Theme 5
Safety Testing, Validation and Risk Assessment

Chairs:
Bob Combes (United Kingdom)
Len Schechtman (USA)

Session 5.1
Strategies for using non-animal methods in relation to HPV, endocrine disruptors and REACH legislation

Poster

A comparison of high throughput reporter gene assays as *in vitro* alternatives to *in vivo* endocrine disrupter screening

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Exposure of humans and wildlife populations to increasing levels of endocrine disrupting chemicals (EDC's) in the environment has focused attention of the US EPA on standardisation and validation of *in vivo* screens for EDC's. In this study a comparison of sensitive, rapid and cost effective *in vitro* alternative methods for detection of EDC's was conducted using high throughput yeast and mammalian cell reporter gene assays and results were evaluated against *in vivo* uterotrophic and Hershberger assays conducted in rats. The yeast assay (developed by GlaxoSmithKline) employed *Saccharomyces cerevisiae* containing the DNA sequence of the human oestrogen receptor (hER) and an oestrogen-responsive ERE/lac-Z reporter gene plasmid in a 96 well-plate chromogenic assay measuring absorbance at 540 nm. The mammalian cell assay used human ovarian carcinoma cells containing an oestrogen-responsive ERE/luc 7 reporter gene plasmid with measurement of luciferase expression. A range of oestrogenic and androgenic steroids, plasticisers, organochlorine pesticides and surfactants were screened and assessment of agonist and antagonist actions made. The results reported show a high degree of sensitivity and reproducibility between the *in vitro* reporter gene assays and compare favourably to the *in vivo* endocrine disrupter assays. It is considered that Regulatory acceptance of standardised and validated *in vitro* alternative assays for EDC's, such as described here, will reduce the need for animal testing and provide sensitive, high throughput capacity to facilitate broad and cost effective uptake of tier I screening methods for endocrine disrupters worldwide.

Poster

A novel non-radioactive method for measuring *in vitro* aromatase activity using the H295R, a human adrenocortical cell line

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Certain environmental contaminants are suggested to disrupt endocrine processes with potential effects on reproduction, sexual differentiation, growth and development. Current research has mainly focused to interactions with the sex hormone receptors. However, other mechanisms such as effects on steroid synthesis and metabolism should be considered. Some chemicals have been shown to interact with aromatase (CYP 19) and consequently have a profound effect on hormone function and homeostasis. Aromatase is of particular interest because it is the rate limiting catalyst in the formation of estrogens. It is of importance, not only in cells involved in the *de novo* synthesis of estrogens, but also in brain and adipose tissue, which utilise circulating levels of androstenedione or testosterone as precursors. A novel non-radioactive method for measuring aromatase activity using H295R cells (human adrenocortical carcinoma) was developed through the optimisation and application of an ELISA-method for estrone in cell culture conditions. The aromatase assay was first tested using pure chemical compounds, such as atrazine, prochloraz, tributyltin, forsfokolin. Both induction and inhibition of aromatase could be detected. The induction responses were accompanied by increases in CYP19 RNA levels, determined by real-time RT-PCR. In order to apply this *in vitro* aromatase assay for assessment of environmental exposure, selected chemical compounds were spiked in water and a solid phase extraction method (SPE) was optimised. With an adequate sample treatment method, real environmental water samples are ready to be analysed and the environmental load of chemicals with potential effects on steroid metabolism can be estimated.

Lecture

REACH – CEFIC's conception of a feasible, information- and priority-based approach

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The European Commission's proposal for a new Chemicals Legislation, REACH, presented as of 29 October 2003, is currently under strong discussion in the processes of the first readings in the EU-Council and in parallel in the EU-Parliament.

It foresees in a very formal and systematic manner obligations to industry to register all substances and submit volume dependant information packages on each substance manufactured or imported above 1 ton/year. It further requires comprehensive documentation of the properties, hazard studies and profiles. Furthermore it stipulates substantial analysis of exposure through extensive surveys of uses. Routinely, starting with substances above 10 tons/year a Chemical Safety Assessment is needed. For dangerous substances a Chemical Safety Report has to be submitted.

The European Chemical Industry as associated within CEFIC has engaged itself in the search and development of proposals to improve the present European regulatory schemes on substances. Specifically CEFIC proposes to achieve the objectives of REACH in a less bureaucratic and thus in the long run less burdensome way.

