12th *ecopa* Annual Workshop
"THE FUTURE OF THE 3Rs – FROM INNOVATION TO VALIDATION"
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ABSTRACTS

THE AXLR8 PROJECT

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AXLR8 is a co-ordination project funded by the European Commission Directorate General for Research and Innovation under the Health theme of the EU's 7th Research Framework Programme. It is the aim of AXLR8 to lay the groundwork for a transition in toxicology toward a more pathway-based *in vitro* and computational approach through enhanced networking and collaboration among scientists, regulators and other key stakeholders at European and international levels. A major activity of AXLR8 during the past year was organising the second annual workshop, which brought together representatives of projects funded by the FP6/7 health and environment programmes, the heads of Member State centres on alternatives to animal testing, and the leaders of international efforts to establish advanced molecular toxicology from Japan and the United States. On the basis of workshop presentations and breakout group discussions, the AXLR8 Scientific Panel recommended a 'Roadmap to Innovative Toxicity Testing' in the context of the EU's forthcoming 'Horizon 2020' research funding programme. The Panel envisioned a large-scale public-private partnership to fund six research 'clusters' — encompassing five human health effect areas (cancer, development, immune system, reproduction, specific target organ toxicity) together with a cross-cutting infrastructure cluster — all under the direction of a central co-ordination action. Within each cluster there should be a strong focus on core 'building blocks', e.g. elucidation of modes of action and critical pathways, developmental of experimental and systems biology models, kinetic modelling, etc. The AXLR8 Progress Report 2011 & Workshop Report on a Roadmap to Innovative Toxicity Testing is available online at axlr8.eu.

INCONSISTENCIES IN EU DATA REQUIREMENTS

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Present and future EU legislation on the protection of animals used for scientific purposes (directives 86/609/EEC and 2010/63/EU) requires that, wherever alternative methods recognised by Union legislation are available, they have to be used instead of animal tests. Unfortunately, this principle is not implemented to its full extent when it comes to risk assessment that chemicals and new products have to undergo prior to their authorisation and placing on the market.
In a recent study funded by the *set* foundation (the German *ecopa* member), the Animal Welfare Academy screened data requirements of relevant EU laws and provisions regarding chemicals (REACH), biocides, pesticides and food safety and found that test methods as part of the risk assessment do not reflect the state-of-the-art of science and technology. Most of the data requirements we investigated still require testing on animals for many toxicological endpoints, even though 40 alternative testing methods accepted on EU- or OECD-level (ICCVAM, Mar 2011) are at hand. This unacceptable state of affairs is due to a multitude of reasons. These may range from shortage of manpower to implement existing knowledge and expertise in the field of alternative methods to unclear and misleading statements on the applicability and state of validation of alternative methods.
In conclusion, we strongly suggest a homogeneous EU-wide strategy for all areas involving risk assessment of substances with the aim to better implement the 3Rs and comply with the directives 86/609/EEC and 2010/63/EU. As a positive side-effect, this would clearly simplify data requirements, save costs on various levels and improve product safety for consumers.

THE FUTURE OF VALIDATION – SCRUTINY AT STAKE?

