

Workshops

Workshop 1: Are *In Vitro* Tests Meeting The EU Commissions Deadlines?

W01-01

An *in vitro* test strategy for predicting human acute toxicity

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Presently, data on the acute toxicity of compounds are based on animal studies and expressed as LD50s in mg/kg body weight. Although the classical determination of LD50, making use of large numbers of animals and a statistical analyses of the data, is now phased out and replaced by a number of animal studies using considerably less animals. However, the total replacement of animal studies for determining acute toxicity is not yet reached.

The A-Cute-Tox integrated 6th framework programme is aiming at providing the methods and the strategy to estimate acute toxicity of chemicals on the basis of non-animal data. In previous programmes, the feasibility of the use of *in vitro* cytotoxicity data for the prediction of *in vivo* lethal doses showed that basal cytotoxicity gave reasonable estimates for only 70% of the compounds, i.e. chemicals could be classified in the appropriate LD50 classes. The remaining chemicals were misclassified.

Deviations from a simple linear relationship between effective concentrations *in vitro* and toxic doses *in vivo* can be the result of the fact that the effective concentration *in vitro* are irrelevant for the concentrations that may cause toxicity in target organs *in vivo*. These deviations may be caused by at least two factors:

1. basal cytotoxicity is not the basis of acute toxicity; more specific effects on specialized cells may be much more important.
2. the toxic concentration in the *in vitro* system needs to be extrapolated to the toxic dose *in vivo* on the basis of the biokinetic behaviour of the compound.

The A-Cute-Tox programme consists of work packages aiming at finding alerts for these deviations and to estimate correctors. To this end, about 95 compounds were studied in a wide variety of test systems for its basal and specific toxicity (neuro-, hepato-, nephro- and hema-toxicity) as well as its biokinetic behaviour. The results are now being evaluated with the aim of developing a strategy on how to test an unknown compound in a transparent and simple strategy of tests.

The results show that specific toxicity as well as kinetic considerations will improve the estimates of acute toxicity. This could lead to a classification scheme of chemicals that would be comparable to the LD50 values in rodents. Furthermore, it could also result in a better prediction of acute toxicity in man. The final stage of the programme will be the (pre-) validation of the approach.

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W01-02

ReProTect: Hazard assessment of reproductive toxicity

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Reproductive toxicity testing will have the highest impact on animal use and costs for regulatory safety testing under REACH. Integrated testing strategies using *in vitro* tests for prediction of reproductive toxicity are therefore becoming of high relevance. In 2004 a new Integrated Project, sponsored by the 6th Framework Program of the European Union, called ReProTect (www.reprotect.eu) started, with the aim to identify a novel approach in reproductive toxicity hazard assessment. The project involves 33 partners from industry, academia, small medium enterprises and governmental institutes. The project was set up in order to develop/optimize *in vitro* models that are able to detect adverse effects associated with reproductive toxicity. Due to the complexity of the mammalian reproductive cycle, the project has been structured in three research areas, covering aspects such as: male and female fertility, implantation and prenatal development. Furthermore, a fourth research area Cross-Cutting Technologies has been created in order to explore the applicability of innovative methods. Within the last four years, ReProTect explored the predictive power of a range of pioneering *in vitro* tests. 128 peer-reviewed reproductive toxicants with different toxicological mechanisms have been selected and tested in order to support the optimization process of test protocols developed. Scientific information on the analyzed toxicological mechanism and the biological relevance as well as SOPs for each tests are now available and the statistical analysis, sponsored by ECVAM, is finalized for most of the tests. It is planned to design appropriate testing batteries by using tests developed in ReProTect.

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W01-03

Sens-it-iv: Novel testing strategies for *in vitro* assessment of allergens

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The scientific approach of the Sens-it-iv project aims at an improved appreciation of the biological processes occurring when tissue is exposed to sensitizing materials, and to relate this with cellular markers involved in these reactions.

The *in vivo* players and mechanisms in lung sensitization were studied using the precision cut lung slices (PCLS) technology. Promising differences in cytokine regulation upon exposure to either sensitizers or irritants were observed.

A catalogue of epithelial (EC) and dendritic (DC) cells was finalized. As yet, the DC line showing the most *in vivo*-like phenotype and functionality is the MUTZ-3 cell line. Studies on the role of

innate immune responses in sensitisation have identified new markers discriminating sensitizers from non-sensitizers. An effective system was established for determination of T cell frequencies for several allergens. Preliminary data on effects of exposure of cells or proteins to sensitizers were obtained. Proteome analyses in primary keratinocytes and DC resulted in the identification of numerous, partially cell-specific hapten-binders. A combined bio-analytical and cellular immunological platform was established for characterization of the rates of appearance and disappearance of starting material and products and determination of extents of irreversible protein binding. *Sens-it-iv* expanded the knowledge about human cells and cell lines, and their responses to selected compounds, to the point that a first set of test systems ($N = 7$) could be transferred to the technology module for further development.

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W01-04

Where we are and what we still need: A realistic perspective on the validation of alternative methods

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Over the last 12 years, the European Centre for the Validation of Alternative Methods (ECVAM), in cooperation with international experts, has set up guidelines for validation, and five stages in the evolution of a new test were defined: development, pre-validation, validation, peer review and regulatory acceptance. The process proved to be successful and led to the regulatory acceptance of several alternative tests. Ensuring a high level of protection for human health and the environment is the central feature of the new policy REACH. Testing for 30,000 existing chemicals will not only require a very large number of animals, but it will also be costly, laborious, and time-consuming, and will thus represent a bottleneck for the completion of the chemical dossiers. Besides the ethical aspects and the public concern, economical considerations also require the timely development and validation of alternative testing strategies that can refine and/or reduce animal use. Many parallel activities in all of the key areas identified by ECVAM have to take place. The overall goal of these initiatives is to more efficiently evaluate chemicals for toxic effects, by using a broad array of test systems and testing strategies and to generate data that strengthen the scientific foundation on which hazard and risk assessments are based. The challenge for the years ahead is to incorporate the mechanistic knowledge generated by cellular and molecular studies into the vast inventory of *in vivo* data to provide a more complete description of toxicological mechanisms as well as to establish a paradigm by which *in vitro* data may be used to predict toxicity *in vivo*. It will only be through the concerted efforts of toxicologists and government representatives that the inherent value of these *in vitro* methods can be realized.

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Workshop 2: Mechanisms of Toxicity in Risk Assessment

W02-01

Molecular clues to the aetiology of Balkan endemic nephropathy-associated urothelial cancer

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Balkan endemic nephropathy (BEN), which occurs in certain rural areas of Bosnia, Bulgaria, Croatia, Romania and Serbia, is a chronic renal interstitial fibrosis with slow progression to end-stage renal failure associated with a high risk of urothelial cancer. Substantiated by the investigations on aristolochic acid nephropathy (AAN) the proposal has been put forward that the primary cause of BEN is exposure to food crops contaminated with seeds of *Aristolochia* spp., which contain high levels of the genotoxic carcinogen aristolochic acid (AA). On both clinical and morphological grounds, AAN is very similar to BEN. Recently, tumour DNA samples from patients with BEN were found to harbour principally A to T mutations in the *TP53* tumour suppressor gene (Grollman et al., 2007). A to T transversions are typical mutations observed in experimental animal models after AA exposure and are consistent with AA-DNA adduct formation primarily at adenine residues. Using a novel *in-vitro* mutation assay that detects and select mutations in mouse embryo fibroblasts expressing human *TP53* sequences, we found A to T mutations were elicited by AA at sites in *TP53* that are rarely mutated in human cancers in general, but which have been observed to be mutated in urothelial tumours from BEN patients. This concordance of specific mutations in human tumours and in AA-exposed cells strongly support the argument that AA has a direct role in the aetiology of BEN-associated cancer.

Reference

Grollman, A.P., et al., 2007. Proc. Natl. Acad. Sci. U.S.A. 104, 12129–12134.

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W02-02

The use of humanised mice in the risk assessment of non-genotoxic carcinogens

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Mice humanised for genes involved in xenobiotic metabolism and its regulation may be used to understand species differences in toxicity and contribute to more reliable risk assessments.

Nuclear hormone receptors such as pregnane X receptors (PXR) and constitutive androstane receptors (CAR) regulate hepatomegaly and xenobiotic metabolising enzymes and variety of chemicals interact with these receptors. For example, phenobarbital (PB), a non-genotoxic carcinogen that in mice induces hepatomegaly (hypertrophy and hyperplasia) and, following long-

term treatment, hepatocellular tumours, has also been shown to activate murine and human CAR and PXR. Indeed, CAR is essential for PB-mediated hepatomegaly and hepatic tumour promotion.

