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

Session I-4: Oral presentations

I-4-530

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

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

I-4-130

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

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The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently convened an international peer review panel to assess the validation status of the BG1Luc ER TA test method (also known as the LUMICELL™ assay)

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

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

I-4-054

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

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

I-4-155

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

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

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

I-4-272

Supporting the implementation of the EU Community Strategy on Endocrine Disrupters

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

I-4-287

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

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

I-4-308

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

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

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

Session I-4: Poster presentations

I-4-288

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

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

I-4-454

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

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

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

I-4-473

Comparison of alternative screening approaches for potential estrogenicity

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