya-1 cells expressed ICAM-1, VCAM and TNF-α, this expression was not enhanced when the cells were exposed to FIV or TNF-α. These studies suggest that the synergistic effects of TNF-α and FIV on BBB function could be mediated through VCAM, rather than ICAM expression on FBEC, and the mechanism is not the result of further TNF-α production.

PO27: Environmental science

ID ABS: 201

Detection of aquatic pollution in the Meriç river by a measure of developmental instability, i.e. fluctuating asymmetry, in the fish *Cyprinus carpio*

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A deviation measure of bilateral symmetry, developmental instability (fluctuating asymmetry, FA), was estimated with two different indexes (namely, FA1 and FA11) from samples of common carp, *Cyprinus carpio*, from different collection sites in the highly polluted Meriç River system of Turkey. We used three traits of the carp, i.e. head length, eye diameter, and barble length, to estimate an index of fluctuating asymmetry separately for each trait (FA1s) and for the all the three traits combined

(FA11s). Only FA1 of the eye diameter resulted in significant deviation from symmetry when the mostly organochlorine pesticide polluted site was compared to the others. Our results suggest that developmental instability may be a relevant indicator of pollution next to the classical markers. We observe that a choice of multiple traits would be helpful to reveal possible candidates of fluctuating asymmetry for further use in other model organisms.

ID ABS: 328

Conservation of *Amazilia castaneiventris*

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The population densitiy of the Chestnut Bellied Hummingbird (*Amazilia castaneiventris*) was studied in the Eastern Cordillera of Colombia. Patterns of spatial distribution and population density of the species depend on their intrinsic properties, interactions with other species, and availability and distribution of resources. Patterns of space use and population densities of hummingbirds, particularly montane species, are poorly documented. *A. castaneiventris* is considered rare and is classified as Critically Endangered. Between July and November 2007 we conducted monthly surveys along three transects, and made *ad libitum* observations to estimate population densities and obtain information on spatial distribution, breeding period and diet of the hummingbird. This study was carried out in a 100 ha forest in Soata, Colombia. We estimated a total density of 2.1 ind/km^2. We did not observe fluctuations in abundance or evidence of altitudinal migration. The available information suggests that the *A. castaneiventris* has low population densities and that its habitat is dramatically reduced. These characteristics make this species extinction prone. It remains to be investigated whether this species is rare at larger scales and throughout its distribution range

ID ABS: 330

Dissemination of alternative *in vitro* methods with fish cell lines to regional environmental protection agencies of Italy

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The European Regulation REACH requires that all chemical substances with a volume of production higher than 1 t per year are tested for their ecotoxicity. In addition to tests with algae and crustaceans, a large number of toxicity tests with fish must be conducted.

Owing to the good correlation between *in vitro* and *in vivo* fish data (Castaño and Gómez-Lechón, 2005) the use of established fish cell lines can represent an alternative to the acute fish bioassay for toxicity screening of chemicals.

In response to the need of reducing and replacing *in vivo* animal tests, a project of the Environmental Metrology Service of ISPRA, entitled "*In vitro* methods for ecotoxicological assessment of chemicals: use of RTG-2 (Rainbow Trout Gonad) cell line", is presented. The main goal of this project is the development and dissemination of alternative *in vitro* methods to the laboratories of the Regional Environmental Protection Agencies of Italy (ARPA/APPA) involved in REACH application. The utilization of the RTG-2 cell line has many practical advantages: the cells can be incubated at room temperature; they can be stored for long periods at 4°C without any medium change; and the method is a rapid and inexpensive tool for routine basal cytotoxicity testing.

This project could become part of a National Network of Reference Laboratories for the application of the REACH procedures.

Reference

Castaño, A. and Gómez-Lechón, M. J. (2005). Comparison of basal cytotoxicity data between mammalian and fish cell lines: a literature survey. *Toxicology in vitro 19*, 695-705.

ID ABS: 375

Quantifying susceptibility of rainbow trout cell lines to oxidative stress by dichlorofluorescein assay upon exposure to prooxidants

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The detection and quantification of reactive oxygen species (ROS) is receiving a great deal of interest because of the importance of ROS in a wide range of physiological and pathogenic events. The present work examines the sensitivity and reproducibility of the rainbow trout (*Oncorhyhnchus mykiss*) cell lines more frequently used in aquatic toxicity testing in the dichlorofluorescein (DCF) assay for *in vitro* oxidative stress assessment of chemicals.

To establish a DCF assay protocol adapted to rainbow trout cell lines, we tested different test conditions by applying chemicals or samples extracellularly to the fibroblast-like RTG-2 (gonad tissue), the epitheloid RTL-W1 (liver tissue) and the RTH-149 (hepatoma tissue) cell lines. The tested substances were 3-morpholinosydnonimine hydrochloride (SIN-1), H_2O_2, PCB 153, $CdCl_2$, $CuSO_4$, fluoxetine, clofibrate, paraquat and alachlor. Under the conditions optimized in this study, the fluorescence varied linearly with increasing concentrations (between 0.15 μM and 500 μM) of SIN-1, H_2O_2, paraquat and alachlor. The comparison of the cell lines revealed that the RTG-2 cell line was slightly more sensitive than the RTH-149 cell line and four times more sensitive than the RTL-W1 cell line. Quantifying cellular ROS by the DCF assay using rainbow trout cell lines is an easy and efficient method with high sensitivity and low variability which can be used to quantify the oxidant potency of compounds.

ID ABS: 517

Improving *in vitro* fish cell line assays: role of dosing, solvents and physico-chemical properties of the test chemicals

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Due to REACH it is required to test chemicals produced or imported above one tonne per year with respect to their impact on humans and the environment. This will lead to a dramatic increase of animal tests, and therefore alternative methods are desired. The most widely applied test in environmental risk assessment is the acute fish toxicity test (OECD 203), which requires a large number of fish and uses death as the integrative and crude endpoint. One promising alternative approach is the use of fish cell lines. However, several studies indicate that fish cell lines are less sensitive than fish. It is the goal of the CEllSens project, funded by CEFIC-LRI/UK-DEFRA, to investigate if the cell line approach can be improved to be more widely accepted as an alternative to the acute fish test. First studies were aimed at the identification of the causes of the seemingly lower sensitivity. We assumed that limiting factors may include the test set-up itself. There, escape routes for the chemicals, which are not present in the fish test, are present, e.g. sorption to plastic and evaporation. Both of these factors are driven by the physico-chemical properties of the test chemicals themselves. To test this hypothesis we used a) different test chemicals with broad spectra of logP and logHLC, b) different solvents and solvent concentrations and c) different dosing methods. We could show that these different parameters exhibit an enormous impact on the predictive power of the fish cell lines, particularly for volatile and lipophilic compounds.

PO28: Animal welfare science

ID ABS: 49

Animal use in research in Brazil

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Animal experimentation is an important issue; however, in Brazil there are few studies about it. This work aimed to study the animal use in research in Paraná, a southern Brazilian State, to understand the possibilities to reduce animal suffering, according to the sections: (I) Animal use control in research, describing the regulations in different countries and comparing them to the situation in Brazil and in Paraná. The legislation of Germany, Australia, Canada, United States, France, Netherlands, Japan, United Kingdom, Sweden and Switzerland was described and may be a basis to improve the control of animal experimentation in Brazil. (II) Animal use in research according to bibliographic sampling in scientific journals from Paraná, investigating the animal use in 2006. This study used 18 journals published in health, animal science, biology, environment and food technology. Brazil is important in the global context of animal experimentation and although the bibliographic sampling is useful, the construction of a system to register animal use is urgent. (III) The Animal Use Ethics Committee of the Agrarian Science Sector – CEUA-SCA – of the Federal University of Paraná-UFPR, regarding the reduction of animal suffering. The description of composition and work methodology showed that the CEUA-SCA collaborated to control the animal use under the SCA-UFPR responsibility. Therefore, the animal use control in research, the availability of information regarding the animals used and CEUA's actuation are concrete tools to reduce animal suffering and also have an important function in ethics and animal welfare studies.

ID ABS: 86

A broad scope on implementing the 3Rs in academic research

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The 3Rs contribute to animal welfare and quality of science simultaneously (http://www.nc3rs.org.uk/news.asp?id=212). A questionnaire distributed among university researchers demonstrated that the search for the 3Rs, required for each application to the animal ethics committee, is usually only performed in Pubmed. Because the 3Rs are to be found in about 100 databases with different information and search strategies, this makes the 3R search complex and time-consuming (Leenaars et al., *ATLA*, 2009). The 3R Research Centre has been established (www.umcn.nl/3rrc), in order to give support to researchers in searching, finding and implementing the 3Rs. Questionnaires have now been sent out to animal welfare officers, members of ethics committees and researchers in academia and industry within the Netherlands. Special lectures and workshops have been introduced within the FELASA category C courses in order to prepare future researchers with a broader scope and skills on retrieving 3R literature. A good literature search is not only of importance for implementing the 3Rs, such as avoidance of unnecessary duplication, but is also necessary for the best choice of an animal model (translational value) and the best basis for designing human trials and patient safety (Pound et al., 2004). Education is focusing on the awareness that the 3Rs lead to better quality science and need to be fully integrated at the start of new scientific projects. In line with the medical field, systematic reviews (SRs) need to become the standard routine when planning animal experiments. We are currently developing guidelines for SRs and executing SRs.

ID ABS: 98

Refining rabbit care – a resource for those working with rabbits in research

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A new report (Hawkins et al., 2008) providing practical guidance on improving rabbit husbandry has been produced by the UFAW/RSPCA Rabbit Behaviour and Welfare Group. The report sets out the welfare needs of the rabbit, based on the current laboratory animal science and welfare literature, and explains how these can be addressed in research facilities.

Topics addressed within the report include: enclosure size, social housing, solid flooring, raised areas, refuges, gnawing objects and dietary enrichment, positive interaction with humans, toys, and special needs of breeding does.

Each topic sets out factors to take into account when changing husbandry, how to introduce the refinement without causing stress or aggression, and what to do if the rabbits do not use a resource or there are behaviours that give cause for concern. Special consideration has been given to minimising the risk of aggression when changing to social housing. Guidance is also provided on making sure that the animals will benefit, by observing rabbit behaviour and monitoring the effects of refinement.

The report will help facilities to comply with, and improve upon, the guidelines on rabbit housing and care within the new Appendix A to Convention ETS123. It is intended for use by animal technicians, facility managers, veterinarians and scientists and is available free of charge from the RSPCA; see www.rspca.org.uk/researchrabbits or email rabbits@rspca.org.uk

Reference

Hawkins, P. et al. (2008). *Refining Rabbit Care: A Resource for Those Working With Rabbits in Research*. RSPCA: Southwater, UK

This poster is presented by Barney Reed on behalf of the UFAW/RSPCA Rabbit Behaviour and Welfare Group

ID ABS: 115

Enhancing laboratory rodents' well-being by introducing environmental enrichment

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Recent studies describe that laboratory animal facilities are often focussed too much on economical and ergonomic aspects and do not consider the animal well-being enough. The design and proper maintenance of facilities influence the level of excellence of husbandry practices. In consideration of these issues, the laboratory animal facility of the College of the Pharmaceutical Sciences and the Chemistry Institute of the University of São Paulo in Brasil was modernized and improved to accommodate the production of defined animals in its units.