PB (80 mg/kg i.p. 4 days) was administered to double “humanised” CAR and PXR (huPXR/huCAR) mice and wild type C57BL/6J mice. Mice which were devoid of both receptors (PXRKO/CARKO) were used as controls. Mice were implanted with osmotic pumps containing BrdU to allow determination of replicative DNA synthesis. Hypertrophy and P450 induction were observed in WT and huPXR/huCAR mice but not in PXRKO/CARKO mice. PB increased the hepatocellular labelling index (S-phase) by approximately 5-fold in the WT mice. No changes in S-phase were detected in huPXR/huCAR or PXRKO/CARKO mice. Studies with another non-genotoxic hepatic carcinogen, chlordane, have yielded similar results.

These data demonstrate that the human receptors are able to support chemically induced hypertrophy but not hyperplasia. Thus, if increased cell proliferation is essential for hepatocarcinogenesis, it is unlikely that exposure to PB poses a hazard to humans.

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W02-03

Role of mechanistic information in the risk assessment of agrochemicals

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There is an increasing demand by regulatory authorities to provide relevant data to explain the mechanism of action responsible for the adverse effects induced by chemicals. For some chemicals such data can provide conclusive evidence that the mechanism of action responsible for the observed adverse effects is not relevant to man. For example, herbicides acting as HPPDase inhibitors induce severe tyrosinemia in rats, which results in ocular effects. Subsequent mechanistic studies have demonstrated that catabolism of tyrosine via HPPDase is the major catabolic pathway in rats; however other species such as mouse and man are able to convert the substrate for HPPDase to other phenolic acids before excretion into the urine. As a consequence it is predicted that tyrosine concentrations in man will never reach those levels which would trigger the adverse eye effects reported in the rat and this is accordingly taken into consideration during the risk assessment of HPPDase inhibitors. In contrast, with the advent of the new 91/414 directive for pesticides, Industry finds itself in a precarious position particularly with respect to chemicals with endocrine disrupting properties. Whilst mechanistic studies, which can elucidate a threshold mechanism of action for endocrine related tumor formation, are useful for conducting appropriate risk assessment of chemicals in the USA, the same mechanistic data can lead to the non-authorization of the very same chemicals in Europe. The impact of this disparity will be discussed.

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W02-04

Risk assessment of genotoxic impurities in pharmaceuticals

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In 2006 the EMEA guideline on limits of genotoxic impurities in new drugs was published. This, together with a more recent EMEA Question and Answer document, recommends limits for such impurities based on the duration of dosing of the drug in man, often referred to as the staged Threshold of Toxicological Concern (TTC). This was followed in late 2008 by a draft FDA guideline on the same topic. The presentation will cover in brief the key points from the two guidance documents as well as highlighting some outstanding issues. This will include:

- Limits for genotoxic impurities in drug development and new products.
- Application of guidance to existing drug products.
- Limits for drugs used to treat life-threatening diseases.
- Use of compound-specific risk assessments or default limits.
- Practical experience with regulatory authorities.

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W02-05

Ethyl methanesulfonate (EMS): In vivo genotoxicity, cross species pharmacokinetic evaluation, and extrapolation to man

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The presence of ethylmethanesulfonate in tablets of a HIV medication triggered non-clinical studies into the dose–response for mutation induction. Despite a multitude of studies on the genotoxic activity of EMS, no lifetime carcinogenicity studies, repeat dose mutation data or exposure analysis is available to serve as solid basis for risk assessment. For alkylators like EMS it is generally assumed that the dose–response for mutagenicity (and by default for carcinogenicity) is linear—indicating that no ‘safe’ dose does exist. A recent in vitro study (Doak et al., 2007) provided evidence, however, that the dose–response curve for mutagenic and clastogenic activity was thresholded. We sought to verify the existence of thresholds for mutagenic and clastogenic activity in vivo. Dose levels ranging from 1.25 to 260 mg/kg/day were applied for up to 28 days. The studies were further supported by metabolism and exposure analyses and a general toxicity study in rats.

Our studies showed that daily doses of up to 25 mg/kg/day did not induce mutations in the lacZ gene in the three organs tested (bone marrow, liver, GI tract, liver) or micronuclei in bone marrow. Only at higher dose levels the genotoxic activity of EMS became apparent. DMPK assessment predicts human AUC and C_{max} values of 350 $\mu\text{M h}$ and 315 μM , respectively, orders of magnitude higher than the maximal exposure of the patients. On this basis we could provide reassurance for exposed individuals that they do not carry a toxicological risk.

To our knowledge, our studies constitute the first solid evidence for a thresholded dose–response for a genotoxin with a directly DNA damaging, alkylating mode of action.

Reference

Doak, Shareen H., et al., 2007. *Cancer Res.* 67, 3904.

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W02-06

Mechanisms of carcinogenesis: Regulatory perspective

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Carcinogenic risk extrapolation to low doses and standard setting should consider the mechanism of action of a given chemical. There is agreement on distinguishing between genotoxic and nongenotoxic carcinogens. However, further differentiations seem appropriate. There are numerous, apparently genotoxic carcinogens where possible thresholds are a matter of scientific discussion: e.g. positive data of chromosomal effects only, in the absence of mutagenicity, may support the characterization of a compound that produces carcinogenic effects only at high, toxic doses. There is consensus that for non-DNA-reactive genotoxicants, such as aneugens, thresholds should be defined. Specific mechanisms of clastogenicity, such as topoisomerase II poisons, or mechanisms based on reactive oxygen, are considered to have practical thresholds. At the level of the European Union, the Scientific Committee of Occupational Exposure Limits has adopted a strategy for the derivation of occupational exposure limits (OELs) that integrates mechanistic information in assessments of carcinogenic risk, and for the purpose of standard setting, considers four groups of carcinogens: nonthreshold genotoxic carcinogens (for low-dose risk assessment, the LNT model appears appropriate); genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported (in these cases the LNT model is used as a default assumption, based on the scientific uncertainty and backed by the precautionary principle); genotoxic carcinogens for which a practical threshold is supported; and nongenotoxic carcinogens and non-DNA-reactive carcinogens (compounds where a true threshold is associated with a clearly founded NOAEL). Examples will highlight current discussions on possible thresholds of carcinogens in standard setting.

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Workshop 3: Environmental Chemicals and Food Contaminants: Are They a Risk to Public Health?

W03-01

Application of OMICS data to human health risk assessment of environmental chemicals

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More than 30,000 new chemicals are produced yearly. Environmental exposure is world wide and so there is a great need for toxicological evaluations. The methods currently used to study the toxic effects of environmental chemicals rely mainly on histopathological or morphological studies; these are time-consuming, costly and require the use of many experimental animals. Therefore, there is an urgent demand for alternative methods and safety testing

strategies which can improve the prediction of the long-term toxic potential of chemicals in short to medium term studies. Previous studies have demonstrated the potential of OMICS technologies to predict specific endpoints of toxicity after short-term *in vivo* exposure in animals because these methods permit the simultaneous analysis of thousands of genes, proteins or metabolites before the morphological changes occur. To apply quantitative toxicoproteomic analysis to the evaluation of toxicity of environmental chemicals for human health risk assessment, we have developed an integrated toxicoproteomic platform. This platform has been applied to the analysis of the effects of several environmental tumorigens, including arsenic, bromate, benzo[a]pyrene, and conazoles. The endpoints studied include protein expression, phosphorylation, and oxidation in mouse and rat liver, lung, thyroid, and kidney and human cells. My presentation will focus on the development of proteomics technologies and applications, to the identification of mechanisms of toxic action or modes of action which are required and routinely used for human health risk assessment of environmental chemicals.

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W03-02

Exposure-driven safety assessment strategies; Application of the Threshold of Toxicological Concern-concept in safety assessment of chemically complex matrices

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The toxicological safety assessment usually is very time consuming, expensive and costs many animals. Improvements in this respect remain limited as long as we stick to the same concepts of toxicological risk assessment. New concepts would therefore be needed to achieve real innovations in risk assessment. The Threshold of Toxicological Concern (TTC) potentially is such a new concept. Its application however still is limited.

We have drafted a framework for the application of the TTC-concept in safety assessment of chemically complex food matrices to address the issue referred to as the Forest of Peaks (FoP). When chemically complex food matrices are analyzed by chromatographic methods a FoP results. The safety of such products is difficult to assess because usually there are many unknown substances present. An full assessment involving animal studies usually is needed to assess the safety of such products. However, in case most of the substances would only be taken in through the food in low quantities, the TTC-concept might in theory be applicable. However, the TTC-concept is designed to assess substances of which structural information is available, but toxicological information is lacking. To apply the concept efficiently to a FoP, a strategy is needed to deal with large numbers of substances of which structural information is lacking. The framework we drafted addresses this issue and proposes a stepwise approach for the application of the TTC-concept in safety assessment of chemically complex food matrices.