In doing so the CEFIC proposal focuses on defining the appropriate scope of REACH, on priority setting according to risks likely going along with substances' uses, and on improving the work flow between all actors.

The paper will highlight the key elements of the industry proposal. The prerequisites for a more efficient system shall be explored.

Poster

ICCVAM recommended reference chemicals for validation of *in vitro* estrogen and androgen receptor binding and transcriptional activation assays

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In 1998, the U.S. EPA's Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the standardisation and validation of several assays for identifying possible endocrine-disrupting (ED) substances. Included among these assays are estrogen (ER) and androgen receptor (AR) binding and/or transcriptional activation (TA) assays. NICEATM and the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) convened an independent scientific expert panel to evaluate the status of these assays and proposed reference chemicals for validation studies. Based on the expert panel's recommendations and public comments on a draft list of substances, ICCVAM prepared a final list of 78 substances for use in future ER/AR binding/TA validation studies. ICCVAM recommends testing a minimum of 53 substances for ER-based assays and 44 substances for AR-based assays; each set includes at least 25% negative or presumed negative substances. The use of this standard list of reference substances in future validation studies will facilitate determination of the acceptability of *in vitro* and *in vivo* assays and test batteries for inclusion in screening programs for ED substances. However, to comprehensively assess the usefulness of ER/AR binding/TA assays as individual components of the EDSTAC Tier 1 screening battery, and to facilitate development of more predictive *in vitro* ED assays, ICCVAM recommends that all 78 substances be tested in the four types of assays. This will generate a high quality *in vitro* database to facilitate future validation efforts and comparison of performance among different test methods and protocols.

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Lecture

Intelligent testing strategies for REACH

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The proposed REACH system for chemicals risk assessment has prompted several proposals for intelligent testing strategies (ITS) which comprise (Q)SAR modelling, read-across (chemical) and other non-biological approaches. The potential advantages and limitations of such ITS will be reviewed. It will be argued that it would be premature to base a testing strategy mainly on chemical approaches and computational modelling, at least until such time as criteria to validate them for their reliability and relevance by using independent and transparent procedures have been agreed. This is due to the inherent problems with validating (Q)SARs for regulatory acceptance and using procedures that have been developed and applied to the validation of *in vitro* tests. Until this issue has been resolved, it is recommended that testing strategies should be developed and applied in a cautious and judicious way, incorporating computational and read-across approaches, along with information from available *in vitro* tissue culture methods and metabolism and biokinetic studies. Such strategies should be intelligently applied by being driven by exposure information (based on bioavailability and not just production volume) and specific hazard information needs, in preference to a generalised tick-box approach. In the meantime, there should be increased efforts to develop improved (Q)SARs, expert systems and new *in vitro* methods. Ways to expedite their validation and acceptance should also be found, and prospectively agreed with all major stakeholders.

Poster

A practical implementation of Three Rs approaches to REACH

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The EU REACH (Registration, Evaluation and Authorisation of Chemicals) system aims to combine existing regulations covering chemical safety into one policy, and to pass the "burden of proof" from the regulators to industry, so that companies must evaluate the safety of substances and satisfactorily manage risks to humans and the environment. It is estimated that of all substances produced and marketed in the EU, 30,000 lack sufficient safety data to fulfil these new requirements and will therefore require further assessment. The number of animals which will be involved in this extra testing is currently estimated to be in the region of 2-4 million over the 11 year implementation period. FRAME is reviewing how the practical implementation of the Three Rs could minimise the number of animals required by REACH. The project focuses on the major endpoints within REACH, and includes a review of *in vitro* alternatives, their organisation into testing schemes for each major toxicity endpoint, and their potential inclusion into overall intelligent testing strategies for risk assessment. Where alternatives will not be available, either now or in the foreseeable future, we indicate how existing animal testing protocols could be improved both scientifically and from an animal welfare perspective. Our findings and recommendations will be presented. This work is linked to a DEFRA-funded project in collaboration with Liverpool John Moores University that includes an assessment of the potential for using (Q)SAR modelling and expert systems in REACH.