In the breeding of these animals we used two systems: open cage and I.V.C (Individual Ventilated Caging). This way, in order to implement a more complete well-being, we are introducing environmental enrichment in the open cage system. Considering the difficulty to acquire national products and the lack of resources to import them, we opted for PVC tubes and cut polypropylene bottles. We used thirty breeding pairs of outbred Wistar Hannover WH.FCF/IQ. They were divided into two groups: the experimental cages with environmental enrichment and a control group, where we introduced neither PVC tubes nor cut bottles. We assessed the influence of these through reproductive indexes and body-weight gain. We observed that the group in which the enrichment was introduced showed a tendency to increase their reproductive indexes. However, in order to get more significant results, we are implementing new studies by using other species and strains in both open cages and I.V.C.

ID ABS: 128

Human and animal health: risk associated with *Mycobacterium bovis* infections detected in selected study herds and slaughter cattle, a Nigerian case study

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Human and animal tuberculosis is wide spread in Africa with very close genetic similarity between the causative organisms, *Mycobacterium tuberculosis (M. tuberculosis*) and *Mycobacterium bovis (M. bovis)*. The presence of *M. bovis* in cattle can pose a serious health risk for man, since close contact between people and animal and consumption of raw milk form part of the society characteristics. In sub-Saharan Africa nearly 2 million tuberculosis (TB) cases per year occur in man, and the genus Mycobacteria has pathogenetic and zoonotic importance (Daborn et al., 1996). It is estimated that in countries where pasteurisation of milk is rare and bovine tuberculosis (BTB) in cattle is common 10% to 15 % of human cases of TB are caused by *M. bovis* (Ashford et al., 2001). In West Africa, BTB has been reported in domestic ruminants in Senegal, Burkina Faso, Mauritania, Ghana and Nigeria (Benkirane, 1998; Bonsu et al., 2000; Dusai et al., 1995; Delafosse, 1995) and has been suspected in all other countries of the region. Man can acquire tuberculosis of bovine origin directly by the aerogenous route and indirectly by consumption of milk and meat. As the main route of entry is the oral route, BTB in man is mainly extra pulmonary, resulting in abdominal, bone and joint tuberculosis as well as infection of the cervical and mesenteric lymph nodes (Dabron and Grange, 1993; Edelstein, 1995; Ashford et al., 2001).

ID ABS: 150

The influence of environmental enrichment on clinical pathology and cardiovascular parameters in rats

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Over the last years and especially since the release of the revised Appendix A of the European Convention ETS 123 in 2006 it has been argued that laboratory animals should be housed under environmentally enriched conditions. A number of papers, especially within neurobiology, have raised the concern that environmental enrichment may increase uncontrollable variation in the animals, and thereby induce the need for a higher number of animals. However, even though this may be used as an argument to deny environmental enrichment, it is unclear whether there is any basis for concern within other research areas. The aim of this study was to study whether clinical pathology and cardiovascular parameters were influenced by housing rats under environmentally enriched conditions. Rats were housed under three different conditions in commercially available housing systems: non-enriched, standard enriched according to the revised Appendix A, and extra-enriched with a shelf and increased height of the cages. A total of 41 different parameters were monitored by clinical pathology, telemetry and coagulation tests, and practically no differences were observed between the three different housing conditions. The uncontrollable variation observed was compared to within-strain variation data supplied from the breeder and was quite low in all three types of housing conditions. We conclude that so far there is no basis for being concerned that environmentally enriched housing will lead to increased group sizes when using rats for research within clinical pathology and cardiovascular research, and as such there is no reason not to enrich the environment.

ID ABS: 167

A characterization routine for improved animal welfare in pre-weaning mice

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The increasing use of mutant mice raises ethical issues about producing/maintaining animal models which involve severe suffering. The need to monitor those animals for development and welfare has been recognised. Pre-weaning development is extremely fast in mice, and pre-weaning monitoring allows detection of abnormal development.

We created a protocol for pre-weaning monitoring of mutant mice. This study aimed to evaluate its efficacy to detect development/welfare problems.

36 litters were screened, including four wild-type strains (C57BL/6, 129S6, BALB/C, B6CBAF1), two genetically modified lines (Nestin-PDGFB, GAD67-GFP) and one spontaneous mutant (Kv1.1-null mouse). Pups were monitored on postnatal days 1, 3, 7 and 14, according to a score-sheet comprising several morphological and reflexologic observations. At weaning (day 21) a clinical examination was performed, evaluating physical and neurological condition and emotional reactivity.

For wild-type mice, development was strain dependent. During the first week of life, C57BL/6 mice had a lower weight gain (2.34 ±0.06 g) than all other strains (129S6: 2.93 ±0.14 g; BALB/C: 3.62 ±0.09 g; B6CBAF1: 2.84 ±0.06 g; all $p<0.001$), and BALB/C had a higher weight gain than all other strains (all $p<0.001$). Fewer C57BL/6 pups (63%) developed the righting reflex on day 3 compared to all other strains (129S6 and BALB/C: 90%; B6CBAF1: 87%; all $p<0.01$).

Within litters of mutant mice, individual cases of abnormal development were found, including decreased body weight (n=24), absence of response to touch (n=21) and sound (n=7), abnormal gait (n=4), dehydration (n=8) and altered emotional reactivity (Nestin-PDGFB mice).

The protocol is effective to detect welfare problems/developmental deviations that may require improved husbandry or humane endpoints.

ID ABS: 170

Sustained release buprenorphine solution for prolonged analgesia in rodents

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Purpose: Buprenorphine is the most widely used narcotic analgesic for rodents because of its excellent analgesic activity and long duration of action. We developed a long-acting formulation of buprenorphine capable of maintaining analgesia in rats and mice for 3 to 5 days following a single subcutaneous administration of drug.

Methods: The solubility and stability of buprenorphine base in TEC, ATEC, TBC, ATBC was determined. An IV pharmacokinetic study was conducted in rats and mice to determine the drug plasma concentrations and corresponding analgesic effect of buprenorphine. Solutions of buprenorphine in TEC, ATEC, TBC, ATBC or their mixtures were injected subcutaneously to rats or mice. Analgesic effect was measured by tail flick method. Blood was collected at intervals to determine corresponding drug concentrations in plasma using a validated LC/MS/MS assay.

Results: Buprenorphine base was stable in TEC, ATEC, TBC, ATBC at 40°C for 30 days. Buprenorphine solution prepared with TBC was able to maintain 50% analgesia for 3 days in rats and mice after a single subcutaneous injection. The maximum plasma concentration in rats was 3.4 ng/ml at 4 hours after the injection; the plasma concentration at 120 hours was 2 ng/ml. The maximum plasma concentration in mice was 36 ng/ml at 3 hours after the injection and at 120 hours, the plasma concentration was to 2.5 ng/ml.

Conclusion: A solution of buprenorphine base in TBC could maintain at least 50% analgesia for at least 72 hours (3 days) in both rats and mice after a single subcutaneous injection.

ID ABS: 171

The benefits of post approval monitoring

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Monitoring of animal use protocols for compliance is a requirement of the Guide for the Care and Use of Laboratory Animals. There are many ways in which to accomplish this monitoring, one of the more popular being Post Approval Monitoring. Post Approval Monitoring is a program through which an institution can ensure and document program integrity, compliance with the regulations and guidelines, and adherence to protocols. At our institution, Post Approval Monitoring is conducted on individual protocols. Procedures are observed with focus on the following areas of the protocol: personnel, study procedures, anesthesia, surgery, post-surgical care, euthanasia, record keeping and the laboratory environment. The benefits of Post Approval Monitoring are numerous and include the ability to identify any problems that may occur and deal with them before they get to a point where they are escalated to regulatory authorities. The program also helps foster an environment of openness, encourages the investigative staff to engage the animal facility and regulatory personnel when then need assistance, and provides an educational and training forum for the research staff. Such programs refine techniques in animal research, thereby reducing the number of animals used while, at the same time, enhancing the welfare of those animals.

ID ABS: 220

Development of evidence-based housing and husbandry guidelines for animals used in teaching and scientific research

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In Australia, the NSW Animal Research Review Panel began developing evidence-based housing guidelines for commonly used research animals about 10 years ago. The objectives are to provide research establishments with guidance on animal housing that meets the physical and behavioural needs of animals and promote good science. This paper describes the process and some of the pitfalls. Commencing with a comprehensive literature search of refereed scientific journals and reference books, findings are grouped into topics including: Normal animal behaviour; Cage or enclosure design; Animal care and management (including social environment and environmental enrichment) and Environmental variables (including light, temperature and ventilation). Points of agreement and matters of conjecture are discussed. Significant background information is documented and used to develop principles and recommendations, citing relevant literature. Draft guidelines are referred to international experts for comment and edited accordingly. Edited drafts are widely circulated to Animal Ethics Committees, animal welfare groups, national and international bodies such as Australia's National Health and Medical Research Council and the Canadian Council on Animal Care, and people with internationally recognised expertise in housing the respective species. Comments received are collated and assessed. The guidelines are amended where appropriate. To date, guidelines have been published for dogs, rabbits, rats and guinea pigs and draft guidelines circulated for mice and sheep. They will be revised periodically with advances in the understanding of animal physiology and behaviour, technological advances, and changes in community attitudes about animal welfare. The guidelines are available on the Animal Ethics Infolink website: http://www.animalethics.org.au/

ID ABS: 227

Rumen simulation technique (RUSITEC) – an *in vitro* substitute for experimentation in ruminant animals

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RUSITEC, a laboratory device for simulating the rumen (cattle stomach) environment was utilized to assess lipid supplementation on Conjugated Linoleic Acid (CLA) production. It is a semi-continuous culture system which simulates rumen in temperature, pH, anaerobiosis, microbial culture and mixing movement. It consists of eight reaction vessels, filled with 650 ml strained rumen liquor and 150 ml artificial saliva, and is immersed in a water bath at 38°C. Nylon bags containing 80 g rumen digesta and 10 g test diet were put into the reaction vessel. Continuous salivary infusion was provided into the reaction vessel and, as fermentation proceeded, effluent and gas were collected in separate containers. Experimental diets were 1) control (C), 2) control diet with 4.5% sunflower oil (SF4.5), 3) with 6% sunflower oil (SF6). They were given an adaptation period of ten days followed by a measurement period of 3 days. Rumen fluid pH in C, SF4.5 and SF6 diets were 6.97 ±0.01, 6.95 ±0.01, 7.06 ±0.002, and CLA concentrations were non-detectable, 0.53 ±0.02, 0.67 ±0.25 respectively. The current study using RUSITEC precludes surgical fistulation, associated trauma, stress and the appalling appearance of the animal. Therefore RUSITEC can be considered as an alternative to live animal experimentation.