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The first concepts for the validation of alternative toxicological test methods have been developed in Europe in 1990 in the Amden I and Vouliagmeni Workshops, then refined in the Amden II Workshop in 1995, and later gained supra-national recognition in two OECD Workshops in Solna 1996 and 2002 in Stockholm. It took another three years until full consensus was reached and OECD Guidance Document (GD) No. 34 on principles and procedures of validation and acceptance of new toxicological test methods was adopted in 2005. Interestingly, during the process of harmonization at the OECD level finally everyone agreed that the principles of OECD GD 34 - originally defined to assure only the validity of new alternative methods – are valid for all new methods, regardless whether *in vitro* or *in vivo*, and whether used for human or environmental health. OECD GD 34 is based on the experience of various successful validation approaches, both prospective validation studies and retrospective validation assessments.
Six years after publication of GD 34 the world has changed. The new EU chemicals legislation REACH and the 7th Amendment of the Cosmetics Directive 76/768/EEC are both calling for reduction and avoidance of animal testing, in the latter case even for a total ban of animal testing. Moreover, publication of the new concept of "Toxicology of the 21st Century" of the US Research Council in 2007 has led to research funding programs with a drastic increase of *in vitro* testing intended to be used in the regulatory context. Finally, new toxicological concerns have created new toxicological endpoints of interest, like endocrine disruption and developmental neurotoxicity, both leading to the development and validation of numerous new alternative tests.
What is the impact of these new developments on the principles of validation and acceptance defined in GD 34? The political pressure in Europe has definitely speeded up consolidation processes, both at the EU and OECD level by shortened commenting periods and consolidations throughout the year in written procedures. However, is the pressure in Europe only streamlining a so far too slow process, or is the process of thoroughly evaluating the validity of new assays at stake? My personal view is "*yes, scrutiny is possibly at stake*".
For the new approach of assessing the validity of evidence based "puzzle toxicology" we do not have an answer yet. However, though this shift of paradigm seems to be the only way forward to non-animal based toxicology, an appropriate application of these techniques is more demanding of careful experimental design than ever, as the potential to generate incomplete and misleading data is great.
In summary (and restricted to the current situation in the EU framework programs), the current exclusive funding of R&D of new *in vitro* methods without a solid funding of projects assessing their validity for predicting human and environmental health is unacceptable. In contrast to evaluating the accuracy of new analytical methods in ring trials, validation of alternative methods is a scientific process, as it includes the assessment of their relevance and limitations.

THE PROCESS OF VALIDATION: THE POINT OF VIEW FROM THE INDUSTRY

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Validation is the process by which the adequacy, reliability and relevance of new or revised methods are established. Several approaches of validation can be considered:

(1) In-house studies to assess the validity of a new method to detect specific toxicity findings. These new methods can help the industry to make decisions for compound selection and can be tuned to screening tests.
(2) Pre-validation studies which are essential for a good validation procedure later on.
(3) International retrospective and/or prospective validation of methods to secure regulatory acceptance.

Of utmost importance for industry is that new validated methods are regulatory accepted on a worldwide basis. For this purpose, OECD has published, based on existing validation processes at ECVAM, ICCVAM and JaCVAM, an international guidance on validation (OECD 2005). Recently, the process of validation has been adjusted at ECVAM. The whole process and new approaches in the process of validation will be considered during the presentation.
In the Pharmaceutical Industry, once a new drug is released on the market, a pharmacovigilance phase is installed for follow up of the new drug to deal with problems which might be encountered due to 1) broad use, and 2) misuse of the drug. Similarly for newly validated methods, a comparable post-validation phase should be installed for new regulatory accepted alternative methods. For this post-validation phase, one of the "VAM's" should take the task to collect the new information and to fine tune the applicability domain.

THE POINT OF VIEW OF THE CRO
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The general trend of industry is to contract out most of the non-clinical development. This tendency is even more marked for small-size and mid-size companies which prefer to rely on established techniques and trained personnel rather than investing valuable resources, time and money in internally set-up and standardize procedures which would be carried out only seldom.
To meet the expectations from different Sponsors, non-clinical CRO must comply with regulatory requirements, ensure an updated knowledge and standardized procedures and build historical data to be used as reference. One hurdle for the CRO is the balance between the immediate investments and the delayed validation project payback in terms of both rate and time. For a CRO it is pivotal to make a wise selection of services which are more likely to be required by the market. The CRO must keep in mind that industry may have a conservative approach driven by the risk aversion: fear that results from alternative studies might be not readily accepted by regulatory authorities as the ones from conventional studies. Furthermore alternative tests are not necessarily cheaper than long-established ones and it poses a challenge for CRO to promote new tests. On the other hand the CRO involvement in the validation process represents an excellent occasion to gain experience regarding opportunities and challenges of the new method both from the technical and result interpretation points of view.
In this presentation the author will share with the audience some real experiences RTC went through in the past years.