Poster

In house validation of a yeast-based assay to determine (anti-) estrogenic and (anti-) androgenic potential

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The potential effects of so-called "endocrine disrupting chemicals" on environment and man are controversially discussed by scientists and the public. We established *in vitro* screening systems based on genetically modified yeast cells to detect the (anti-) estrogenic potential (YES) and the (anti-) androgenic potential (YAS) of test substances. Genes, encoding for the human estrogen receptor alpha, and the human androgen receptor, respectively, have been integrated into the yeast genome of the strains used. Additionally, the cells contain a plasmid carrying the lac Z gene, which is receptor-dependently expressed and used as reporter gene (1, 2). The YES and YAS assay is conducted on 96 well plates using a standard protocol to determine agonistic as well as antagonistic effects over a concentration range of seven magnitudes on the same plate. Positive control substances used for estrogen and androgen agonistic activity were Estradiol and Dihydrotestosterone, for antagonistic activity Hydroxytamoxifen and Hydroxyflutamide, respectively. Both assays have been validated with more than 60 literature known substances, covering synthetic and natural agonists and antagonists, industrial chemicals, pesticides as well as substances expected to be cytotoxic and hormonally inactive. The results show very high reproducibility and good concordance with the literature data. As shown for different substances, an advantage of these assays is to detect cytotoxicity simultaneously with the endocrine modulating activity, to avoid artifacts due to cell death. In conclusion, the YES and YAS turned out to be robust systems, easy to handle and satisfying the requirements for screening systems.

Literature:
1. Routledge, E. J. and Sumpter, P. (1996). *Environ. Toxicol. Chem. 15*, 241-248.
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Lecture

ToxCast: A strategy for the categorisation of chemicals

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The US EPA is often faced with assessing the hazards and risks of large numbers of chemicals (e.g. High Production Volume Chemicals, potential endocrine disrupting chemicals, pesticidal inerts, the Candidate Contaminant List) without necessarily having the full context of toxicological information desired for analysis. Tools are therefore needed to help the Agency prioritise and categorise chemicals for evaluation. Partly in response to this need, the EPA initiated a computational toxicology program (see www.epa.gov/comptox) and established the National Center for Computational Toxicology (NCCT) to carry out supporting research. "ToxCast" is a concept being developed by the NCCT to aid in the prioritisation process. It is based on the assumption that toxicological hazard is a result of chemical-biological interactions, and that information pertinent to such interactions can be derived from a number of domains. These information domains include physical-chemical properties, predictions of reactivity by structure-activity analyses, interactions with specific cellular macromolecules, reactions of cellular based assays, and "omic" information derived from cells in culture or whole animals. These domains would be populated to the extent of economic and technological feasibility with data for individual chemicals. Informatic tools would then be applied within and across these information domains to cluster chemicals exhibiting similar properties or patterns of activity. Using an initial set of chemicals whose toxicological profile is well characterised will allow a proof of concept demonstration of the ability to categorise chemicals based on potential biological activity. Examples from the areas of proteomics and genomics suggest that the approach is feasible. This is an abstract of a proposed presentation, and does not necessarily reflect agency policy.

Poster

Endocrine disruptors – new challenges on phytoestrogens

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Plant substances with oestrogenic-like characteristics are called "Phytoestrogens". The isoflavones contained in the soy plant are among the most important because they are often found at high, and variable, levels in most "traditional laboratory diet formulas". Phytoestrogens will influence any body functions that involve estradiol. They will influences many research projects.

Because of their low estrogenic activity, dietary phytoestrogens have strong agonistic and antagonistic effects on estrogen receptors. Phytoestrogens can be thus considered selective estrogenic receptor modulators (SERM). They have effects not only on the reproductive tract but many, and perhaps more significant, effects on non-reproductive functions.

Phytoestrogens in GLP studies: Pytoestrogens may play a significant role in GLP studies. The "rodent uterotrophic bioassay" is a well-known test procedure. In an extensive validation study of the procedure by the OECD, the effect of the most important soya phytoestrogens genistein, daidzein and coumestrol has been examined. "Traditional standard diets" with a level of TGE (total genistein equivalent) between 99 and 513 mg/kg were evaluated. The study confirmed that the phytoestrogens had a uterotrophic effect in rats and might mask a mild endocrine disruptor at the higher end of the concentration range.

Solution for research: Not only may traditional laboratory diets contain high levels of phytoestrogens, but the levels may vary significantly from batch to batch. The phytoestrogen content of laboratory diets can be sustainable reduced by substitution of soy components. This low level should be monitored regularly.