ID ABS: 236

New protocols to assess the welfare of transgenic pigs

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Genetically modified pigs are increasingly used as models of human diseases. However, there is legitimate concern with regard to the welfare of the involved animals. In contrast to laboratory rodents welfare assessment protocols have not been published for large animal species such as pigs.

Based on existing methods used in laboratory mice and farmed pigs such a protocol was developed and has been applied in an ongoing study on pigs expressing GFP (green fluorescent protein), a study aimed at improving techniques for generating transgenic animals.

Heterozygous transgenic (tg) and wild type (wt) offspring (littermates) of heterozygous tg (n= 9) and wt (n=8) primiparous sows and reciprocal boars are being compared focusing on health, early development, suckling, social behaviour and reaction to novelty and humans. Additionally, reproduction data was recorded and the animals were filmed post partum.

Preliminary data suggest that sow genotype does not influence mean gestation length (113.9 in tg and 114.6 days in wt sows, p=0.365) and number of liveborn (10.7 in tg and 9.4 in wt sows, p=0.206) or stillborn piglets (0.9 in tg and 1.88 in wt sows, p=0.102). Data from the first batch of weaned piglets shows no difference in growth of tg (n=34) and wt (n=36) offspring from birth to weaning at 4 weeks of age (p=0.219).

If the results confirm that green pigs do not suffer from reduced welfare, this will be no big surprise. But the protocol will be useful for future monitoring of transgenic pigs serving as models of serious human diseases.

ID ABS: 306

A novel approach for the assessment of psychological suffering among animals: chimpanzee case study

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The association of pathological and detrimental behaviors with captivity in nonhuman primates has been noted for decades. Despite compelling similarities between humans and nonhuman primates, it is uncommon to study psychopathology using the terms and tools of clinical psychology in primates other than humans. Instead, detailed descriptions, inventories and rates of "abnormal" or "non-adaptive" behaviors are used. When the etiology, sequelae and epidemiology of psychopathologies are viewed in this narrow, fragmented way, there is little potential for coherence with clinical approaches or the modern scientific

understanding of behavior and biology. Here, we detail a pilot study of chimpanzees previously used in research (n=116) using reports of respondents with specialized training and experience. This novel method was used to quantify the prevalence and range of behaviors in chimpanzees consistent with depressive, compulsive and anxiety disorders, including post-traumatic stress disorder (PTSD). In total, 28.4% of chimpanzees fulfilled symptomatic criteria for depression. Bivariate analysis demonstrated a correlation between duration of captivity and the number of depression symptoms (r=+0.24, p=0.01). Data also showed a high prevalence of anxiety disorders, including compulsive behaviors. If similar rates generalize to other facilities, psychological disorders could be at epidemic levels among chimpanzees currently and previously used in research. The study's findings underscore the failure of existing regulations to prevent psychological suffering. We discuss our results in the context of international regulations and imperatives for proper assessment and treatment of psychological conditions.

ID ABS: 321

Regulation of animal use in research, testing and teaching: comparison of New Zealand and European legislation

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The use of animals in research, testing and teaching (RTT) in New Zealand and the European Union is strictly regulated to ensure that detrimental impacts to animal welfare are minimised wherever possible. This paper discusses one area of RTT, regulatory testing procedures, in which the promotion of replacement, reduction and refinement techniques and introduction of these principles into the current legislative framework can significantly affect the welfare of the animals utilized in this area. The paper also discusses current limitations in determining and comparing the extent of animal usage within the two jurisdictions and steps being taken to increase the accuracy of collected data through the introduction of new legislative procedures. The necessity to encourage the use of alternative methodologies through recognition of these techniques as valid regulatory testing procedures, and inclusion of these techniques within legislative material, is emphasised.

ID ABS: 362

Screening of haemolytic potential on an alternative insect

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Insects appear to be possible good alternative models for screening tests of adverse haematological effects of xenobiotics. Oxygen is, however, transported by different mechanisms in vertebrates and insect tissues: insects have no red blood cells to be used for an assessment of toxic-haemolytic potential. On the contrary, insect haemocytes seem to be phylogenetically close to mammalian leukocytes. Thus, our findings may come as a surprise: we present a new screening test for haemolytic potential on *Spodoptera* larvae *in vivo*. We found that insect haemocytes can be influenced by the agent with haemolytic potential *in vivo*. Haemocyte disintegration causes changes in total haemocyte counts and differentials. It is shown that only qualitative evaluation of panoptically stained haemolymph smears is sufficient, fast and cheap to detect haemolytic potential. The presented assay also prevents unneeded, non-ethical use of vertebrates.

ID ABS: 373

Examination of the scientific validity and necessity of chimpanzee research, and the psychological impact of such research on chimpanzees

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Research on captive chimpanzees incurs considerable animal welfare, ethical and financial costs. Advocates claim these costs are outweighed by biomedical progress and critical contributions to combating human diseases. To assess these claims, we performed a citation analysis of chimpanzee publications, and conducted systematic reviews of chimpanzee use in HIV/AIDS

and cancer research. Citation analysis revealed half of published chimpanzee papers had never been cited. Just 15% were cited by (a total of 27) papers associated with human disease research, yet none of these cited chimpanzee studies contributed towards human clinical practice. In HIV/AIDS research, despite protective and/or therapeutic responses elicited via many vaccination methods in chimpanzees, similar effects have not been demonstrated by any vaccine to date in humans. In cancer research, chimpanzees have been used scarcely, and chimpanzee tumours are extremely rare and biologically diverse from human cancers. Papers describing potential new cancer therapies note significant concerns regarding the chimpanzee model. Many interventions have not been pursued clinically despite promise in chimpanzees, and available evidence indicates chimpanzees are not essential in the development of therapeutic monoclonal antibodies. Claims of the importance of chimpanzees in areas of research including major human killer diseases such as AIDS and cancer are therefore without foundation and scientifically unjustifiable. Finally, papers examining Post Traumatic Stress Disorders in chimpanzees rescued from laboratories illustrate the enormous suffering and toll that research takes on chimpanzees. Together, these papers present effective and substantiated arguments for the necessary and urgent need for non-animal alternatives in the life sciences.

ID ABS: 379

Transportation as major life event in rats, effect on welfare and limits of adaptation

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Transportation is a major stressor in the life of a laboratory rodent. Nevertheless, very little is known about the size of the effect of transportation on the animal and how long it takes for the animal to restore. Most experiments make use of an acclimatization-period after transport to decrease the influence of transportation on results, but the duration of this acclimatization-period is scarcely based on scientific research. This research project aims on the physiological and ethological effects of transportation on small laboratory rodents.

In general, animals subjected to the environmental changes occurring during transportation (housing in transport boxes, several hours of travel, final placement in a new facility, exposure to new animal caretakers and procedures) react with changes in their physiology, such as body weight, plasma hormonal levels, heart rate and blood pressure changes.

To foster good scientific practice, animals should only be used in experimental procedures after adaptation to their new situation and stabilization of their physiological parameters.

By getting more information about transportation stress, we aim to decrease the variation in research results and thereby decrease the number of animals needed. Secondly, we aim to increase the welfare of laboratory rodents during and after transportation.

The current research involves both physiological and behavioural measurements in laboratory rats before and after van transportation. Data acquired with transmitters are blood-pressure, heart rate, respiratory rate and activity. Besides these parameters, homecage-behaviour, bodyweight and faecal and plasma-corticosteron were measured. Measurements are performed before and after van transportation and in transported and non-transported animals.

ID ABS: 412

Evaluation of cage enrichment for guinea pigs in the pharmaceutical industry

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Guinea pigs are commonly used in the pharmaceutical industry, not only in R&D but also in QC toxicology testing.

We evaluated the acceptance of wooden shelters (made of Aspen). The well being of guinea pigs was monitored through the general activity level of the animals and food consumption. The health status and possible negative test interference was evaluated through monitoring of the body weight and determination of organ weight after fixed time intervals. To completely exclude the possibility of negative test interference, we additionally analyzed the blood parameters using a validated test setup appropriate for GMP QC testing.

After a short period of time most animals placed their nest under the wooden shelter. The animals with shelters also showed less nervous behaviour. Although during the observation period no significant difference in food consumption between the guinea pigs with a shelter and the reference group could be observed, the guinea pigs with shelter gained significantly more weight during a three month period. When analyzing the blood

parameters a reduction in the white blood cell count was observed in the groups without shelters, indicating again that these groups are more susceptible to stress. No differences could be found in blood parameters or in the organ weights.

From these experiments it can be concluded that cage enrichment clearly improves the long term health status of guinea pigs. This should improve our ability to measure the effects of test substances by minimizing the effect of negative environmental influences.

ID ABS: 456

Ethological observation of rats and mice as a support for the strengthening of the Three Rs

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The use of animals in scientific experimentation causes a dual reaction between those who defend the use of non-human animals to certify practices and experimental processes and those who are against of the use animals based on the ethical principle of respect for other forms of life. The implementation of alternatives to the use of animals for experimentation has found its point of equilibrium in the Three Rs. Behaviour study has become the main tool to transform the animal-world vision, especially of those animals used in scientific experimentation, which were before seen as machines; and in a slow but firm manner, such a conception has been turned into ethical principles for animal handling in research. Rats and mice are currently the most commonly used animals in scientific research. Behavioural parameters are known of wild animals, but studies on the behaviour of specific strains are scarce in Colombia. This is the reason an ethological evaluation program on the species *Rattus Norvegicus* and *Mus musculus*, animals of the laboratory of biological reagents of Biotechnology Institute from Colombia, was initiated.

The collected information is unified in ethograms. With this information, patterns of improvement and attention are generated for the animal's well-being in our laboratory to guarantee a responsible and ethical management and implement the Three Rs principles.

ID ABS: 467

Managing wild ungulates in their natural habitat: the vicuñas (*Vicugna vicugna*) experience in Jujuy, Argentina

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Sustainable use of wild species could become a good source of income and social commitment to local communities that have protected the vicuñas for decades.

The program of sustainable use of wild vicuñas considers the chasing, rounding, capture and restraining for shearing, and takes biological samples in the animals' natural habitat under animal welfare standards.

The system of capture and release borrowed from Inca tradition and several modifications to minimize injuries, stress and mortalities caused by the captures have been included. Time of capture, distance and herding time, capture facilities, restraint time, handling, sampling procedures and shearing methods were improved.

A sustainable use programme based on capture and release of wild vicuñas, maintaining animal welfare standards to minimize the stress produced by the activity, should be considered as a method of harvest in a non-lethal way in an endangered species.

ID ABS: 557

Humane euthanasia for laboratory mice?