IN VITRO BBB MODELING: FROM RESEARCH TO HIGH-THROUGHPUT SCREENING
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The market for neuropharmaceuticals is regarded as one of the potentially largest sectors of the global pharmaceutical market owing to the increase in average life expectancy. But the value of many promising CNS drug candidates is diminished by the presence of the Blood-Brain Barrier (BBB) located at the level of brain capillaries.
Since the early 90's, our goal is focus on the development of relevant in vitro Blood-Brain Barrier (BBB) models. Our first BBB model consisting of a co-culture of bovine brain capillary endothelial cells (BBCE) together with rat glial cells has been successfully used for medium-throughput screening and mechanistic purposes for more than a decade. By modifying this highly predictive model, a procedure has been developed to obtain a differentiated endothelial cell monolayer after only 4 days and without using primary glial cells to substantially reduce the set-up time and the use of animals. It's the only in vitro BBB model suitable for High-Throughput Screening (HTS) of compounds (drug, chemicals, cosmetics and consumer products). Now, our last development consists of a BBB in vitro model available in frozen ready-to-use format. This model user-friendly considerably reduces the technical needs to obtain in a quick time a functional in vitro BBB model that mimics in vivo situation.
All these models are developed to answer of the growing needs for identifying compounds that have lowest risk for toxicity and highest probability of success in accordance to the concept of 3R's.

MODULATION OF ENDOTHELIAL CELLS MORPHOGENESIS BY GLIAL CELLS IN BLOOD BRAIN BARRIER *IN VITRO* MODELS.
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The design and discovery of drugs that can readily cross the blood brain barrier (BBB) is a major bottleneck in the development of drugs targeting the Central Nervous System. On the other hand, nowadays there is a growing interest to minimize the use of animal experimentation mainly due to ethical issues and high experimental cost. Therefore the development of *in vitro* BBB models that preserve *in vivo* transporter functions to assess drug toxicity, permeability and safety at the earliest stages of drug discovery is of utmost importance for the Pharma industry. The aim of the present study was to evaluate the role of astrocytes on the modulation of paracellular permeability and morphogenesis in different BBB cell culture-based models.
Two different cell lines, Caco-2 and MDCKII, were cultured alone or co-cultured with rat primary astrocytes. The development of the barrier was monitored by the measurement of the transendothelial electrical resistance (TEER).

Moreover, Lucifer Yellow permeability assays and tight junctions cytometry studies were carried out to detect functional differences on the BBB models. In the presence of glial cells, co-cultures developed elevated TEER respect to cell cultures without astrocytes. Likewise, paracellular transport studies indicated lower Lucifer Yellow absorption values and therefore, an increase in selective permeability. Higher tight junction protein expression was observed in co-cultures compared to cells grown as monolayer.
Our results showed that the presence of astrocytes in the co-cultures enhances the adhesion and permeability properties of the BBB models. Future efforts should be directed towards improving existing models.

TOLERANCE OF FLURBIPROFEN LOADED POLYMERIC AND LIPID NANOPARTICLES FOR TOPICAL ADMINISTRATION

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Polymeric and lipid nanoparticles made from biodegradable components, have successfully used as topical drug delivery systems. Flurbiprofen, a water-insoluble NSAIDs drug, is a nonselective inhibitor of prostaglandin biosynthesis, by inhibiting cyclooxigenase, and is indicated for the acute or long-term treatment of the sings and symptoms of gout, osteoarthritis or rheumatoid arthritis. Furthermore, it is currently used as a first-line ophthalmic medication for the inhibition of miosis induced during the cataract surgery. Nanoparticles of non-steroidal anti-inflammatory drugs, such as flurbiprofen, has been reported as a promising approach to improve the drug's bioavailability and biocompatibility, avoiding the characteristic side effects of these drugs.
Alternative tests, based on the different physiological changes associated with ocular irritation have been developed. In vitro tests differ widely in their requirements and ability to predict irritancy potential.
The main goal of this study was the determination of potential topical irritation induced by flurbiprofen loaded polymeric and lipid nanoparticles by in vitro test: the chick chorioallantoic membrane (HET-CAM) assay and the Eyetest™, which evaluate the conjunctival inflammatory response and the increase of corneal opacity respectively and skin test to evaluated skin tolerance. In vitro tests displayed a good correlation with the in vivo data, evaluated by Draize test. The results obtained demonstrated that the tested nanoparticles were well tolerated and both, the ocular and skin surface tissues, remained normal after in vitro/in vivo exposure. These results support the potential use of these colloidal systems as drug carriers to treat topical (ocular or skin) surface disorders.