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W03-03**Role of genotoxic and non-genotoxic mechanisms in furan carcinogenicity**

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Furan is found in various cooked food products and produces liver tumors in rats. To improve the risk assessment of furan, an EU STREP project (FURAN-RA) aims to understand the mechanism(s) of action and the dose-response relationships. Although there is some evidence of genetic toxicity of furan and of a major metabolite cis-2-butene-1,4-dial, the findings in various systems are equivocal. In vivo, oral doses as low as 2.0 mg/kg bw per day for 5 days cause a proliferative response in specific lobes of the liver. A reversible change in expression of cell cycle and apoptosis-related genes was measured at 14 days but no alteration of the methylation status of the regulatory region of a range of genes analysed could be found. A higher dose (30 mg/kg bw daily for 3 months) caused an initial extensive centrilobular necrosis and a subsequent sustained biliary cell proliferation and intestinal metaplasia even after a 1-month off dose period. This change was found to be associated with (1) a sustained inflammatory response, (2) alteration in expression of a range of genes including those associated with DNA damage, (3) a change in the methylation of the regulatory region of the Grap-2 gene (but not that of a range of other genes) and (4) secondary oxidation of cellular hepatic DNA. The combined data on furan in the rat, though still leaving uncertainty regarding genotoxicity, suggest that epigenetic events may contribute through alteration of gene expression, sustained alteration of hepatic architecture and secondary genotoxicity.

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W03-04**Genotoxicity and carcinogenicity of acrylamide**

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Acrylamide is carcinogenic in rodents, producing tumors at multiple organ sites in rats and mice by several routes of administration. Acrylamide induced an increase in lung tumors and skin tumors in mice, while in rats acrylamide administered in drinking water has been shown to induce testicular mesotheliomas, thyroid follicular cell tumors, and mammary gland tumors, as well as primary brain tumors in some studies following chronic exposure. An additional rat study reported an increase in adrenal pheochromocytomas, pituitary and clitoral gland adenomas, oral cavity papillomas, and uterine adenocarcinomas. In humans and experimental animals, ingested acrylamide is metabolically converted to the genotoxic epoxide, glycidamide, which is likely to be important in the carcinogenicity of acrylamide. Acrylamide exposure in rodents has also produced epigenetic effects in some target tissues by acute and subchronic exposures. These data, taken in concert with the genotoxicity results suggests that multiple mechanisms of action may contribute to the carcinogenic effects of acrylamide in rodents. In contrast to the rodent findings, epidemiologic studies (occupationally exposed workers or populations where acrylamide was present

in foodstuffs) of possible health effects from acrylamide exposures have not produced consistent evidence for an increased cancer risk at any site. While an apparent doubling of risk for pancreatic cancer was reported in the most highly exposed workers within the occupational cohort, no consistent exposure related response relationships were identified. Acrylamide, therefore, is a multi-organ site rodent carcinogen that appears to possess both genotoxic and epigenetic properties. Its potential carcinogenic activity in humans remains unresolved.

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W03-05**The role of flat dysplastic aberrant crypt foci induced in the colon by food contaminants**

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The diet itself, several of its constituents or dietary contaminants such as heterocyclic amines and others appear to be related to the risk of colon cancer. In order to facilitate the study of dietary factors, short term animal models have been developed. The development of biomarkers of colon cancer risk in such models requires the identification of early changes or stages in colon carcinogenesis. The classical ACF, which has been used extensively, consist of enlarged crypts, elevated above the surrounding mucosa. However, the relationship of the classical ACF to tumour development is not clear as they are induced in a number much larger than that of tumours, they display mostly hyperplasia, occasionally mild dysplasia, never severe dysplasia and they tend to regress. We first observed flat dysplastic ACF in Min mice that spontaneously develop colonic tumours. The flat dysplastic ACF appear bright blue upon transillumination of the intestine following staining with methylene blue, are not elevated above the surrounding mucosa and show severe dysplasia and β -catenin accumulation. They show dysplasia already at the monocryptal stage and appear to gradually grow and develop and into tumours. Usually they lack goblet cells. We recently showed that in rodents there is a great overlap between the flat dysplastic ACF and so-called mucine depleted foci (MDF) as described by Caderni et al. We have used flat dysplastic ACF as an early biomarker or colon carcinogenesis following exposure to food mutagens such as heterocyclic amines, acrylamide and other food mutagens and as an effect biomarker for other component in the diet that might modulate colon carcinogenesis.

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W03-06**EFSA's risk assessment on PFOS and PFOA in the food**

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Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two important classes of compounds of perfluoroalkylated substances, which are environmentally persistent and can be bioaccumulated in humans. The European Food Safety Authority's Panel on Contaminants in the Food Chain (CONTAM) evaluated these compounds regarding the importance of food and non-food sources to the human exposure and established health based guidance values.

The indicative estimate of dietary exposure to PFOS is 60 and 200 ng/kg bodyweight (b.w.) per day, for average and high consumers in Europe, respectively. For PFOA the indicative estimate of dietary exposure is 2 and 6 ng/kg b.w. per day for average and high consumers, respectively. Fish seems to be the major source of human exposure to PFOS and PFOA. Non-food sources can contribute <2% and up to 50% for PFOS and PFOA, respectively.

For PFOS a no-observed-adverse-effect-level of 0.03 mg/kg b.w. per day was derived from a subchronic monkey study reporting changes in lipids and thyroid hormones. Based on this study the Panel established a Tolerable Daily Intake (TDI) for PFOS of 150 ng/kg b.w. For PFOA a 95% lower confidence limit of the benchmark dose for a 10% increase in effects (BMDL10) of 0.3 mg/kg b.w. per day was derived. This was based on effects in the liver of rodents. The Panel established a TDI for PFOA of 1.5 µg/kg b.w.

The Panel identified the need for further data on the levels of these compounds in food and humans and recommended such investigations to be promoted.

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Workshop 4: Immune Function Testing: Pros and Cons of the KLH Assay as an Alternative for the PFC Assay**W04-01****Comparison of immune function tests using T-cell dependent antigens: A meta-analysis**

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Increased expectations from regulatory agencies for evaluation of potential adverse effects on the immune system have led to the increased use of the T-cell dependent antibody response (TDAR) as a main functional test of immunotoxicity. TDAR measurements include both well established tests, e.g., anti-sheep red blood cell Plaque Forming Cell (PFC) assay, and newer models,

e.g., anti-keyhole limpet hemocyanin (KLH) antibody ELISA. These tests vary in the study design, antigen application and analytical methods. In attempt to evaluate impact of the method differences on the outcome of immunotoxicity studies, the comparative analysis of TDAR data has been undertaken using meta-analysis of results generated by several laboratories using multiple TDAR methods. In these analyses different primary TDAR assay formats that used either SRBC or KLH antigens in two strains of rats by multiple routes of immunization were compared. Although limitations imposed by the scale of the data set and the need to convert some data to an ordinal scale to allow comparisons restrict the scope and strength of the conclusions that can be drawn, the results of this study showed that the two antigens, the three assay formats (SRBC-PFC, SRBC-ELISA or KLH-ELISA) and two routes (intravenous and subcutaneous) of immunizations give comparable results. Similarly, both antigens and assays formats showed the same pattern of response to strong immunotoxicants. These results indicate that standardizing the choice of antigen, assay format or route of immunization may not be critical in the evaluation of immunotoxicity potential of chemicals or drug candidates.

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W04-02**Regulatory view on the relevance of the TDAR in immunotoxicity testing**

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The immune system is a very complex system in human body and the consequences of damaging its function may be life-threatening.

During the preparatory discussions for the ICH Guideline S8 it was agreed that a cause for concern approach would be sufficient to assess the risks associated with disturbances in the immune system. The focus was given to (1) findings from Standard Toxicity Studies (2) the pharmacological properties of the drug, (3) the intended patient population, (4) structural similarities to known immunomodulators, (5) the disposition of the drug, and (6) clinical information.

Changes in various parameters could reflect immunosuppression or enhanced activation of the immune system. Because of the complexity of the immune system a test that reflects the integrity of the system as broad as possible (innate and adaptive immune system) is preferred. T-cell-dependent antibody response (TDAR) is the preferred functional test.

A survey has been published which forms the basis of the decision in the ICH Expert Working Group to accept a cause-for-concern approach instead of the EU guideline of routine requirement of additional immunotoxicity studies (Weaver et al., 2005). Part of that survey was the review of TDAR studies, and the data provided showed a high variance. This variance hampered the value of these TDAR studies as follow-up studies, and conclusions about the immunotoxic influence of various compounds were difficult.