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The most common method of euthanasia of small laboratory rodents is exposure to carbon dioxide (CO_2), but recent evidence suggests that rodents find this gas aversive. The aim of our study was to use approach-avoidance testing to evaluate mouse responses to CO_2 and to the alternatives argon, carbon monoxide (CO), and isoflurane. Mice were trained to access the bottom cage of a two-cage apparatus to gain access to shredded coconut; the experimental gas or air (as a control) was turned on at

a pre-determined flow rate as soon as mice started eating. We scored the latency to leave the test cage, the gas concentration in the cage when mice left, and the time until recumbency with CO and isoflurane during forced exposure. Mice always chose to leave the cage before losing consciousness when tested with CO_2, argon or CO. The average highest CO_2 concentration tolerated was (mean ±S.E.M.) 14 ±1.7%, and this concentration decreased with increasing flow rates ($F1, 19=28.11$, $P < 0.001$). The lowest oxygen concentration tolerated when exposed to argon was 8.4 ±0.5%. The average (±S.D.) CO concentration tolerated was 2.4 ±0.7%, but recumbency occurred at 5.1 ±0.4%. Two mice became recumbent in the cage when tested with isoflurane. Mice remained in the cage closer to the time of expected recumbency with higher concentrations ($F1, 33=10.13$, $P=0.003$). We conclude that CO_2 euthanasia is aversive to mice and that isoflurane is a more humane alternative.

PO29: Immunology

ID ABS: 33

Prevalidation of a human T cell activation assay to identify immunosuppressive compounds

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The REACH legislation may increase animal testing if alternative methods do not become available soon. The immune system is a possible target for many chemicals, and alternative assays for immunotoxicity testing are not available yet. Previously, we have compared the sensitivity and specificity of a number of *in vitro* lymphocyte stimulation assays to evaluate chemicals for their immunosuppressive effects. Based on the results, the human T cell activation assay was identified as the most promising. This assay is based on CD3/CD28-mediated T cell activation using proliferation and cytokine release (TNF-α and IFN-α) as read-out parameters.

A prevalidation of the human T cell activation assay has been started within three different laboratories. To meet the requirements of the prevalidation, the project will be conducted in 3 phases. Phase 1 will determine the influence of human inter-individual variability on the test outcome, by comparing the effects of rapamycin (immunosuppressive) and D-mannitol (non-immunosuppressive) on the CD3/CD28-mediated T cell activation of 10 donors. During the second phase, the standardized procedure (obtained in phase 1) will be used to compare the effects of at least 10 immunosuppressive and 10 non-immunosuppressive compounds on T cell activation. In the third phase data will be analyzed on sensitivity, specificity and predictivity. Results will be reported to an international group of representatives from regulatory agencies, industrial parties and academia and, if successful, results will be submitted to ECVAM for consideration of formal validation of the human T cell activation assay.

This project is funded by ZonMw (project number: 11.400.0096) and ECVAM

ID ABS: 37

Development of a human lung model system for the identification of chemical respiratory allergens *in vitro*

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No validated methods exist for the evaluation of chemicals inducing respiratory allergy, neither *in vivo* nor *in vitro*. Therefore, the development of tests is of great concern, especially considering the new legislation of chemicals by the European Union (REACH). Attention should be directed to *in vitro* models, because the REACH guidelines clearly state that tests on animals should be avoided, and a complete ban on animal testing for cosmetic ingredients will be implemented in Europe by 2013.

To establish an *in vitro* test system for the identification of chemical respiratory sensitizers, we developed an immunocompetent, three-dimensional co-culture system representing the proximal alveolar region of the human lung. The model contains epithelial cells, alveolar macrophages, and monocyte-derived dendritic cells as antigen-presenting cells. During optimization of the cell model for respiratory allergen exposure, integrity of the epithelial cell layer was determined, and characteristic markers were analyzed employing flow cytometry and immunohistochemistry. The final triple cell co-culture enables crosstalk between the cells as well as assessment of the ability of the agents to penetrate the epithelial cell layer before coming in contact with the antigen-presenting cells.

Next, we will expose the model to well-known respiratory sensitizers and irritants in the form of aerosols or particles employing the Cultex® *in vitro* inhalation system. By exposing the cells at the air/liquid interface, the system mimics the *in vivo* situation in the lung as closely as possible.

We hope to identify predictive endpoints and biomarkers for the identification of the respiratory sensitization potential of chemicals.

ID ABS: 74

Comparison of contact allergen-induced gene expression changes in human PBMC-derived dendritic cells and DC-surrogate cell lines

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To help identify predictive endpoints for use in an *in vitro* cell based assay(s) for assessing the skin sensitization potential of chemicals, we focused on the identification of genes that are regulated in human peripheral blood-derived dendritic cells (PBMC-DC) following chemical allergen exposure. A list of 29 potentially predictive genes derived using PBMC-DC have been evaluated with 26 chemicals using a multiplexed bead assay. With PBMC-DC, 14 out of 29 genes are consistently positive in response to 5 mM DNBS, with very little donor-to-donor variability seen among 27 donors. Excluding DNBS, no genes were consistently positive for all allergens tested, and some allergens induced changes in only a few genes. These same genes have been evaluated in several DC-surrogate cell lines, including THP1 and U937, with a smaller number of chemicals. These cell lines show few changes in the 29 genes following allergen treatment. U937 cells had few positive responses, which were minimal with only 2-6 fold changes over control. Slightly more gene changes were observed in the THP1 cells. One gene was positive in THP1 cells with all of the allergens tested, i.e. AKR1C2. There are clear differences between PBMC-DC and the DC-surrogate cell lines in the allergen-induced expression changes of these 29 genes. PBMC-DC appear to be the cells of choice for use in this gene expression-based method.

ID ABS: 130

Results of a Japanese ring study of h-CLAT (6th report): a study for evaluating oxidation hair dye skin sensitization potential using h-CLAT

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We are now conducting the Japanese ring study to develop the human Cell Line Activation Test (h-CLAT). The aim of this study is to test whether h-CLAT can predict the skin sensitization potential of oxidation hair dyes. In addition, we analyzed the effect of autofluorescence on prediction performance. Six oxidation hair dyes in cosmetic use, which had been previously evaluated for skin sensitization potency *in vivo*, were selected. Aniline and Bandrowski's Base were also selected, with a view of comparison with p-Phenylenediamine. CD86/CD54 expression on THP-1 cells was analyzed after 24 h exposure to each chemical with 8 doses based on CV75. All experiments were independently performed in two laboratories. Good reproducibility was obtained between the two laboratories. Except for 2,5-Diaminotoluene sulfate, which has poor solubility, concordance was obtained with the LLNA result for the study chemicals. p-Phenylenediamine, which exhibits fluorescence around 530 nm, which is in the h-CLAT measurement area, was correctly evaluated as positive. It was possible to compensate for the influence of fluorescence in CD86/54 expression measurement, suggesting that even substances with autofluorescence, such as oxidation hair dyes, can be estimated. A notably concentration-dependent high CD54 expression was found with Bandrowski's Base, but not with p-Phenylenediamine. This result suggests that the sensitization behavior of Bandrowski's Base may differ from that of p-Phenylenediamine. In conclusion, it is suggested that h-CLAT is useful for evaluating the skin sensitization potential of oxidation hair dyes. This study was supported by a Grant-in-Aid from MHLW.

ID ABS: 139

Development of a high-throughput keratinocyte-based standard assay to detect skin sensitizers based on ARE-dependent gene activity

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We have shown that skin sensitizers can be identified by their ability to induce antioxidant response element (ARE) dependent genes in a breast cancer cell line. Gene chip analysis of sensitizer challenged dendritic cells also showed that ARE-regulated genes such as AKR1C2 are up-regulated by sensitizers. The *in vivo* relevance of this finding has recently been confirmed in Nrf2-knockout mice. Here we report a novel stable cell line derived from a keratinocyte cell line: A single copy of a 56-base-pair region containing the ARE sequence from AKR1C2 was inserted upstream of a luciferase gene. An SOP was developed for the high-throughput testing: Chemicals are simultaneously tested in triplicate at 12 concentrations, and significant induction of gene activity is evaluated. We report results from this assay on (i) an ECCVAM list of reference chemicals, (ii) the ICCVAM list of chemicals for validation of variants of the LLNA and (iii) on a list of 65 chemicals derived from the ICCVAM database with both LLNA and human/guinea pig evidence. The results indicate a good predictive value of this approach for hazard identification. By measuring full dose-response curves, an indication on the potency of chemicals can be given. Unlike other proposed assays the read-out is not related to cytotoxicity, as gene induction occurs at subtoxic concentrations. Its technical simplicity, the high-throughput format and the good predictivity make this assay a candidate for efficient validation to meet the tight deadline to replace animal tests for skin sensitisation by 2013 set by European authorities.

ID ABS: 147

Results of a Japanese ring study of h-CLAT (7th report): a study for evaluating fragrance skin sensitization potential using h-CLAT

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We are currently conducting a Japanese ring study of the human Cell Line Activation Test (h-CLAT). The aim of this study is to test whether h-CLAT can predict skin sensitization potential of fragrances that have low solubility or high volatility.

Seven fragrances with various properties (different solubility, volatility), which had been previously evaluated for skin sensitization potential *in vivo* (LLNA), were selected. In addition, to investigate whether the volatility or solubility of test chemicals influenced the result, we examined whether or not sealing the plates or sonicating a solution of the fragrance influences the outcome. All experiments were performed independently in 2 laboratories.

It was confirmed that sealing the plate had little effect on the CV75 and RFI values of CD86/54. Sonicating a solution of test fragrance altered the turbidity of the solutions and the CV75 values, but did not affect the RFI values of CD86/54. Therefore, it appears that sonicating the solution of fragrance also has little effect on the result.

The results of CD86 for two fragrances were different between the two laboratories, though they were evaluated as positive, because CD54 was positive in both cases. Therefore the reproducibility between the two laboratories was 100%. The accuracy with respect to LLNA was 86%: diethyl phthalate was false positive in both laboratories. In conclusion, it is suggested that h-CLAT is useful for evaluating the skin sensitization potential of fragrances, some of which have low solubility or high volatility. This study was supported by a Grant-in-aid from MHLW.

ID ABS: 218

Comparison of flow cytometry and immunohistochemistry in the evaluation of the skin sensitization potential by nonradioactive LLNA using BrdU

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Although para-phenylenediamine (PPD) has been widely used as a hair-dye precursor, a skin sensitization potential was reported through conventional guinea pig maximization test and mouse local lymph node assay (LLNA) using radioactive thymidine. In this study, we investigated the skin sensitization potential of PPD using non-radioactive LLNA with 5-bromo-2'-deoxyuridine (BrdU) using flow cytometry (LLNA:BrdU-FC) and immunohistochemistry (LLNA:BrdU-IHC). Female Balb/c mice were applied topically with increasing concentrations of PPD, DNCB or SLS on the dorsal areas of both ears. After sacrifice, auricular lymph nodes were isolated and underwent lymphocyte preparation and tissue processing for immunohistochemistry. The proliferative response in draining lymph nodes was assessed by evaluating BrdU incorporation into cells using flow cytometry and immunohistochemistry. As a result, contact dermatitis reactions, consisting of increased ear and lymph node weight and the presence of inflammatory infiltrates, were also observed in the animals treated with 1% PPD. PPD treatment significantly increased the stimulation index (SI) determined by LLNA:BrdU-FC assessment in a dose-dependent manner and SI of 1% PPD was more than three times the threshold level for the determination of sensitizer. The number of BrdU positive lymph nodes by LLNA:BrdU-IHC in the mice treated with 3% and 10% PPD increased significantly compared to vehicle control group. These data were comparable to the previously reported EC3 value of PPD tested in the LLNA assay using the radioisotope. With this result, we suggest that the non-radioisotopic LLNA detecting BrdU incorporation with flow cytometry and immunohistochemistry can be a useful model for the evaluation of the skin sensitization potential for hair-dyes.