RAPID PROTOTYPING AS A METHOD FOR DEVELOPMENT OF STRUCTURE-ACTIVITY RELATIONSHIPS

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In the development of alternative methods, the utilisation of existing knowledge using in silico methods is a complimentary approach to in vitro studies. The development of structure-activity relationships (SARs) to predict the potential toxicity of new compounds provides the user with predictive tools, which are based on mechanistic understanding supported by the published literature. Despite the advantages of using expert-derived SARs, the knowledge acquisition and development methodology is a time consuming process. We investigated a number of strategies in an effort to speed up this process. Methods for optimising the process for assessing structure-toxicity data by rapid prototyping of SARs for both studied and new endpoints are described. Data sets covering a number of toxicological endpoints including hepatotoxicity, nephrotoxicity, bradycardia, mitochondrial dysfunction, and bone marrow toxicity were analysed. The compounds were clustered using automated methods or by visual analysis depending on the size of the data set and the proportion of positive compounds. The clusters were then assessed according to specific biological criteria for each endpoint to identify suitable SARs. This resulted in the development of over 150 rapid prototype SARs. The advantage of this approach is that with each data set it allows efficient coverage of chemical space and an overview of the potential SARs. Where required, investigations of the literature can also be carried out to identify supporting evidence. The rapid prototypes identified in this step can then be assessed against new data and investigated in depth for development as complete expert-derived SARs.

USE OF BIOMARKERS OF DIFFERENTIATION FOR IMPROVING THE EMBRYONIC STEM CELL TEST
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We have recently published a procedure to improve the performance of the embryonic Stem cell Test (EST) (Journal of Toxicology, doi:10.1155/2011/286034). The EST is a validated assay for testing embryotoxicity *in vitro*. The total duration of this protocol is 10 days, and its main end-point is based on histological determinations. It is suggested that improvements on EST must be focused toward molecular end-points and, if possible, to reduce the total assay duration. Five days of exposure of D3 cells in monolayers under spontaneous differentiation to 50 ng/mL of the strong embryotoxic 5-fluorouracil or to 75 *μ*g/mL of the weak embryotoxic 5,5-diphenylhydeantoin caused between 20 and 74% of reductions in the expression of the following genes: *Pnpla6*, *Afp*, *Hdac7*, *Vegfa*, and *Nes*. The exposure to 1mg/mL of non-embryotoxic saccharin only caused statistically significant reductions in the expression of *Nes*. These exposures reduced cell viability of D3 cells by 15, 28, and 34%. We applied these records to the mathematical discriminating function of the EST method to find that this approach is able to correctly predict the embryotoxicity of all three above-mentioned chemicals. Therefore, this work proposes the possibility of improve EST by reducing its total duration and by introducing gene expression as biomarker of differentiation, which might be very interesting for *in vitro* risk assessment embryotoxicity..