Lecture

Animal testing will be minimised. REACH – the new EU chemicals policy and the German position

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The decisions taken to date on the modernisation of EU chemicals legislation are outlined, with a particularly thorough discussion of the policy objectives being pursued with the REACH regulation. REACH will require a considerable amount of data on the intrinsic properties of substances and current levels of exposure to be gathered. This will succeed in ensuring that human beings and the environment are better protected against chemical risks. It also means that REACH will contribute to the conservation of wildlife and the restriction or prevention of the unintended animal experiments taking place today due to the ubiquitous application of xenobiotics.

REACH will lead to a temporary increase in the number of animal tests for a period of around 10 years. Scientists cannot dispense with data from animal testing when the risks of complex effects, such as long-term toxicity, are being evaluated. Nevertheless, the Commission's draft regulation contains various measures to limit the number of animal tests. These measures are set out individually in detail:

The regulation itself and the proposals concerning it put forward by the member states will make data sharing obligatory in relation to animal testing. This will prevent duplicate or multiple animal tests having to be carried out.

Researchers will also be allowed to make use of older data that were not obtained with current standard methods or in compliance with Good Laboratory Practice, provided these data are valid.

Alternative methods that do not involve animal testing will also be deployed wherever available. An account is given, in particular, of the scientific efforts undertaken in Germany over recent years to develop alternative methods of this kind.

The introduction of structural activity relationship analysis techniques (SAR, QSAR) into risk analysis raises the prospect of a further minimisation of animal experiments.

Finally, there is still a defined time window before REACH enters into force during which ongoing research projects on alternative methods can be completed and further options for cutting down on animal testing developed.

Lecture

ECVAM activities for alternative methods to animal tests for the detection of chemicals with (anti)-estrogenic and (anti)-androgenic activity. ECVAM validation study

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Over the last 40 years, there have been constant reports concerning synthetic chemicals that were introduced into the environment that might pose risks to human health and wildlife. Within these xenobiotics, there exists a particular type with hormone-like activity and putative interference with the endocrine system.

Scientific organisation and international regulatory bodies agreed to establish special activities to address the issue of endocrine disruption, promoting initiatives to develop screening programs for identifying chemicals with endocrine disrupting activities with the scope to develop new Test Guidelines.

The European Centre for the Validation of Alternative Methods (ECVAM), created for the protection of animals used for experimental and other scientific purposes, is leading a project to validate test methods tailored for rapid *in vitro* identification of compounds for their potential to induce hormone-related health effects.

Poster

Mandatory data sharing and flexible testing strategies to prevent animal testing under the new EU Chemicals Policy

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The European Commission is currently revising its chemicals policy, aiming to cover both existing and new chemicals under a common system, called REACH (Registration, Evaluation and Authorisation of Chemicals). According to official estimates, this will result in an increase of up to 45 million toxicological and ecotoxicological animal tests. From the point of view of animal welfare animal testing should be prevented both for ethical and scientific reasons, and this goal can be met without impeding human health and environmental protection. Apart from the prerequisite to intensify the development and validation of new non-animal test methods and to include all available non-animal test methods in the REACH Regulation, mandatory data sharing and flexible testing strategies can play significant roles in preventing animal testing. Nevertheless, the draft Regulation does not go far enough to ensure that these requirements will be met. Concrete proposals are presented how to amend the provisions to ensure that all existing information on the effects of chemical substances is made available and shared without exception so that for every single substance only one dossier is submitted. The significance of mandatory data sharing is underlined by the results of a survey performed by the German Animal Welfare Federation in Germany, where such an obligation has been implemented in the national Chemicals Act. Additionally, a concept for a step by step testing strategy is discussed that ensures that only such data is collected on a given substance that is necessary for its safe handling.