From a regulatory point of view we fully support further development of a robust testing strategy in this field, as public health protection is the important goal.

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W04-03**Sensitivity of the KLH-assay versus PFC-assay in rodents**

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For several decades the T-dependent antibody response (TDAR) to sheep erythrocytes (SRBC), also known as the Plaque Assay or Plaque Forming Cell-assay (PFC), has been used as a sensitive and predictive functional immune assay for detecting immunomodulatory compounds. The PFC-assay has been validated in multiple ring studies in the United States and in international ring studies. Recently, various laboratories have chosen to use ELISA-based assays for evaluating the primary immune response in rodents. The ELISA-based assays offer several advantages over the Plaque Assay, which make them attractive for use in immunotoxicological evaluations. Among the most popular antigens used in the ELISA-based assays are SRBC and more recently KLH. Depending on the drug or compound evaluated, different effects and degrees of sensitivity can be seen with the PFC-assay and KLH-assays. Recent reports have shown that the sensitizing dose of KLH used in the KLH ELISA differentially affects the responses observed in rodents. Even within the same species, different strains of mice and rats produce different magnitudes of responses to the same sensitizing dose. One of the areas of concern has been the sensitivity of the well studied PFC-assay as compared to the relatively new KLH-assay. Using known immunosuppressive agents, which have different mechanisms of action, cyclosporine A, cyclophosphamide, azathioprine, and dexamethasone, head-to-head studies were conducted to compare assay sensitivity. The finding in these studies was that for the primary IgM immune response the PFC-assay was more sensitive than the KLH-assay in rodents.

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W04-04**Validation of the KLH assay in rats: A European collaborative study**Steven Spanhaak^{1,*}, Andre Penninks²¹ Johnson & Johnson PRD, Tox/Path, Beerse, Belgium, ² TNO Quality of Life, Toxicology and Applied Pharmacology, Zeist, The Netherlands

The Immunotoxicity Inter-Laboratory Project (IILP) aimed to obtain a properly validated and well-defined alternative for the Plaque Forming Cell (PFC) assay. The developed method is not intended or proposed as the single alternative TDAR but as one of the alternatives for the PFC assay. However, part of the effort in this joint venture was aimed at creating a reference framework enabling an improvement in the ability to evaluate the results obtained with this method or comparable designs. Thus the specific goals of the IILP collaboration were: (1) to establish a common design for a TDAR assay using KLH; (2) to validate and test the robustness and sensitivity of the design by inter-laboratory testing using model immunomodulatory compounds; (3) to compare the obtained results with those obtained in PFC assays, and; (4) to make the design of this first inter-laboratory study available for general use and acquire acceptance by regulatory authorities. Main studies with 4 reference compounds (azathioprine, cyclophosphamide, cyclosporine A and levamisole) using the KLH testing protocol were performed in 9 laboratories in either Wistar or Sprague Daw-

ley rats. In parallel the model compounds were tested in the PFC assay. Obtained result indicate that the applied KLH methodology is robust, well reproducible and sensitive. Results for reproducibility, sensitivity and comparison of KLH with PFC results will be presented.

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Workshop 5: Lung injury, inflammation and repair: Fundamental aspects of lung toxicity**W05-01****Inflammasomes—Danger sensing platforms controlling IL-1beta activation**

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Caspase-1 plays an important role in innate immunity and host defence against microbe-dependent and microbe-independent danger. Caspase-1 is activated by the intracellular assembly of multiprotein complexes called inflammasomes. Cleaved, activated caspase-1 can then cleave the inactive precursors pro-Interleukin-1 β and pro-Interleukin-18 to activate the potent proinflammatory cytokines IL-1 β and IL-18, and then promoting their secretion.

Inflammasomes contain NOD-like receptor (NLR) family proteins, which are, in contrast to Toll-like receptors, cytosolic pattern-recognition receptors that can be stimulated in response to exogenous and endogenous danger signals.

Currently three prototypes of inflammasomes are distinguished: the NALP1, NALP3 and the IPAF-inflammasome, with the NALP3-inflammasome being the best characterised.

Due to their cytosolic localization, the inflammasomes detect intracellular danger signals, such as ATP or uric acid crystals, which can be released during cell death.

In the lung and the skin, two major boundaries between the body and the outside world, danger signals such as asbestos, silica dust or UV-light can also activate the inflammasome, leading to the secretion of highly active IL-1 β to initiate an immune response.

Apart from exogenous or endogenous danger signals, gain-of-function-mutations of NALP3 lead to autoimmune hereditary periodic fever syndromes with fever, serosal and synovial inflammation and rashes due to increased IL-1 β -secretion.

The remarkable progress in the field of NLR-family proteins also offers new hope to patients, as IL-1beta antagonists are already used as efficient therapy in hereditary fever syndromes and in clinical studies in rheumatoid arthritis and gout.

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W05-02**Effects of fine atmospheric particles on human respiratory epithelium: The role of organic compounds in oxidative stress, inflammatory and repair responses**

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The fine and ultra fine airborne particles generated by the burning of fossil fuels contain a large amount of organic compounds includ-

ing polyaromatic hydrocarbons (PAH) and are the most abundant components of PM_{2.5} (particulate matter 2.5 µm) in urban areas such as Paris. Most of them are produced by diesel engine-powered cars. In a kerbside station in Paris more than 50% of particles were close to the ultra fine range ($\leq 0.26 \mu\text{m}$) likely due to the influence of the traffic (Baulig et al., 2004). Chemical analysis between PM_{2.5} collected in a kerbside and a background station in Paris revealed that PAH are twice as important in the kerbside station. The presence on particles of organic compounds able to be associated to oxidative stress and inflammatory response raises the question of their bioavailability and their metabolism in the lung. We have shown, using a human bronchial epithelial cell line (16 HBE) and primary cultures of nasal and bronchial human epithelial cells, that DEP, PM_{2.5} and their respective extracts act as activators of the cytosolic aryl hydrocarbon receptor AhR, inducing CYP1A1 expression and activity whereas carbon black particles have not such an effect (Bonvallot et al., 2001). The genes of Phase II metabolism (GST, NQO-1) are regulated in a concerted manner at the transcriptional level through the antioxidant-responsive element (ARE). The transcription factor Nrf2 is central to ARE-mediated gene expression. DEP induce the translocation of Nrf2 to the nucleus of HBE cells, increase nuclear protein binding to the ARE as well as NQO-1 expression. These results give evidence that organic compounds are bioavailable as they induce phase I (CYP 1A1) and Phase 2 (NQO-1) gene expression. Many recent data have shown that organic compounds are a source of ROS. Indeed, we have measured a pro-oxidant status using various specific fluorescent probes in airway epithelial cells treated either with DEP, PM or their corresponding organic extract. A pro-oxidant status is known to induce cellular specific responses in order that cells face oxidant insult. By a genomic approach, the expression profiles of proinflammatory genes induced by DEP, PM or their organic extracts show a differential expression of cytokine genes such as IL 1 α , GM-CSF and ligands of the EGF receptor such as amphiregulin (AR), transforming growth factor α (TGF α) (Blanchet et al., 2004; Rhumelard et al., 2007). The organic fraction of particles is mainly involved in these responses. The release of proinflammatory cytokines induced by PM or DEP occurs after triggering transduction pathways including nuclear factor (NF) κ B activation and mitogen activated kinases (MAPK) phosphorylation (Bonvallot et al., 2001; Baulig et al., 2003). Moreover, transduction pathways as well as cytokines secretions were inhibited by the antioxidants such as N-acetyl-cysteine or DMTU suggesting the role of an oxidative stress. AR secretion is mediated through the activation of the EGFR and Erk MAP kinase pathway. In addition, AR secretion was also inhibited by antioxidants suggesting an EGFR transactivation via oxidative stress. The ability of PM_{2.5} to induce the expression and secretion of an EGFR ligand *in vitro* may reflect an important mechanism for sustaining the proinflammatory response and tissue repair after injury.

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W05-03

Oxidative stress by environmental and host derived sources leading to lung inflammation

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The airways of the lung are constantly exposed to noxious stimuli including pathogens, particles and oxidants. Furthermore, alveoli cells are exposed to pathogens and oxidants from the pulmonary circulation. In each case these insults can overwhelm endogenous anti-oxidant systems resulting in increased oxidant stress which directly induces inflammation and amplifies responses induced by cytokines. We and others have shown that bacterial pathogen associated molecular patterns (PAMPs) including LPS induce nitric oxide synthase (NOS) II, oxidant stress and inflammation in the lung. LPS induces NOSII and inflammation in the lung when given via either an inhaled or systemic route of administration, which illustrates the importance of blood borne insults to lung injury. In addition to PAMPs, our group and others have shown that oxidants, including those present in cigarette smoke, activate immune cells *in vitro* as well as induce inflammation in the lung *in vivo* via a number of signaling pathways including Toll like receptors (TLRs). Moreover, we and others have shown that oxidants synergize with PAMPs amplifying NF κ B surrogates such as CXCL8. The role of oxidants in lung inflammation associated with various conditions, including asthma, COPD and sepsis will be discussed. The mechanisms by which oxidants and PAMPs interact to amplify inflammation will also be highlighted.