ID ABS: 312

Culture of dendritic cell-like cells: impact of extracellular matrix

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There is interest in the development of *in vitro* methods for the identification of contact sensitizers, including those that use cultured dendritic cells (DC), key players in cutaneous immunity. However, these assays often lack sensitivity and dynamic range. Therefore, and with a view to enhancing the responsiveness, the impact of growing a DC-like cell line (THP-1) on extracellular matrix is being examined. Primary human foreskin fibroblasts (HFF) and the keratinocyte HaCaT cell line have been used for the preparation of cell-derived matrices. Cells have been grown on pre-treated glass cover slips for up to 14 days, the matrices have been denuded of cells and stained for the presence of fibronectin, type I collagen and laminin. Fluorescence microscopy of HFF-derived matrices revealed long fibrils of both fibronectin and type I collagen and the expression of laminin at fibril junctions. Experiments with HaCaT-derived matrices have demonstrated the presence of laminin (with a marked speckled appearance), but fibronectin and type I collagen expression was undetectable. HaCaT and HFF matrices are therefore representative of epidermal and dermal extracellular proteins, respectively. Culture of THP-1 cells for 24 h on HFF matrices resulted in HLA-DR expression, a molecule responsible for antigen presentation to T cells. In the absence of matrix cells failed to express this membrane determinant. These results demonstrate that THP-1 cells express a more DC-like phenotype when grown on extracellular matrix and we speculate that it may be possible to exploit this phenomenon to improve the sensitivity of *in vitro* skin sensitization assays.

ID ABS: 329

Assessment of contact sensitivity potency by using IL-8 release and p38 activation

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The local lymph node assay (LLNA) has been established for assessing the skin sensitization potential of compounds by using the EC3 as "threshold" for sensitization, but it can also be used to evaluate their potency. Therefore, development of *in vitro* reliable alternatives to LLNA assay should not only consider the identification of hazard risk but also the relative potency of chemicals. Among the different assays proposed, we have recently described selective release of interleukin (IL)-8 by the THP-1 cell line in the presence of allergens and a significant modulation of its secretion by activation of the common pathway p38 mitogen-activated protein kinase (p38 MAPK). Now, we used this approach to establish dose-response relationships with the amount of secreted IL-8 by treating the THP-1 cell line with increasing concentrations of different chemicals. The panel of compounds includes nonsensitizers (sodium lauryl sulphate, salicylic acid, phenol, propylenglycol, lactic acid); weak sensitizers (citral, imidazolidyn urea, penicillin G); moderate sensitizers (2-aminophenol, isoeugenol, cinnamaldehyde, tetramethylthiuram disulfite, 2-mercaptobenzaothiazole, glyoxal); and extreme sensitizers (benzoquinone, 2,4-dinitrochlorobenzene, p-penylenediamine, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one). Calculation of the concentration of allergen that induced the release of 100 pg/ml IL-8 by linear regression analysis of data allows us to rank the chemicals in terms of potency. When compared with the available *in vivo* LLNA EC3 values the correlation observed in the current study was consistent with our previous observations (r=0.924, p=0.0248)) and thus supports the potential use of this *in vitro* approach to assess the potency of skin sensitizers.

Acknowledgment: 2008 European animal welfare and alternatives award

ID ABS: 340

ECVAM activities on sensitisation

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Both the 7th Amendment to the Cosmetics Directive and REACH require the availability of alternative methods to animal testing for skin sensitization hazard and safety assessments. At research level, ECVAM is strongly involved in the EU sponsored integrated project Sens-it-iv, which aims to develop over a period of five years, strategies to replace animal experimentation with *in vitro* assays able to identify skin and respiratory sensitisers. Our main contribution to the project is the identification, purchase and distribution of the compounds to be used for test development and optimization. Emphasis is also put on the refinement and reduction of existing animal tests. Criteria (Performance Standards) to be used for the assessment of the validation status of modified versions of the standard local lymph node assay (LLNA) encompassing non-radioactive endpoints have been developed in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and were endorsed as valid by the ECVAM Scientific Advisory Committee (ESAC). Partial replacement test methods to be used as components of a testing strategy for the full replacement of the animal tests are currently under assessment for their eligibility to enter a formal validation study.

ID ABS: 355

Comparison of lymph node cell count and ^{3}H-Thymidine incorporation for evaluating LLNA results during routine testing

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

We present here additional data from routine studies with a variety of chemicals and mixtures proving the correlation of the two endpoints. When using a SI of 1.5 for cell count cut off to predict a positive response in CBA/J mice, equivalent estimated concentrations (ECs) for the prediction of skin sensitizing potency are obtained in the majority of cases with both measurements.

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ID ABS: 360

A strategy to establish relevance of immunological models for biopharmaceutical evaluation

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The biopharmaceutical market has grown steadily since the early 1980's. Unlike most chemical drugs, these products often exhibit immunotoxicity that often only comes to light during human studies. The main forms of immunological risk are hypersensitivity, immunogenicity that results in autoimmunity, immunostimulation resulting in cytokine release and serum sickness and immunosuppression that can increase the incidence of neoplastic lesions or infections

Regulatory agencies acknowledge that the immunotoxicological risk assessment of biopharmaceuticals cannot readily be accommodated within the small chemical drug development paradigm. Of particular concern is the identification of relevant animal models. Hence, it is perhaps not surprising that the evaluation of a single biopharmaceutical can require the use of three or more animal models.

A flexible strategy for immunological evaluation of protein biopharmaceuticals is discussed within which the roles of SAR and antigenic mapping, human and test species cell-based comparative studies and innovative lymph and peripheral tissue models in informing species and strain selection for the evaluation of immunotolerance, autoimmunity, hypersensitivity and immunogenicity are examined. The aim is to provide a strategy that will reduce the unnecessary use of animal models.

ID ABS: 368

Validating alternatives to the LLNA: what is the gold standard?

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In vitro alternatives to in vivo assays for skin sensitisation such as the LLNA are reaching the stage of formal evaluation. To ensure the performance of such approaches is correctly evaluated as well as for maintaining the present level of protection of human health or enhance it, it is essential that *in vitro* alternatives are assessed against an accurately classified database of an appropriate scale and scope. Existing datasets, notably those used in the validation of the LLNA, and more extended lists of tested chemicals published since then are valuable in this respect. Nevertheless, past validation reported the concordance of guinea pig and LLNA sensitisation test results as being approximately 90%, and neither alone fully reflected human sensitisation. This means that, where available, other relevant evidence must be taken into account in making classification decisions, including structure activity relationships, clinical data and other human evidence. For example, if sodium lauryl sulphate (SLS) or nickel chloride were new substances, both would be classified incorrectly by the LLNA; it is via our greater body of knowledge that they are correctly regarded as a non-sensitiser and a skin sensitiser respectively. In the presentation, we give further examples and details on this topic, as well as addressing how this knowledge can be used to more effectively validate new methods.

ID ABS: 386

The pathogenic role of increased hyaluronan production and decreased E-cadherin expression by cytokine-stimulated keratinocytes in spongiosis formation

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The pathogenesis of spongiosis, which is a well-known hallmark of acute eczema, is not fully understood. We think that it is crucial to clarify the mechanism for the influx of tissue fluid into the epidermis and the loss of cohesion between keratinocytes in acute eczema that result in spongiosis. By immunochemical staining using hyluronan binding protein (HABP) and by *in situ* hybridization, we demonstrated increased intercellular accumulation of hyaluronan in the spongiotic epidermis and augmented HAS3 mRNA expression by spongiotic keratinocytes, respectively. We also showed that the epidermis, where the intercellular space was strongly stained with HABP, showed weaker expression of membrane E-cadherin. Next, we demonstrated that, among various cytokines, only IL-4, IL-13, and IFN-γ increased hyaluronan production, enhanced HAS3 mRNA expression, and decreased membrane E-cadherin expression by normal human epidermal keratinocytes in both low and high Ca media by a sandwich assay using HABP, real-time PCR, and flow cytometry. Finally, we demonstrated IL-4, IL-13, their combination, and IFN-γ could induce intercellular space widening of the epidermis with increased hyaluronan accumulation and decreased E-cadherin expression in the organotypic culture. These results suggest that the augmented production of hyaluronan and the decreased E-cadherin expression by keratinocytes stimulated with IL-4/IL-13 or IFN-γ cause spongiosis in acute eczema.

ID ABS: 426

Current database of the *in vitro* skin sensitization test; human cell line activation test (h-CLAT)

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We have been developing an *in vitro* skin sensitization test, the h-CLAT, using THP-1 cells (human monocytic leukemia cell line). This test is based on the augmentation of CD86 and CD54 expression in THP-1 cells following 24 hours exposure to skin sensitizers. We previously showed that the h-CLAT protocol is easy to transfer and that good inter-laboratory reproducibility was observed in an inter-laboratory study in Japanese, European, and US laboratories. Now, we updated the database of h-CLAT to include 106 chemicals. The accuracy of the h-CLAT vs. LLNA is 84%. Most chemicals were evaluated correctly, but there were some false negative (isoeugenol, phthalic anhydride, etc.) and false positive (1-bromobutane, diethylphthalate, etc.) outcomes. On the other hand, the accuracy of the h-CLAT vs. human data is 81%. This test especially shows good positive predictivity for LLNA or human data (89.2% and 90%, respectively). From the current database we also estimated the applicability domain. Pro/pre-haptens, which require metabolism to become allergens *in vivo*, might not be evaluated correctly due to a lack of a metabolism system in THP-1 cells. Moreover, chemicals for which the test dose exceeds the limitation of solubility in the medium may also not be evaluated correctly. Additionally, auto-fluorescent chemicals and extremely weak allergens tend to be false negative. In conclusion, there were good correlations between h-CLAT and LLNA or human data. Furthermore, a possible applicability domain of h-CLAT is suggested. We are advancing development of this method to clarify the applicability domain and to predict the strength of skin sensitization.

ID ABS: 453

Functional characterization of reconstructed human epidermis integrating Langerhans cells (rhe-LC)

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The most common manifestation of immunotoxicity in humans is allergic disease resulting from industrial or environmental exposure to sensitizers. A range of different *in vitro* models has been used in order to understand the mechanisms through which chemical allergens induce allergic contact dermatitis in humans and to develop *in vitro* assays to assess the potential of a chemical to induce skin sensitization without the need for animal testing. However, none of them efficiently simulate the natural tridimensional microenvironment of the skin in which the main cellular actors of skin sensitization, dendritic cells (DC) and keratinocytes (KC) co-exist.