Lecture

Data availability and needs for a precautionary assessment of chemicals for endocrine-mediated toxicity

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Various frameworks have been proposed for the testing and assessment of chemicals for possible endocrine-mediated effects, including a two-tier testing battery by the US, a "toolbox" model by the OECD, and entirely animal-free approaches by alternatives and animal advocacy organisations. The former two models identify an "enhanced" two-generation reproduction study in rats (OECD 416) as the "definitive" test for adverse, endocrine-mediated effects in humans. This raises a number of substantial science-policy considerations, including whether the addition of new endocrine endpoints to the current OECD 416 will enable the detection of adverse chemical effects at lower doses; the approach to verifying this empirically (i.e., validation process); and if the enhanced protocol does prove to be more sensitive, the implications for use of existing data from OECD 416 and other sub/chronic toxicity studies in a risk assessment. Some officials in government and industry have asserted that existing evidence of adverse reproductive effects may be insufficient to classify a substance as an endocrine disruptor, and that such a classification would depend on an OECD 416 study being repeated using an "enhanced" protocol. The animal welfare, economic, and regulatory implications of this position will be examined in the context of standard data requirements and availability for different substance classes (e.g., pesticides, pharmaceuticals, food additives, and new/existing chemicals), as will strategies for avoiding new and/or duplicative animal testing (e.g., chemical grouping, read-across, and mining existing data from relevant toxicological studies).

Poster

Animal welfare implications of proposed data requirements under REACH: Opportunities for the 3Rs

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Extravagant data requirements outlined in the European Commission's proposal for a new regulatory regime for chemicals (REACH) could overshadow the laudable aim of the legislation – to protect human health and the environment from harmful substances – and undermine its sustainable implementation. According to the current REACH proposal, a new high production volume (HPV) chemical could be required to undergo up to six times more animal testing than is required elsewhere in the world in order to be marketed in Europe. Data requirements outlined in Annexes V-VIII of the Commission's proposal could likewise trigger a dramatic increase in animal testing for all existing substances manufactured or imported into Europe in volumes greater than 10 tonnes. Some of the tests proposed consume as many as 2,500 animals and cost up to € 1 million per chemical, yet most have never been properly validated according to modern standards. Substantial amendments to the REACH testing annexes have been proposed by animal protection and other stakeholders in order to promote maximum use of validated alternative methods and testing strategies available now or in the foreseeable future. Sustainable REACH implementation can be achieved, but only if industry, regulators, and Europe's political leadership fully embrace the cost- and animal-saving efficiencies offered by modern computing and cell-based techniques, together with a more precautionary approach to chemical regulation.

Poster

Integration of the 3Rs in regulatory toxicology testing at Huntingdon Life Sciences

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Under the mammalian EU VIIA toxicology package, it is necessary to evaluate chemicals for their potential to cause harm as part of the registration process. At HLS we have embraced the 3Rs making full use of the increasing acceptance of *in vitro* data by the regulators.

A Weight-of-Evidence (WoE) analysis is performed before doing any *in vivo* skin and eye work, which relies on client information, together with relevant database searches. This together with test substance physical attributes will determine whether an animal exposure is necessary. If the above analysis suggests that the test substance may be a strong irritant or corrosive, then an *in vitro* alternative assay is performed, such as the EpiDerm corrosivity assay. In the absence of such an alert, sentinal animals are used before exposing the main study animals. Similarly for eye irritation, no animal exposures are performed if there is known potential for eye irritation from the WoE or prior knowledge of the skin irritating potential.

In addition, the 3Rs are applied throughout the regulatory package. For instance, the data from the skin irritation and the acute dermal studies is used to decide whether there is a need to evaluate dose tolerability in a preliminary LLNA study and to assist with setting dose levels. Together, these approaches have resulted in a reduction both in the number of animals exposed and the severity of the findings.

Lecture

ICCVAM's role in validating *in vitro* test methods for endocrine disruptor screening

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Evidence linking exposure to natural and man-made substances in the environment to adverse effects on the endocrine and reproductive systems of a variety of animal species resulted in concerns about the possibility of similar adverse health effects in humans. The U.S. Congress enacted provisions to safeguard public health from exposure to pesticides in foods and drinking water and required the EPA to develop and validate a screening and testing program to identify substances with endocrine disrupting activity. There are an estimated 87,000 chemicals produced today which have insufficient scientific data to allow evaluation of their potential for endocrine disruption. The EPA is developing a two-tiered screening and testing process. Estrogen receptor (ER) and androgen receptor (AR) binding assays and transcriptional activation assays (TA) have been proposed as part of the Tier 1 screening battery. ICCVAM comprehensively reviewed all the *in vitro* ER and AR binding and TA assays and concluded that none were adequately validated. Minimum procedural standards such as dose selection criteria, number of replicates per test, appropriate positive and negative controls, and criteria for an acceptable test were proposed that should be incorporated into standardised protocols for each of the four types of assays evaluated.

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