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

Session I-9: Oral presentations

I-9-657

Predicting reproductive and developmental toxicity: A tiered strategy

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

I-9-258

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

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

I-9-300

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

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

I-9-647

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

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

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

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

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

Session I-9: Poster presentations

I-9-201

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

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

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

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

I-9-261

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

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

I-9-330

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

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

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

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

I-9-376

Towards automation of the Embryonic Stem Cell Test

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

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

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

I-9-523

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

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

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

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

References

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I-9-538

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

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

Reference

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I-9-550

Integrated testing strategy for reproductive toxicity

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

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

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I-9-588

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

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

I-9-590

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

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

I-9-627

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

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

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

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

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

I-9-628

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

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

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