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W05-04

Inflammatory processes in a mouse model of chronic pulmonary diseases by cigarette smoke inhalation

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Laboratory animal models can help to improve our understanding of the mechanistic basis of chronic smoking-related diseases such as emphysema and lung cancer. A/J mice were whole-body exposed to fresh air or to cigarette mainstream smoke from a research reference cigarette in various combinations of inhalation and post-inhalation periods of up to 18 months at daily doses of total particulate matter of up to 3000 mg/m³ × h. Genes involved in xenobiotic metabolism (e.g., *cyp1A1*) and oxidative stress (*hmox1*) were found to be transiently induced during inhalation periods, returning to control levels directly after cessation of smoke exposure. Genes involved in inflammatory processes (*mip-1 α*) or proteolytic processes (*mmp12*) displayed sustained induction. Similarly, inflammatory cells and several protein markers in bronchoalveolar lavage showed either a transient induction pattern (PMNLs, TNF- α) or a sustained induction pattern (lymphocytes, KC). After 5 months of inhalation, altered lung mechanics (higher tissue elastance and partial loss of elastic recoil) and emphysematous changes (increased mean chord length, decreased bronchial attachments) were observed. In studies of up to 18 months, a dose-dependent increase in the multiplicity of adenomas and ade-

nocarcinomas in the lungs was observed. The causal role of the inflammatory effects in the chronic pathogenesises developing in this mouse model and their relevance to human smoking-related diseases remain to be established.

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W05-05

Mechanistic aspects of in vivo and in vitro testing of allergic agents

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A wide range of industrial chemicals can induce respiratory allergic reactions. At present, there are no widely applied or fully validated test methods to identify respiratory allergens, i.e., compounds that are considered capable of inducing allergic asthma. Hence, there is an urgent need for methods identifying and characterizing the biological action of chemicals in the lung. To discriminate and classify the allergic potential of the different compounds, several in vivo and in vitro models were utilized. In order to test rather low doses of chemicals in vivo, various methods, different application routes, i.e., intradermal, topical or inhalation exposure, different duration and several species, i.e., guinea pig, rat or mouse were used. In vivo physiological parameters like respiratory functional response (specific or unspecific), tests in lymph nodes, and cellular response in B- and T-cells, macrophages and epithelial cells were performed. In addition reliable alternative ex vivo and in vitro methods are to claim. In primary epithelial cells, B- and T-cells, and dendritic cells and different cell lines structural cell changes, expression and release of proteins and mediators (like TH1 and TH2 cytokines) were monitored to determine the ability of compounds to provoke allergy. As the lung consists of more than 40 different cell types, respiratory allergens are hard to define in single cell types. Viable lung slices represent an alternative method to test functional and immunological responses to chemical compounds.

Herein, an overview of the different methods and their mechanistic responses to the industrial chemicals trimellitic anhydride (TMA) and 2,4-dinitrochlorobenzene (DNCB) is given.

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W05-06

Lung fibrosis is uncoupled from inflammation in response to toxic nano- and micro-particles

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The paradigm that inflammation drives pulmonary fibrosis has been recently challenged. We observed that nano- and micro-particle-induced lung fibrosis in animals was unpaired to inflammation. The fibrotic disease was also resistant to several anti-inflammatory therapies and occurred in mice deficient for pro-inflammatory cytokines (i.e., IL-1, IL-12p35, IL-17 and type I

IFN). Interestingly, silica-induced lung fibrosis in mice was characterized by a chronic anti-inflammatory response accompanied by macrophage-derived anti-inflammatory cytokine overexpression (i.e., IL-10, soluble TNF- α receptors and TGF- β). We showed that IL-10 (like TGF- β) has a detrimental activity during the establishment of lung fibrosis despite its capacity to control certain macrophage functions and the inflammatory process. IL-10 deficient or over-expressing mice developed reduced or enhanced silica-induced lung fibrosis, respectively. The profibrotic activity of IL-10 *in vivo* appeared to be mediated by its ability to stimulate the expression of TGF- β 1 while suppressing the expression of cyclooxygenase-2 and thus production of the anti-fibrotic eicosanoid PGE2. The anti-inflammatory pathway associated with silica-induced fibrotic lesions in mice was not restricted to macrophages since immunosuppressive regulatory T cells, estimated by Foxp3 lung expression, were also accumulated during the development of lung fibrosis. In conclusion, a pronounced anti-inflammatory reaction may also contribute to particle-induced lung fibrosis and represent an additional etiopathogenic pathway of lung fibrosis.

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Workshop 6: Evidence-based Decisions and Toxicovigilance in Human Toxicology

W06-01

Overview on evidence based clinical toxicology

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During the last decade, the evidence from scientific research has become an important determinant of clinical decision making, coined as 'Evidence-based medicine' (EBM). It has been proposed to translate this concept into toxicology. However, the evidence of causation (important in toxicology, referred to as 'evidence-based toxicology') has to be distinguished from the evidence of treatment effects (called 'evidence-based medicine' in clinical toxicology). Human studies in toxicology are difficult to perform: volunteer human studies for the testing of toxicity have ethical limitations, and clinical trials to test treatment effects have limitations due to small numbers and the heterogeneity of patients and poisoning circumstances.

Recently it has been tried to apply the idea and the methodology of evidence-based medicine to the field of toxicology, and to review the processes in the scope of hazard, risk, causation and uncertainty. These are important challenges to current regulatory toxicology programmes like REACH.

In clinical toxicology there is still little hypothesis-testing research. Randomized clinical trials (RCT) are an exception. Observational studies are often performed on highly selected patient groups thus producing marked bias. In most instances, clinical decisions are taken based on expert opinion and guidelines deduced from known pharmacological or toxicological effects, generalization from substances within the same therapeutic or chemical class, animal data, and case reports. While RCTs will remain rarely applicable, improved and more sophisticated methods of epidemiological research in the collection of data on human poisoning are feasible to produce data capable to address the clinical and public health aspect of poisoning.

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W06-02**Antidotes for poisonings: More need of evidence to improve clinical practice**

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Question: An antidote is a pharmaceutical with assessed mechanism of action, able to modify either the toxicant pharmacokinetics or pharmacodynamics and whose administration reliably improves the patient. However, evidence regarding the current antidote efficiency is lacking.

Methods: Data review regarding antidote evaluation.

Results: Prospective trials establishing antidote benefit remain limited to fomepizole in toxic alcohols and hyperbaric oxygen in carbon monoxide poisonings. A randomised trial showed multiple-dose charcoal non-usefulness in self-poisonings. Antidigoxine Fab-fragment interest to improve survival in digitalis poisonings was assessed. Cumulative data allow hydroxocobalamin reliable use in cyanide and octreotide interest in sulfonylurea poisonings. In contrast, several antidotes are routinely used based on theoretical benefits, animal studies or single cases. Glucagon efficiency in beta-blocker and insulin-glucose in calcium-channel-antagonist poisonings remain questionable. Based on animal data and successfully treated anaesthetic overdoses, lipid rescue has been encouraged to treat sodium-channel-blocker poisonings without evidence. Continuous flumazenil to avoid intubation in benzodiazepine poisonings has never been validated. Naloxone to reverse buprenorphine toxicity is unknown. Aims of calcium-salts in calcium-channel-blocker and 8.4%-sodium bicarbonate in sodium-channel-blocker poisonings are undetermined. Patients to treat with *N*-acetylcysteine based on Rumack's nomogram need clarification. Concerns regarding pralidoxime utility (regimen, concentrations, patients to treat) and safety are raised in organophosphorus poisonings. Although combined ventilation, epinephrine, and diazepam have been demonstrated beneficial in chloroquine poisonings, diazepam role remains mysterious.

Conclusions: Controlled trials in toxicology are needed to improve practice. Antidote interest should be evaluated based on expected benefits, adverse events, and cost. Optimal administration should result from evidence-based bench-to-bedside assessment.

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W06-03**Role of poison control centres in human data collection and dissemination of new treatment recommendations**

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Although intoxications cause 2–10% of all cases managed by emergency medicine services, the frequency of severe poisonings treated in intensive care units has decreased substantially over the last two decades in Europe. This is mainly caused by successful safety measures at home and at workplaces and by substitution of dangerous drugs and dangerous chemicals (in consumer products) by less toxic agents. Today, treatment units with medical personnel particularly trained on poisonings are available only in very few hospitals. Most

medical doctors treating poisoned patients in prehospital settings or in hospitals are not particularly trained in clinical toxicology. They frequently use the service of a poison control centre (PCC) to choose the appropriate diagnostic measures and the best treatment for the poisoned patients.