We present here a full characterization of such a model, consisting in a reconstructed human epidermis integrating $CD34^+$ derived Langerhans cells, and we assess its response after exposure to TNF-α or to contact sensitizers. This model allows us to study the cross-talk between KC and DC during chemical exposure and to determine the role of each cell type in the response to a sensitizer.

ID ABS: 469

Results of a Japanese ring study of h-CLAT (5th report): a study to evaluate preservative skin sensitization potential using h-CLAT

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We are currently conducting the Japanese ring study to develop the human Cell Line Activation Test (h-CLAT). The aim of this study is to investigate whether the h-CLAT can predict the potency of preservatives to induce skin sensitization. In addition, we analyzed the effect of two solvents (physiological saline and dimethyl sulfoxide) on the prediction performance. Sixteen

preservatives used in cosmetic products, which had been previously evaluated for their skin sensitization potency *in vivo*, were selected as test chemicals, including 13 sensitizers and 3 non-sensitizers. CD86/CD54 expression on THP-1 cells was analyzed after 24 h exposure to each chemical with 8 doses based on CV75. In addition, 5 sensitizers out of 16 chemicals were tested using two solvents. All experiments were performed in 2 laboratories independently. As a result, 13 out of 16 chemicals were predicted correctly in laboratory A (Accuracy: 81%) and 12 out of 16 chemicals were predicted correctly in laboratory B (Accuracy: 75%). One false negative result and 2 false positive results were found at two laboratories. Among these chemicals, benzyl alcohol was predicted as false positive in h-CLAT, but the skin sensitization potential may be suggested in HRIPT data. Meanwhile, 5 sensitizers tested with two solvents were predicted as positive by h-CLAT in both laboratories. Therefore, it is indicated that the solvent type may not affect the predictive performance of the h-CLAT. In conclusion, it is suggested that h-CLAT is useful for evaluating skin sensitization potential of preservatives.

This study was supported by a Grant-in-aid from MHLW.

ID ABS: 486

A plasmacytoid dendritic cell-based assay to screen the allergenicity potential of chemicals

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An *in vitro* assay system that utilizes human cells to predict the allergenicity potential of chemicals will have utility throughout industry to monitor products for contact sensitization. Development of such a non-animal alternative assay system for hazard assessment directly addresses REACH (Registration, Evaluation, and Authorization of Chemicals). In this study, we investigated whether CD86 expression in plasmacytoid dendritic cells (pDC) can be used to identify contact allergens. Human DC were generated from $CD34^+$ progenitor cells and the pDC fraction ($CD123^+/CD11c^-$) was harvested using FACS sorting. The pDC were exposed to chemical allergens (n=23) or irritants (n=18). Sub-toxic concentrations of each chemical were determined using FACS analysis of propidium iodide stained cells. Allergens were identified based on stimulation index (SI) calculated by the fold increase in CD86 expression. A material that had an SI >1.5 in at least 50% of the pDC donors (n=2-5 donors) was considered an allergen. Using this methodology, CD86 expression increased >1.5 fold for 23 of 23 allergens but not for 14 of 18 non-allergens. Based on these results, a preliminary prediction model was developed to identify chemical allergens (sensitivity=100%, specificity=78%, accuracy=89%); these results were similar to those obtained using the mouse local lymph node (LLNA) assay (sensitivity=85%, specificity=83%, accuracy=84%). In conclusion, CD86 expression in pDC appears to be a sensitive and specific predictor of allergenicity. The assay is advantageous because high throughput screening of chemicals is possible, donor-to-donor variation can be monitored, the cells are of human origin, and the assay is cost effective.

ID ABS: 526

In vitro evaluation of propoxur induced oxidative stress, immunosuppression and apoptosis in avian lymphocytes

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Chemical pesticides have become an indispensable part of modern agricultural industry though not without deleterious effects on humans, animals and birds. Propoxur (2-isopropoxyphenyl N-methylcarbamate) is a carbamate insecticide of moderate mammalian toxicity. It is used in homes, hospitals, factories and stables as a potent insecticide. The present communication reports propoxur induced immunosuppression, oxidative stress and apoptosis in an avian lymphocyte cell culture system employing cytokine assay, annexin V assay, oxidative stress assay, DNA fragmentation assay and electron microscopy.

The cytokine assay revealed significant reduction in IL-1 and IL-2 levels. There was significant increase in oxidative stress in propoxur treated cells as compared to control cells as revealed by nitric oxide estimation. The annexin V assay showed an increased number of cells under going apoptosis, which was further confirmed by a typical ladder pattern as revealed through DNA agarose gel electrophoresis. Both TEM and SEM showed prominent ultrastructural changes in the cells exposed to propoxur, which showed distinct features of apoptosis, further strengthening the evidence of propoxur induced immunotoxicity in avian

lymphocytes. Thus, our findings reveal that a low level dose of propoxur induces significant immunosuppressive effects, higher oxidative stress and apoptosis in avian lymphocytes. Further, it is suggested that the *in vitro* lymphocyte cell culture system can prove helpful in preliminary screening of low level pesticide exposure and reduce experimental animal testing.

ID ABS: 543

Understanding signaling pathways induced by contact sensitizers in U937 cells

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Contact sensitizers induce several phenotypic and functional changes of 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. This marker has proven its relevance to evaluate the sensitizing potential of chemicals as demonstrated by the development of two assays: the MUSST based on U937 cells and the h-CLAT based on THP-1 cells. Beside CD86, other markers characterizing the response of DC (or DC-like cells) to haptens have been described: p38MAPK phosphorylation, apoptosis induction, Nrf-2 activation, etc. Some of these markers have also been used in cell-based assays in order to evaluate their capacity to predict the sensitizing potential of chemicals.

In the present study we aim to better understand the different signaling pathways induced by contact sensitizers in our U937 based model and to identify the links between the different biomarkers cited above. This analysis of complementarities between the different endpoints used in *in vitro* assays will give mechanistic insights that will allow us to predict skin sensitization potency of cosmetic ingredients by integrating multiple *in vitro* data.

ID ABS: 567

Co-culture systems between U937 or THP-1 and EpiSkin as new *in vitro* skin sensitization models

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In vitro skin sensitization tests focused on U937 (MUSST) or THP-1 (h-CLAT) cell activation have been developed. In such models, cells are directly treated with test chemicals in culture medium, which is highly simplified as compared to the skin sensitization process. Therefore, such submerged culture systems have some limitations. We are developing new models consisting of cell and EpiSkin co-cultures as a means of circumventing some of these limitations and taking into account the skin barrier and metabolic capabilities as well as topical application.

U937 (L'Oréal) or THP-1 (Shiseido) cells were seeded underneath EpiSkin inserts, and chemicals were applied onto the EpiSkin. After 18 h or 2 h incubation, respectively, the EpiSkin inserts were removed and the cells were cultured separately for 24 h or 22 h respectively. Cell surface expression of CD86 (and CD54 for h-CLAT) was measured by flow cytometry and EpiSkin viability by MTT assay.

In the co-culture models, two non-sensitizers, i.e. SDS and lactic acid, were found negative in both cell lines. Reference sensitizers (DNCB, PPD, Eugenol, Isoeugenol and HCA tested with THP-1; PPD, Eugenol, Isoeugenol and Ethylenediamine tested with U937) were found positive. This is a proof of concept showing the co-culture systems are functional.

Isoeugenol and HCA were false negative in h-CLAT, and SDS was false positive in MUSST, suggesting the co-culture model is useful for evaluation of various chemicals. These are first elements showing the added value of co-cultures to complement direct assays.

Further research is necessary to confirm the added value of co-culture systems.

ID ABS: 579

Internationally harmonized performance standards (PS) for the murine local lymph node assay (LLNA)

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ICCVAM, in conjunction with ECVAM and JaCVAM, developed internationally harmonized PS for the LLNA that can be used to evaluate modified versions of the LLNA. These PS include essential test method components, a minimum list of reference substances, and standards for accuracy and reliability. Essential test method components are structural, functional, and procedural elements of a validated test method that should be included in the protocol of a mechanistically and functionally similar proposed test. Essential components of the LLNA include topical application of the test substance to the mouse ears, measurement of lymphocyte proliferation in lymph nodes draining the application site, and use of the maximum soluble dose that does not result in systemic toxicity or excessive local irritation. The list of reference substances includes 13 sensitizers and 5 non-sensitizers. Four optional substances are included to demonstrate superior performance relative to the LLNA. The accuracy and reliability standards are based on the performance of the LLNA as compared to human and guinea pig results. An update to OECD TG 429 has been proposed to include these PS, which will facilitate more rapid and efficient validation/acceptance of modified LLNA protocols. New improved versions of the LLNA that offer other advantages are expected to result in broader use of the LLNA, which will further reduce and refine animal use for allergic contact dermatitis assessments. ILS staff supported by NIEHS contract N01-ES-35504. This abstract reflects the views of the authors and has not been approved by the US CPSC or other agencies.

ID ABS: 580

The updated ICCVAM recommended murine local lymph node assay (LLNA) protocol

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In 1999, ICCVAM recommended the LLNA as a valid test method to assess the skin sensitization potential of most substances. ICCVAM concluded that the LLNA provided several advantages compared to guinea pig methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. In 2002, the LLNA was adopted by OECD as TG 429, assuring its international acceptance as a test for skin sensitization potential. In March 2008, ICCVAM and NICEATM convened an international independent peer review panel (Panel) on the LLNA to evaluate alternative versions that are not based on radioactivity and new applications. ICCVAM considered the Panel's conclusions and recommendations, comments from SACATM (ICCVAM's advisory committee) and the public, and recommended updates to the protocol. These included: reducing the required number of animals from five to four per group; adding rationale for collection of individual animal data; adding guidance for use of a concurrent positive control group; and adding guidance on evaluating local irritation and systemic toxicity to establish the appropriate highest dose to test. The updated ICCVAM recommended test method protocol for the LLNA has been forwarded to US Federal agencies for their consideration as a standardized protocol for the purposes of skin sensitization hazard classification. A proposal to update OECD TG 429 has been submitted. ILS staff supported by NIEHS contract N01-ES-35504. This abstract reflects the views of the authors and has not been approved by the US CPSC or other agencies.