CHANGES IN THE GENE EXPRESSION OF MOUSE EMBRYONIC STEM CELLS AFTER 12HOURS EXPOSURE TO CHEMICALS. I.e.: CHLORPYRIFOS AND ITS METABOLITE
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We are working in the development of an improved short test of embryotoxicity test based on molecular gene expression endpoints in the cell differentiation process of D3 mouse embryonic stem cells. In this work it is applied to the example of an organophosphate pesticide chlorpirifos (CPF) and its metabolite chlorpyrifos-oxon (CPO).This pesticide is not classified as toxic to development although there are some doubts concerning the potential effects on development. D3 cells seeded in monolayer and in differentiation were incubated during 12 hours in presence of CPF and CPO. The treated cells were collected after 12 hours. The expression of different genes was quantified using qPCR. The expression of α-fetoprotein, gene marker of the differentiation to visceral endoderm was significantly increased ($p<0.01$) by 32 and 12 times after exposure to 100 μM CPF and 400 μM CPO, respectively. These exposures were the maximum tolerable concentration under citotoxicity criteria, causing only slight cytotoxicity with reduction of cell viability in both cases lower than 20%. The same exposure to CPO caused significant ($p<0.05$) increases (by 1.9 times) and reductions (by 89%) in the expression of Nanog (marker of pluripotentiality) and Flk1 (marker of differentiation to mesoderm), respectively. In addition, Pnpla6, gene codifying the protein NTE was assessed as well as longer exposure periods.

THERMOLUMINESCENCE AS A USEFUL TECHNIQUE IN TOXICOLOGY
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A model was developed to evaluate the alterations produced by environmental chemicals on the physiology of photosystem II thanks to standard thermoluminescence of B band. In addition, high temperature thermoluminescence was applied, quantifying HTL_2 band as a biomarker of lipid peroxidation on vegetables. Thermoluminescence probes the emission of luminescence associated with the de-trapping of a radical pair at specific temperatures. In photosynthetic organisms, most of the thermoluminescence emissions are due to the reverse reaction of charge separation, induced by the light in the photosystem II. The photosynthesis initiates in the photosystem II complex; the light is absorbed and promotes an electronic flow from photosystem II to photosystem I to generate reduction power and a gradient or protons, needed for the vital activity of photosynthetic organisms. Illumination of photosynthetic material at low temperature produces a stable separation of charges among the donor and the acceptor of photosystem II. The different recombinations generate characteristic thermoluminescence bands. The effects of the chemicals bromobenzene, diethanolamine, chloroquine, sodium fluoroacetate, propyl gallate and indium nitrate were studied in the alga *Chlorella vulgaris*. The most relevant effects were observed for the chemical intermediary diethanolamine. A considerable reduction on the functionality of photosystem II and an increase of lipid peroxidation

levels were detected. Furthermore, the study of thermoluminescence emissions is a useful technique to study the effects of environmental chemicals on photosynthetic organisms**.**

LEITAT CENTERS ITS ACTIVITY ON ALTERNATIVE TESTING STRATEGIES IN ORDER TO RESOLVED NEEDS IN HEALTH AND WELFARE

QUINTÀS G, VILÀ M, ROMERO J, ZURBANO MJ, DE LA VARGA M, GOMBAU L

LEITAT is a Technological Center that collaborates with companies and institutions focusing on research, development and industrial innovation. Its experience in different fields (biomedicine, nanomaterials, environment, industrial biotech, etc) creates synergies among research areas. Biomed Division R & D lines include identification of new targets for oncology and generation of New Molecular Entities to modulate them. It eventually fully characterize its *in vitro* & *in vivo* profiles in order to deliver these innovative drugs ready for clinical phases. Industrial Biotech provides biotechnological solutions (microorganisms and enzymes) with application in industrial processes while the NanoHealth & Safety group evalutes the potential risks of nanomaterials to human health and to the environment, and explore their benefits for diagnostics and therapeutics. The BioInvitro Division is the new unit of Leitat with more than 10 years experience in developing *in vitro* models capable of covering both safety and efficacy testing of raw materials or final products as an alternative to animal testing strategies. Skin biology and drug pharmacokinetics (ADMETox) are its main areas of research. Available *in vitro* models for skin care include skin fibroblasts, keratinocytes, reconstructed 3D human tissues and adipocytes, providing information on vitalizing and moisturizing products, effects on firmness, elasticity enhancers, anti-radicals claim and anti-cellulite products among others. BioInvitro also assists clients' problems solving in ADMETox to support their drug discovery and development processes. Ready-to-use Caco-2 and CacoGoblet cells, liver subcellular fractions, engineered cell lines individually expressing CYP450 isoforms and freshly isolated hepatocytes are the model systems available for this purpose.