In most European countries PCC were constituted within the last 50 years. Medical toxicologists of PCC give advice by telephone based on careful evaluations of toxicological data. The scientific community of medical toxicologists is actively creating, sharing and discussing new scientific results. The European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) is one of the worldwide leading organisations in this process. As the majority of the European PCC directors are active EAPCCT members new scientific evidence is rapidly integrated in PCC's clinical management recommendations, which in consequence leads to improvements in treatment of poisonings with only short lag time.

Most PCC treatment recommendations are based on experiences from human poisonings, but most severe poisonings are occurring only rarely in humans. Furthermore, only a small portion of human poisonings is published in scientific literature. Unusual cases are more often published than typical cases or case series. This often causes insufficient and (in parts) distorted datasets on human poisonings, especially for those caused by rare agents.

Therefore, PCC not only give advice in poisoning cases but also collect and document data on the poisonings in which they are involved by follow up calls and collection of written reports from hospitals. Thus, hundreds of case reports are collected in PCC databases in a partially standardized, structured and quality controlled way daily. As most of these cases are not published in the literature this PCC datasets form an valuable source for toxicological assessments: PCC databases are frequently retrieved for retrospective studies, either to support evaluation of recent cases of rare poisonings or for many other purpose, i.e. surveillance of exposure or poisoning trends or detection of new poisoning risks by public health authorities, comparing human poisoning frequencies and detecting rare risk of commercial products (i.e. contracted by industry associations or companies).

Today, data on human poisoning cases only play a minor role in regulatory risk assessment due to the low publication frequency and the often uncontrolled quality of reports available. High quality data may be provided by PCC and this may play an increased role for regulatory exposure assessment and poisoning risk assessment in the future.

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W06-04**The role of clinical toxicology units in public hospitals An important source of data for toxicosurveillance**Ana Ferrer-Dufol^{1,*}, Santiago Nogue Xarau², Sebastian Menao Guillen¹, Rosa Martinez Arieta³, Salome Ballesteros³, Fatima Ramon³

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In 1997 a section of Clinical Toxicology (STC), belonging to the Spanish Association of Toxicology (AETOX) was established, including in its goals the promotion of Functional Units of Clinical Toxicology to improve the standardization of acute poisoning diagnosis

and treatment and to provide an updated epidemiological profile of toxic cases in our country.

Interest in surveying chemical poisonings (gases, solvents, caustics), accounting for 15% of the total acute poisonings in Spain, led to the development of a Toxicosurveillance Program (TSP) by means of a collaboration between the Spanish Health Ministry and the STC, running from 1999 up to now. The other source of data about these chemical incidents is the Toxicology Information Service (SIT).

We are comparing the epidemiological profiles of poisoning by chemical agents in Spain from these two sources to show the similarities and differences related to these two different points of observation.

TSP has accumulated 5259 chemical cases between 1999 and 2007. SIT cases by chemical agents in 2007 were 33,502. Their profiles show some relevant differences that can be explained by the bias produced by the different origin of the SIT and TSP cases: 64% of the SIT cases come from calls from the general public and 100% of TSP cases come from the Emergency Departments.

Therefore we can conclude that different sources of data produce different results for evaluating chemical poisonings. Data from SIT are broader but present some bias and should be complemented by cases from the EDs, more representatives of real poisonings.

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W06-05

The DeNaMiC project: Description of the nature of accidental misuse of chemicals and chemical products

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Purpose: The DeNaMiC project was funded by the European Chemical Industry Council (CEFIC) to determine the availability of information from poisons centres and other sources to characterise the nature of accidental exposures to household chemical consumer products to inform and improve risk management.

Method: The project involved various subcomponents of work and included; developing an analytical tool to compare data on accidental poisoning obtained from various sources and comparing and mapping the data collection and product classification schemes used by three poisons centres, Gottingen, Lille and London. A retrospective analysis of 3 years of enquiry data from Gottingen and Lille was conducted to determine routinely available data on circumstances of exposure and the usefulness of this information for product risk assessment was evaluated. In addition, a meta-analysis and survey of European poisons centres toxicovigilance activities were conducted. Finally, the project explored the feasibility of using poisons centres to obtain additional information

about circumstances of exposure through a prospective follow-up study.

Results: A range of publicly available data on accidental exposures was found; however, there was little information on the circumstances of exposure. European poisons centres collected the same base data set but varied in collecting data relevant for risk assessment. European poisons centres varied in their understanding of toxicovigilance, but most stated that they perform it. It was possible to collect additional data prospectively on exposures to household products relevant for risk assessment and management.

Conclusions: Poisons centres are an important potential source of data useful for product risk assessment and management.

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Workshop 7: Safety and Usage of Herbal Medicines: Issues for Toxicology

W07-01

Pharmacokinetics with herbal medicines with regard to their safety and efficacy

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Bioavailability is defined as the fraction of a given dose of compound that reaches the systemic circulation as intact compound. In this context, ADME parameters (Absorption, Distribution, Metabolism, Excretion) are important indicators of the pharmacokinetic profile of a drug. In contrast to orthodox drugs, herbal medicinal products do not contain pure single active compounds, but a complex mixture of many constituents. Therefore, also pharmacokinetics are much more complex.

Pharmacokinetic data of herbal medicinal products may be important to identify and characterize relevant active constituents, to demonstrate the relevance of *in-vitro* data for clinical settings, and to adjust the dosage. They provide information on *in-vivo* metabolism and may help to compare products with known active constituent/s.

However, from a regulatory point of view they are only reasonable for quantified extracts, because in that case the therapeutically relevant constituents are known. In case of standardized extracts and other extracts, in which the active constituents are not fully known, pharmacokinetic studies are either not applicable (other extracts) or of limited significance.

In case of Echinacea preparations, it could be shown that the alkamides are detectable in the plasma of healthy volunteers already 10 min after oral administration. Therefore also pharmacological *in-vitro* may be of high relevance. Also the bioavailability of bilobalide, ginkgolide A and B could be demonstrated by LC-MS already 15 min after oral administration of Ginkgo preparations.

Also in case of toxicological *in-vitro* data, like with benzophenanthridine alkaloids or with pyrrolizidine alkaloids, the bioavailability must be considered in conclusions for their relevance in the *in-vivo* situation.

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W07-02**Methodologies for the evaluation of safety and standardisation of herbal medicines**

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The intoxications with medicinal plants, edible plants and herbal medicines are becoming more and more frequent. In Switzerland, for example, there are about eight times more intoxications with plants than with mushrooms. This is due to the poor botanical knowledge of many people who collect plants themselves. Confusions between *Allium ursinum* (Liliaceae) and *Colchicum autumnale*, and even *Convallaria majalis*, occur every year. Some people even cannot distinguish *Taxus baccata* (Taxaceae) from Pinaceae species such as *Abies alba* or *Picea abies*. Herbal drugs which are on the market can also be victims of botanical confusions, as exemplified by the Chinese plant *Aristolochia fangchi* (Aristolochiaceae).

Microscopic analysis enables to detect very quickly falsifications of herbal medicines and some examples will be shown. TLC is also an important technique but the sensitivity is low. Hyphenated techniques such as LC/MS and LC/NMR are appropriate tools for the rapid identification of contaminants such as pyrrolizidine alkaloids which are hepatotoxic, anacardic acids which are strong allergens and many other classes of toxic plant constituents. Microcapillary NMR, due to its high sensitivity, will play a more important role in the future. The above-mentioned technique can also be applied to the standardisation of herbal drugs.

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W07-03**Adverse effects and drug interactions of herbal medicines**Nursen Basaran^{1,*}, A. Ahmet Basaran²¹ Hacettepe University, Faculty of Pharmacy Toxicology Department, Sıhhiye Ankara, Turkey, ² Hacettepe University, Faculty of Pharmacy, Pharmacognosy Department, Ankara, Turkey

Since the use of herbal medicines have become increasingly popular in recent years, it is essential to be aware of clinical and adverse effects, doses and potential drug interactions. Although herbal medicines are often believed to be natural and therefore safe, many adverse and even lethal side effects have been reported. They are generally marketed without the proof of efficacy or safety as conventional drugs. Lack of regulation of quality control and of product standardization makes it difficult to establish safe doses of herbal medicines in most countries. Active compounds may vary between batches and manufacturers. Contaminants of heavy metals, such as lead, arsenic and mercury have been found in some traditional Chinese medicines. Some herbal preparations have also been shown to be adulterated with undeclared conventional drugs like paracetamol, diazepam, and indomethacin. Also evidence has emerged that herbal medicines can interact with concurrently taken conventional medicines and cause serious adverse effects. In particular commonly used herbal preparations such as *Hypericum perforatum* (St. John's wort), *Echinacea purpurea*, *Allium sativum* (garlic) and *Ginkgo biloba* which have been shown to inhibit or induce the activity cytochrome P450 (CYP) isoenzymes, have clinically important interactions. Herb–drug interactions may be of clinical significance particularly for patients taking medicines with a narrow therapeutic

range. The majority of suspected herb–drug interactions are identified through case reporting and it is therefore difficult to determine definitely if the observed adverse effect is produced by herb–drug combination or by other factors.