ID ABS: 581

ICCVAM test method recommendations for the reduced LLNA (rLLNA)

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In 1999, U.S. regulatory agencies that require submission of skin sensitization data accepted the LLNA as an alternative to guinea pig tests for assessing skin sensitization potential. In 2007, the U.S. Consumer Product Safety Commission requested ICCVAM to assess the usefulness and limitations of the rLLNA. In the rLLNA, the test substance is tested at the maximum testable dose level only, instead of a minimum of three dose levels used in the traditional LLNA. A retrospective review was conducted of data from 471 traditional LLNA studies (457 unique substances) obtained from 11 sources. Compared to the traditional LLNA, the rLLNA has an accuracy of 99% (465/471), a false positive rate of 0% (0/153), and a false negative rate of 2% (6/318). Based on these data, ICCVAM concluded that the rLLNA is sufficiently accurate to distinguish between skin sensitizers and nonsensitizers. ICCVAM recommended routine use of the rLLNA for determining the sensitization potential of chemicals and products, when dose response information is not needed. ICCVAM recommended a standardized rLLNA protocol, future studies to improve the usefulness and applicability of the rLLNA, and the evaluation of modified rLLNA test methods with LLNA performance standards. An update to OECD TG 429 has been proposed by ICCVAM to include the rLLNA, which should facilitate international acceptance of this modified protocol. Use of the rLLNA should reduce animal use for skin sensitization testing while supporting the protection of human health. This abstract reflects views of the authors and has not been approved by any agencies.

ID ABS: 588

Responses of a human distal lung co-culture model to chemical allergens

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Inhalation of low-molecular weight (LMW) chemicals is a leading cause of allergic respiratory diseases. As a possible tool to identify sensitizing chemicals cultures of the respiratory epithelium are of growing interest.

We assessed the effect of respiratory sensitizers on an *in vitro* model of the air-blood barrier, a co-culture (with 2 compartments) of human lung alveolar type 2-like epithelial cells (NCI H441) and microvascular endothelial cells (ISO-HAS-1). Cells were exposed for 24 hrs to the respiratory sensitizers ammonium hexachloroplatinate IV (HCTp), diphenylmethane diisocyanate (MDI), and trimellitic anhydride (TMA), the irritant salicylic acid (SA), and the skin sensitizer 2,4-dinitrochlorobenzene (DNCB). Following exposure cytotoxicity was determined on the different cellular types in mono- as well as in co-culture by detecting mitochondrial enzymatic perturbation (MTS) and the influence on barrier properties (TEER, TransEpithelial Electrical Resistance). Increases in cytokine levels were measured by multiplex technology. Apically exposed, the irritant SA did not induce any cytokine release in co-culture. HCTp, TMA and MDI induced increased release of the chemokines CXCL-8, CCL-2 from the endothelial cells. TEER-values of co-cultures exposed to subtoxic concentrations of LMW chemicals remained unchanged compared to untreated controls. Although barrier properties were maintained, cytokine release was detected in the lower well (basolateral) after apical exposure (epithelial side) with respiratory sensitizers. In contrast, epithelial DNCB treatment yielded small, nonsignificant increases in basolateral cytokine levels. Our data suggest that measuring a basolateral release of cytokines in apically exposed co-cultures may represent a promising *in vitro* model for the screening of potential chemical respiratory allergens.

ID ABS: 790

Characterization of early events in human dendritic cell maturation induced by contact sensitizers

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Dendritic cell (DC) maturation following exposure to chemicals is a critical step in the skin sensitization process. It is characterized *in vitro* mainly by phenotypical changes and secretion of proinflammatory cytokines. However, early events and cell signalling pathways are not yet fully identified. Previous experiments performed on mono-DCs identified two classes of sensitizers: NiSO4 activated both p38 MAPK and Erk1/2 pathways while DNCB activated p38 MAPK but markedly inhibited Erk1/2 with a cross-talk between oxidative stress and kinase phosphorylation SDS had no effect. In order to establish a predictive model accessible for all laboratories, these investigations were extended to the human promonocytic cell lines, U937 and THP-1, using weak to extreme sensitizers (according to LLNA results) and non sensitizers (lactic acid, glycerol, salicylic acid) and SDS as irritant. Our data demonstrated that in mono-DCs, main sensitizers such as DNCB induced p38 MAPK activation and Erk1/2 inhibition. In the U937 cell line, sensitizers also inhibited Erk1/2 but p38 MAPK activation was not always detected. In THP-1 cells data Erk1/2 was not inhibited but rather activated by DNCB and 4-nitrobenzyl bromide. In all cases, non sensitizers and irritant had no effect. Pretreatment of mono-DCs, U937 and THP-1 cell lines with the antioxidant, N-acetyl-cysteine, blocked kinase modulation induced by DNCB and 4-nitrobenzyl bromide suggesting that p38 MAPK and Erk1/2 are regulated via an oxidative stress. In conclusion, there is more similarity between the kinase activity of mono-DCs and the U937 cell line than with the THP-1 cell line

PO30: Neuroscience

ID ABS: 52

Towards the differentiation of mouse embryonic stem cells into functional neurons for developmental neurotoxicity testing *in vitro*

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

In the present study, we have developed a new and efficient protocol for differentiating murine ES cells into neural cells. This method is based on the formation of neuronal spheres and takes only about 12 to 14 days to produce mature neurons. The differentiation process has been well characterized my means of immunofluorescence staining of selected marker proteins specific for neuronal precursor cells as well as mature neurons and glial cells. To fully investigate the potential of our method we also determined transmitter phenotypes and performed $Ca2^{+}$ imaging to confirm that functional neurotransmitter receptors are present. Currently our efforts are focused on ES cell differentiation into neurons on the surface of microelectrode arrays. This approach will be used for extracellular recording of electrical activity patterns in neural networks that have been formed.

In comparison to previous protocols our method significantly shortens the differentiation time needed for the development of mature neurons from ES cells. At the same time, the number of maturing cells is sufficiently high to be applicable in developmental neurotoxicity testing.

ID ABS: 54

Differentiated human neuroprogenitor cells for neurotoxicity testing

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

Differentiated hNPCs express marker genes for gabaergic, cholinergic, dopaminergic and serotoninergic neurons as well as NMDA receptor subtypes. Moreover, they react to glutamate and acetylcholine with an intercellular Ca^{2+} increase. First results with model compounds show that treatment of differentiated cells with subcytotoxic methylmercury concentrations reduces beta(III)tubulin expression in Western blot analyses. Furthermore, MPP^+ (1-Methyl-4-phenyl-pyridin), which is selectively toxic to dopaminergic neurons, reduces protein levels of dopamin decarboxylase.

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

ID ABS: 62

The human dopaminergic neuronal cell line LUHMES as *in vitro* model for Parkinson's disease

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Parkinson's disease is characterized by a gradual degeneration of dopaminergic neurons in the *substantia nigra*. Dopaminergic neurons are continuously exposed to elevated oxidative stress conditions due to the unstable neurotransmitter dopamine that can easily undergo oxidation to form superoxide and a quinine-form capable to react with cysteine residues in proteins or with glutathione to form dopamine-conjugates. For investigations on the molecular events occurring under these conditions, an experimental human *in vitro* model that closely resembles the characteristics of dopaminergic neurons *in vivo* was established.

LUHMES cells were validated with respect to their response toward the parkinsonian toxin MPP+. A time-dependent degradation of neurites, accompanied by a loss of cellular ATP and GSH, and increased formation of radical species was observed. These effects were only detected in fully differentiated cells, whereas undifferentiated LUHMES demonstrated no significant response to the same toxic insult. The neurodegenerative effects observed were partially prevented or delayed by co-incubation with the mixed lineage kinase inhibitor CEP1347, or by inhibition of poly-ADP-ribose polymerase (PARP). The involvement of dopamine in the neurodegenerative process was further underlined by application of dopamine transporter, or tyrosine hydroxylase inhibitors, which significantly protected against MPP+-induced degeneration.

The herein introduced human neuronal cell line closely reflects the unique properties of dopaminergic cells *in vivo*. This model cannot only serve for basic research on the events occurring in neurodegenerative diseases, but can also be used as a screening system within neurotoxicological testing programs.

ID ABS: 164

An integrated test strategy to detect neurotoxicants and developmental neurotoxicants

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In order to obtain a comprehensive toxicological profile and to cover different steps and mechanisms, we designed a test battery consisting of different model systems. In addition we performed an extensive literature search to select a list of highly 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 or astrocytes. 2. Condi-

tionally-immortalized human neural precursor cells (LUHMES cells) were differentiated within 5 days to a homogeneous population with a complex neurite network and typical biochemical and morphological features of dopaminergic neurons, using GDNF and cAMP.

The toxicity of the compounds for developed cells was assessed using conventional cytotoxicity assays based on the metabolic capacity of the cells and their cell membrane integrity. Additionally, more advanced endpoints like neurite mass, nuclear condensation and cell number were analysed, using high content analysis methods. These endpoints were measured with the objective of establishing more sensitive and in case of the neurite mass also more neuronal specific endpoints.

In order to identify compounds which exhibit a neuronal developmental specific toxicity, the compounds were tested in the two systems during various developmental phases. IC50 values obtained thereby were then plotted against each other, and based on these plots potentially neurotoxic and developmental neurotoxic compounds were identified. Based on the IC50 values in the different systems, additional data from primary cells and simple cell lines, toxicity profiles of the different compounds were established.

ID ABS: 194

Generation of functional astrocytes from murine embryonic stem cells

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Astrocytes are the most abundant glial cells in the brain. They are involved both in the maintenance of physiological homeostasis and in inflammatory responses. Research into the cell biology of these cells would benefit from the availability of astrocyte cultures free of other glial contaminants. One approach to obtaining a reliable and reproducible cell culture system is differentiation of the cells from embryonic stem cells (ESC). We present here a method to generate highly astroglial-enriched cultures from ESC, and characterise them for inflammatory signalling responses. During the differentiation of both embryonic and neural stem cells, astroglial markers (GFAP, S100b, A2B5, CD44) were upregulated while the transient neural markers nestin, NCAM, and bIII-tubulin were downregulated in the final cultures. The presence of astroglial markers in about 90% of the cells of the culture and the absence of the neuronal markers NCAM and bIII-tubulin indicate a successful differentiation process. We demonstrated inflammatory signalling capability of our astrocytes by nuclear factor kB translocation into the nucleus and an increased release of IL-6 and NO into the supernatant upon stimulation with a proinflammatory cytokine mix. ESC-derived astrocytes and neural stem cell-derived astrocytes showed the same response pattern as astrocytes isolated from mouse brain.

ID ABS: 217

Zebrafish (*Danio rerio*) as a versatile *in vivo* screening platform

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Solvay Pharmaceuticals continuously seeks to reduce its animal use within early drug discovery. Accordingly, zebrafish larvae up to 7 day post fertilization were considered as a possible alternative screening platform. Since zebrafish are complex vertebrates, they may provide predictive validity and proof of concept up to lead class selection.

Zebrafish are easy to breed and maintain. Larvae have no blood-brain barrier, and compounds can be added to their biotope. Finding active compounds *in vivo* in an early stage reduces the number of rodents needed for research on novel targets dramatically.

Zebrafish are potentially applicable to any target. Here we investigated zebrafish larvae as a model for Parkinson's disease (PD) both at a behavioral and biochemical level. Zebrafish are sensitive to dopaminergic neurotoxins such as MPTP and 6OHDA. Incubation with these toxins reduced dopamine levels and spontaneous locomotion by 60%. This could be blocked by the selective dopamine reuptake blocker Nomifensine.