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W07-04**Safety of herbal medicinal products—Regulatory approach**

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Herbal medicinal products (HMPs) are highly accepted in many Member States of the European Union, even though their specific regulatory status may be different.

In contrast to other medicinal products HMPs have particular characteristics which should be additionally considered in assessment of safety. HMPs are produced from botanical starting material which may also be part of the regular, dietary and/or environmental exposure. The active substances are complex mixtures with a large number of constituents the quantitative composition may be even varying. Detailed specifications and suitable labelling of HMPs are inevitable to guarantee a safe use.

Generally there are no exceptions in the European legislation with respect to the preclinical safety evaluation of HMPs. Nevertheless, when applying the legislation a reasonable adaptation to cover the particulars of HMPs is inevitable.

A special Committee on Herbal Medicinal Products (HMPC) was established at the European Medicines Agency (EMA) in London to generate a harmonised view on all issues concerning HMPs. The most important task of the HMPC is to create European monographs for herbal substances. Within this work the HMPC adopted a series of guidance documents referring to the safety of HMPs (e.g. general guidance on safety assessment, specific issues like toxicity of defined natural products). Different preclinical testing strategies are required for different categories of HMPs (new active substances, well-established use, traditional HMPs). Especially evaluation of genotoxicity of HMPs and suitable strategies to get reasonable safety data in this field are a current focus of the German national competent authority.

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Workshop 8: Zebrafish as a Toxicogenomic Model of the Effects of Chemicals on the Developing Vertebrate Embryo**W08-01****Zebrafish embryos as a model in general toxicology**

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On January 1, 2005, the 48 h embryo test with the zebrafish (*Danio rerio*; DIN 2001) became a mandatory test for the routine monitoring on whole effluent toxicity in Germany and, thus, replaced the conventional acute fish test as a standard method for effluent

testing. In an attempt to establish an alternative test method for the assessment of chemical toxicity, as well, a proposal for a new guideline on fish embryo toxicity for testing of chemicals has been submitted by the German Federal Environment Agency to the OECD guideline program. A comprehensive statistical analysis revealed that the correlation between the fish embryo test and the acute fish toxicity test is just as good as that between conventional acute fish toxicity tests with different fish species. Comparative studies with the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*) documented that species-specific versions of the optimized zebrafish test protocol could equally be applied to other OECD species. Additional modifications to the standard protocol allowed extending the use for the fish embryo test to, e.g., *in situ* sediment toxicity evaluation, genotoxicity and mutagenicity testing, endocrine disruptor research as well as histopathological studies into, e.g., gonadal or hepatic pathology. Only recently, several approaches have been made to use microarrays for zebrafish embryo toxicity and teratogenicity testing purposes.

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W08-02

In vitro teratogenicity testing with zebrafish embryos combined with a mammalian metabolic activation system: Pro's and con's

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Several alternative methods are available investigating the embryotoxic and/or teratogenic potential of chemicals, which are based either on cell, organ or whole embryo cultures. However, it is highly disadvantageous that some of these tests (i) still need animals, (ii) often don't cover the whole embryo-/feto-genesis and (iii) generally don't address possible metabolic activation of test materials. Recently, the zebrafish (*Danio rerio*) embryo was shown to be a useful tool for the detection of teratogenic effects induced by chemicals: The development of zebrafish is very similar to higher vertebrates, the eggs are completely transparent enabling a detailed observation and the test system is easy to handle. While many direct acting teratogens e.g., retinoic acid can easily be detected in this test system, most proteratogenic compounds are missed due to the absence of an efficient metabolic activation system. In this lecture the successful combination of the zebrafish embryo assay with a metabolic activation system (liver microsomes) is described. A standard protocol including specific parameters, such as incubation time, temperature, agitation, and buffer system is provided. Teratogenicity is scored at 24, 48 and 72 h post fertilization using standardized morphological endpoints. Several examples of substances displaying teratogenic effects in *Danio rerio* only after metabolic activation e.g., cyclophosphamide, ethanol and trimethadione, are presented. Major limitations including the reduced bioavailability of poorly water-soluble substances and the toxicity of the metabolic activation system itself are mentioned. Finally, the relevance of this newly developed teratogenicity assay in the context of scientific and regulatory needs is discussed.

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W08-03

In vivo approaches to define toxic response mechanisms

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The field of toxicology is rapidly evolving from a descriptive to a mechanistic discipline. Zebrafish share a number of similarities with established models at the genomics, cellular, physiologic and behavioral levels. Exposure to dioxins, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a wide array of adverse responses which are mostly considered to be mediated through the inappropriate activation of the aryl hydrocarbon receptor (AHR) signaling pathway. The molecular mechanisms responsible for the adverse outcomes after AHR activation are unclear. To identify events downstream of AHR, which play roles in the toxic response, we employed a caudal fin regeneration model. We previously discovered that AHR activation inhibits tissue regeneration. Using antisense morpholinos and mutant fish lines, we identified that zfAHR2 and zfARNT1 are the *in vivo* dimerization partners that are required for TCDD to inhibit regeneration. Comparative toxicogenomic analysis in adult and larval fin regenerates revealed that TCDD exposure leads to the misregulation of Wnt signaling pathway. R-Spondin1, a ligand for the Wnt coreceptor, was highly induced by TCDD exposure. Antisense repression of R-Spondin1 restored the regenerative response, in the presence of TCDD. This finding demonstrates that inhibition of regeneration by TCDD is mediated by R-Spondin1 misinduction. We further demonstrated that the TCDD-mediated block in regeneration is also LRP6 dependent. Collectively, these results indicate that inappropriate regulation of R-Spondin/LRP6 is absolutely required for TCDD to inhibit fin regeneration. More importantly, these results indicate the utility of the zebrafish model to rapidly elucidate toxicant modes of action.

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W08-04

Analysis of signaling pathways by knock down screens in zebrafish

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Purpose: We are interested in the function of protein-tyrosine phosphatases in early development and we have identified all 47 zebrafish genes that encode protein-tyrosine phosphatases in the zebrafish genome. We have established their expression patterns during early embryogenesis by whole mount *in situ* hybridization.

Method: Morpholino-mediated knockdown of protein expression in zebrafish embryos is a powerful method to rapidly and specifically assess protein function in a whole organism. Antisense morpholinos are designed to target either the start ATG or exon-intron boundaries and they are micro-injected at the one-cell stage. Typically, morpholino injections result in transient knockdown of target protein expression. Morpholino-mediated knockdown can be used to assess the function of many genes in embryogenesis individually or in combinations.

Results: Morpholinos were designed against the exon-intron boundary upstream of the catalytic site of each protein-tyrosine

phosphatase to ensure that no functional protein-tyrosine phosphatase would be expressed following micro-injection of the morpholinos. Knockdown of at least six protein-tyrosine phosphatases induced defects in convergence and extension cell movements during gastrulation. By analysis of epigenetic interactions, we have placed some of these protein-tyrosine phosphatases in signaling pathways upstream of Src family kinases and small GTP binding proteins, in particular RhoA. Morpholino-mediated knockdown has provided insight into the function of protein-tyrosine phosphatase signaling in embryonic development.

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W08-05

In vivo small molecule discovery in zebrafish

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The divide between in vitro and in vivo research remains a major impediment to drug discovery. The in vitro assays that dominate modern discovery efforts are often poor predictors of a compound's ultimate efficacy and safety. As a result, compounds with promising in vitro activity often fail upon transition to the in vivo setting.

The unique attributes of the zebrafish enable in vivo experimentation to be conducted inexpensively and in high throughput. As a result, zebrafish allow in vivo studies to be incorporated into most steps in the drug discovery process, including high-throughput screening. We have used screening in zebrafish to discover compounds with in vivo activity against pathways of diseases ranging from leukemia to anemia and psychiatric diseases. Because the discovery process occurs in vivo, effective compounds can be discovered even in the absence of a validated target. And, because the in vivo screens eliminate compounds with significant in vivo toxicity, the compounds discovered may be less likely to produce unexpected toxicities at later stages of development. Thus, the zebrafish and other emerging model organisms may accelerate drug discovery by reducing dependence on target validation and by providing in vivo data on compound efficacy and safety at the earliest stages of discovery.