Furthermore, the D1/D2 receptor agonist Apomorphine dose-dependently increased locomotor activity. This effect could be antagonized by cis-Flupenthixol (D1/D2 receptor antagonist) and SCH 38393 (selective D1 receptor antagonist).

Our data show that zebrafish display dopamine related behavior and biochemistry which are deemed relevant for PD. Multiple pharmacological effects can be measured and allow for lead class selection at an early stage. Taken together, zebrafish larvae offer a versatile pharmacological platform to assess *in vivo* efficacy of compounds in early drug discovery as an alternative to rodents.

ID ABS: 250

The suitability of BV2 cells as alternative model system for primary microglia cultures or animal experiments of brain inflammation

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The role of microglia in neurodegeneration, toxicology and immunity is an expanding area of biomedical research requiring large numbers of animals. Use of a microglia-like cell line would accelerate many research programs and reduce the necessity of continuous cell preparations and animal experimentation, provided that the cell line reproduces the situation *in vivo* or in primary microglia (PM) with high fidelity. BV-2 have frequently been used as a substitute for PM, but recently doubt has been raised on their suitability. Here, we re-evaluated strengths and potential shortcomings of BV-2 cells. Their response to lipopolysaccharide was compared with the response of microglia in *vitro* and *in vivo*. Transcriptome (480 genes) analysis after stimulation with lipopolysaccharide indicated a reaction pattern of BV-2 with many similarities to that of PM, although the average upregulation of genes was less pronounced. The cells showed a normal regulation of NO production and a functional response to IFN-γ, as would be important for their interaction with T cells and neurons. BV-2 were also able to stimulate other glial cells. They triggered the translocation of NF-κB, and a subsequent production of IL-6 in astrocytes. Thus, BV-2 appear to be a good substitute for PM in many experimental settings, including complex cell-cell interaction studies.

ID ABS: 254

Mouse embryonic stem cells in reproductive toxicology: establishment of a predictive *in vitro* test module for developmental neurotoxicity testing

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Pluripotent mouse embryonic stem (ES) cells are capable of differentiating into many fully specified and functional cell types including neural cells while recapitulating *in vivo* developmental programs. Therefore, these cells offer the unique opportunity to study adverse effects on neural cell development *in vitro* after exposure to toxic compounds.

In the context of a complex modular strategy for developmental neurotoxicity testing, we are currently developing a predictive *in vitro* test module using the mouse ES cell line D3. To this end, we established a robust and fast method for differentiation of D3 cells into neurons, astrocytes or oligodendrocytes suitable for testing of chemicals and other compounds. The protocol was also adapted to a 96-well plate format in order to facilitate higher throughput. Furthermore, to assess adverse effects on neural cell differentiation and proliferation predictive toxicological endpoints have been established using flow cytometry of neuron-specific as well as glia-specific marker proteins and proliferation assays. Here we present the results obtained with selected compounds of known and characterized developmental neurotoxic potency *in vivo* as well as negative control substances. Comparative evaluation of dose response profiles revealed significant differences in the sensitivity of undifferentiated ES cells, 3T3 fibroblasts and ES cells differentiating into neurons. Overall our results suggest that the mouse ES cell model provides trustful and reliable results in the detection of developmental neurotoxicants.

ID ABS: 303

The use of non-invasive brain stimulation techniques in the investigation of visual attention

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Understanding the neural basis of attentional control is one of the great outstanding problems of modern neuroscience and is essential if we are to unravel the mystery of unilateral neglect – one of the most disabling, frequently examined and yet mysterious neuropsychological disorders. The problem of attention is also at the core of many neuroscientific questions ranging from the basis of consciousness to the neural mechanisms of action selection. The principal brain structures involved in the control of visual attention have already been identified. However, the specific role of each brain structure within this attentional network and the way in which these different structures work together is poorly understood. To address these questions we are examining how information flows between these brain structures; how they interact with each other, i.e. how disruption of one brain structure affects the contribution of other structures; and what specific contribution each of these structures provide towards the control of attention. Transcranial magnetic stimulation (TMS) combined with the visual search paradigm provides a highly effective strategy to study these questions without recourse to traditional primate experimentation that would involve lesions, brain cooling or micro-stimulation techniques in the live monkey. Using dual site TMS we are uncovering compensatory mechanisms that could be recruited in the case of damage to other regions.

Acknowledgement: Funded by The Dr Hadwen Trust for Humane Research, the UK's leading medical research charity funding exclusively non-animal research techniques to replace animal experiments.

ID ABS: 315

Conventional and innovative animal friendly technologies for developmental neurotoxicity testing: a model study in rats with MeHg

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An extensive (neuro)developmental study (OECD 415; EPA OPPTS 870 6300, 6800) was carried out in rats with the model developmental neurotoxicant methylmercury (MeHg; 0, 0.1, 0.4, 0.7, 1.0, 1.5, 2.0 mg/kg body weight; GD6-PN10). The study was designed to gather information on the relationship between conventional technologies and indicators for developmental (neuro)toxicity (mainly landmarks, behavior and an extensive neuropathology survey), versus new (innovative) technologies, i.e. stereology, toxicogenomics, *in vivo* PET-imaging and *in vitro* field potentials in hippocampal brain slices. In addition, a comparison was made between a classical NOAEL and a benchmark approach. The innovative technologies were assessed on the control group and some of the MeHg groups. The results showed that, whereas conventional endpoints showed hardly if any effects of perinatal exposure to MeHg, the new technologies all pointed at a delay in neural development and persisting deficits in the brain of the offspring during adulthood resulting from maternal exposure to MeHg. Moreover, the statistical power of the benchmark approach appeared stronger than that of the NOAEL approach since, unlike with the NOAEL approach, a dose response relationship was demonstrated for most parameters. Together, the results demonstrate that modern technologies and statistical approaches may be very sensitive and may reflect serious life-lasting effects. Their discriminative power appears so strong that compared to conventional endpoints the number of animals involved can largely be reduced. The relevance of the findings will be discussed in the context of regulatory testing for developmental (neuro)toxicity and the 3Rs.

ID ABS: 419

mRNA expression is a relevant tool to identify developmental neurotoxicants using an *in vitro* approach

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To date only a few industrial chemicals have been identified as developmental neurotoxicants. Since the current developmental neurotoxicity (DNT) guidelines (OECD TG 426) are based entirely on *in vivo* studies that are both time consuming and costly, there is a high demand to develop alternative *in vitro* methods for initial screening to prioritise chemicals for further DNT testing. Here, the gene expression at the mRNA level was evaluated to determine whether this could be a suitable endpoint to detect potential developmental neurotoxicants. Primary cultures of cerebellum granule cells (CGCs) were exposed to well known (developmental) neurotoxicants (methyl mercury chloride, lead chloride, valproic acid and tri-methyl tin chloride) for different time periods. A significant down-regulation of the mRNA level for the neuronal markers (NF-68, NF-200, NMDA-R and GABAA-R) was observed after exposure to methyl mercury chloride, valproic acid and tri-methyl tin chloride. Moreover, a significant increase in the neural precursor marker nestin mRNA was also observed. The mRNA expression of the astrocytic markers (GFAP and S100β) was unchanged. In contrast, exposure to lead chloride significantly decreased the mRNA level of the astrocytic marker GFAP while the neuronal markers were less affected. These results suggest that gene expression could be used as a sensitive tool for the initial identification of DNT effects induced by different mechanisms of toxicity in both cell types (neuronal and glial) and at various stages of cell development and maturation.

ID ABS: 791

Development of *in vitro* test model for developmental neurotoxicity using embryonic stem cells

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The availability of embryonic stem cells (ESCs) has recently been focused on developing an *in vitro* test to assess toxicity of chemicals as well as cell replacement therapy. In the previous study, we developed an *in vitro* model for developmental neurotoxicity testing (DNT) using mouse ESCs. The model was based on the default differentiation method, one of neuronal differentiation methods of mouse ESCs. Here, we did collaborative study for optimization of the DNT model. We improved the condition of neuronal differentiation of ESCs into N2B27 medium without basic fibroblast growth factor (bFGF) and ascorbic acid. Neuronal markers, microtuble associated protein 2 (MAP2) and ß-? tubulin (TUJ1) were measured for the determination of ID50 value of neuronal differentiation by developmental neurotoxicant using flow cytometry and Taqman® PCR. To investigate the specificity and the transferability of this DNT model among laboratories (Labs), three Labs compared embryonic stem cell test (EST) with the DNT model using developmental neurotoxicants (lead (?) acetate and aroclor 1254) and negative control (penicillin G). In all three Labs, Aroclor 1254 and penicillin G were classified as weakly embryotoxic and non embryotoxic respectively, whereas lead (?) acetate was misclassified as non embryotoxic in EST prediction model. On the other hand, lead (?) acetate was correctly classified as weakly embryotoxic in the DNT model. The results of the other chemicals in the DNT model were the same as those in the EST. There were no variations among the results of three labs. We also tested other chemicals such as valproic acid, methylmercury and arsenic compounds to develop a prediction model specific for this optimized model. These results suggest that this optimized model is specific for developmental neurotoxicity testing and transferable among labs. This optimized model can also be used as a test model of *in vitro* developmental neurotoxicity.

Authors

The Congress Co-Chairs and the Alternatives Congress Trust would like to express their gratitude to all those who have generously supported the Congress:

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ALTEX
www.altex.ch

A journal for new paths in biomedical science

Vol. 26 (Special Issue/2009)

Official organ of: CAAT, Center for Alternatives to Animal Testing, Johns Hopkins University, Baltimore, USA; EUSAAT, European Society for Alternatives to Animal Testing, Vienna, Austria; t4 – transatlantic think tank of toxicology, Baltimore, USA, Konstanz, Germany, Utrecht, The Netherlands; Doerenkamp-Zbinden Chairs in Germany, India, the Netherlands, Switzerland and USA;

Address:
Society ALTEX Edition
Weinmanngasse 86,
8700 Kuesnacht ZH, Switzerland
Phone: +41 44 3800830
Fax: +41 44 3800832
e-mail: editor@altex.ch

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ALTEX Online:
www.altex-edition.org
English summaries: http://altweb.jhsph.edu
ALTEX is indexed in MEDLINE, Current Contents®, SciSearch® and ISI Document Solution® and eMBASe.

Layout: H. P. Hoesli, Zurich, Switzerland

Print: Stuertz GmbH, Wuerzburg, Germany

Distribution:
ALTEX, Weinmanngasse 86
8700 Kuesnacht ZH, Switzerland
Phone: +41 44 380 0830; Fax: +41 44 380 0832
Fax: +49 7531 25833
e-mail: abo@altex.ch

Publisher:
Spektrum Akademischer Verlag.
Springer Science + Business Media.

Print run, Spec. Issue 2009:
1,800

Circulation:
5 times per annum (four English and one German issues) plus supplements

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