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W08-06

Barcode-like toxicogenomic profiles identify several hundred genes that respond to environmental toxicants in the zebrafish embryo

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Early life stages are generally most sensitive to toxic effects. Our knowledge on the action of man-made chemicals on the developing vertebrate embryo is rather limited. We addressed in a systematic manner the toxicogenomic response of the zebrafish embryo by asking whether distinct chemicals would induce specific transcriptional profiles. We obtained specific expression profiles for each of the 11 chemicals and could predict the identity of the toxicant from the expression profiles with high probability. Changes

in gene expression were observed at toxicant concentrations that did not cause morphological effects. The toxicogenomic profiles were highly stage-specific and we detected tissue specific-gene responses underscoring the sensitivity of the assay system. We report on the further characterisation of specific genes that respond to the toxicant methylmercury.

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Workshop 9: Assessment of The Action of Chemical Mixtures and Impact on The Concept of Toxicological Threshold of Concern (TTC)

W09-01

General principles for the toxicological assessment of mixtures

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In risk assessment the presence of a mixture is characterized by an external exposure pattern with several compounds (chemically similar or dissimilar) at the same time. Mixtures may occur as combination therapy/concomitant therapy, as pesticide residues and as contaminants.

Compounds may interact at the level of toxicokinetics or of toxicodynamics. Critical kinetic interactions are characterized by an increased internal exposure and an increased effect size. Critical kinetic interactions are mediated by enhanced tissue binding, by a lower clearance due to inhibition or enhanced bioactivation due to induction of metabolism. Combined effects may be non-interactive; the effects observed being dose addition or response addition. Interactive action may lead either to a greater than dose additive effect (potentiation) or to an effect less than dose additive effect (antagonism).

Grouping compounds may be based on the same mode of action and/or the same identified molecular target. Data is needed for defining key events and dose-response data for benchmarking. Ideally data on time course of toxic effects and data from studies with mixtures would be available.

There are several methods in use to assess mixtures. The Hazard index (HI) may be useful for first level screening but is limited by potential influence of policy on choice of assessment factors. The Point of departure index (PODI) is preferable to HI. The combined margin of exposure (MOET) has the disadvantage that acceptance criteria are not established. Relative potency factors (RPF) are only applicable for simple similar action.

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W09-02

Deviation from additivity in mixture toxicity: Nonlinear dose-response and relevance for genotoxicity

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Nonlinearity in dose-response relationships for toxic effects, including genotoxicity, is the rule rather than the exception. This finding complicates the interpretation of mixture effects because sublinearity (slope increasing with dose) can result in seemingly synergistic combination effects. If two mixture components oper-

ate by the same mode of action, dose addition is the appropriate type of data analysis. This means that one toxicant drives the effect up on the sublinear curve of the other. If the observed effect is induced via different pathways, response addition is appropriate; two dose-response curves follow each other sequentially. Curves of dose addition and response addition embrace an “envelope of additivity”. Synergistic or antagonistic interaction can be postulated only if the mixture effect is outside this area. This situation was investigated experimentally with (i) the Ames test and (ii) in vitro micronucleus tests, using binary combinations of different DNA-alkylating agents, the topoisomerase-II inhibitor genistein, and gamma irradiation. Results showed not only different types of dose-response relationships and combination effects, such as supraditivity, dose addition, response addition, and antagonism, but also mixed effects. We conclude that knowledge of the dose-response curves of all components of a mixture is crucial to postulate synergism or antagonism and that an in-depth mechanistic knowledge is required for appropriate data interpretation.

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W09-03

Use of Cramer classes and additive models of mixture toxicity: Application to real world mixtures and an assessment of factors that minimize the potential for synergy

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Cramer classes and the Munro et al. (1996) database (hereafter referred to as CC), are part of the TTC and conservatively predict chronic toxicity based on structure. In this paper CC are used for providing the noncancer toxicity estimates for mixture components that have missing toxicity data. Without such data, it is not possible to use additive or other models of mixture toxicity. In this presentation, we report on an assessment of mixtures in a series of 48 surface water samples. These samples contained detectable levels of from 2 to 23 chemicals and a total of 44 chemicals were found in one or more of the samples. Toxicity data were found for 31 of the 44 chemicals and CC were used for the remaining 13 chemicals. A screening additive model was used to assess the toxicity of the mixtures. The resulting estimates of mixture toxicity were sufficient to determine that the mixtures were unlikely to pose a risk to human health. Use of the CC was shown to result in 4 to 5000 fold overestimates of mixture toxicity. This suggests that the approach is most appropriate for use in screening assessments. In addition, the additive models constrained exposures to all mixture components to doses below their chronic standards and for most components the doses were more than 100 fold below their standards. This constraint limits the potential for interaction between mixture components and thus the possibility of synergy.

Reference

Munro, I.C., Ford, R.A., Kennepohl, E., Sprenger, J.G., 1996. Food Chem. Toxicol. 34, 829–867.

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W09-04

Critical analysis of literature on low dose synergy for use of TTC in screening chemical mixtures for risk assessment

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Scientists need a better understanding of the consequences of co-exposures to substances at environmentally relevant concentrations. As it is not possible to test all combinations, a default assumption of dose or response additivity, as appropriate, has been used for the joint toxicity assessment of chemicals. From a safety perspective, it is essential to know whether synergistic interactions can occur at these low exposure levels. A literature search was conducted to identify studies of synergism, with emphasis on doses close to the individual chemicals' NOAELs or LOAELs. The search identified 204 chemicals by in-depth critical review of 90 references. A quantitative estimate of low dose synergy was found in only a few studies. Calculations of interaction magnitude were included in 11 articles. While the range of reported values is quite wide, these values are not directly comparable because definitions and quantification of synergy differed across the studies. Methods varied in terms of the null hypothesis, response measured, point of departure (POD), and whether the slope of the dose-response curve was considered. Nevertheless, the magnitude of any low dose synergy reported was generally relatively modest. It is anticipated that this analysis will help in developing an approach for quantitative risk assessment of chemical mixtures, including use of a Threshold of Toxicological Concern for screening and prioritization. Prior to this, it is recommended that: synergy be defined in terms of departure from dose additivity; uniform procedures be developed for assessing synergy at low exposures; and POD for calculating synergy be standardized.

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Workshop 10: Photosensitization: What Makes the Difference?

W10-01

Physicochemical behaviors of photosensitizing drugs

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The absorption of light by a drug generates electronically excited states (singlet or triplet states). In both multiplicities, very fast internal conversion leads to the lowest excited states (S_1 and T_1 , respectively). These states, which have typically lifetimes of nanoseconds for S_1 and microseconds for T_1 , live long enough that a chemical reaction competes with internal conversion to the ground state (S_0). These excited states are electronic isomers of the ground state and can show a different chemistry.

The competition between the chemical reactions and physical decay to the S_0 depends in a complex way on the structure and on conditions. Efficiency and photoproduct distribution of a photochemical reaction can change significantly even among closely

related compounds and further depend on the experimental or applicative conditions. In this context, a satisfactory knowledge of the physicochemical behaviors of a photosensitizing drug is necessary to understand the effect produced by the combined action of the drug and light on patients. Mechanistic aspects of the interaction of drug-derived reactive intermediates and/or reactive oxygen species with biomolecules will be analyzed to explain the desired effects produced by some drugs in the PUVA-therapy or photodynamic therapy (PDT), as well as the undesired effects associated to drugs such as phototoxicity, photoallergy, photogenotoxicity or photocarcinogenicity.

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W10-02

Phototoxicity in the human retina

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Purpose: To characterize the auto-oxidation and photooxidation products of A2E and compare them with that found in lipofuscin *in vivo*.

Methods: Human RPE lipofuscin granules were isolated as described (Feeney-Burns, 1980) from donor globes (Midwest Eye Banks). The organic soluble portion was obtained by extraction with equal amounts of CHCl₃:CH₃OH:H₂O, and the extract was analyzed by LC-ESIMS. A2E was kept in the dark for 3 weeks (auto-oxidation) or irradiated for 1 h (photooxidation) with Phillips "special blue" Bilirubin bulbs. These samples were analyzed by LCMS as described above.

Results: The oxidation of A2E results in a complex mixture of products consisting of 1) lower molecular weight aldehydes and a series of oxygen atom additions. However, the majority of human lipofuscin consists of relatively hydrophobic components corresponding to derivatized A2E with discrete molecular weights of 800–900 *m/z*, 970–1080 *m/z* and above 1200 *m/z* regions. In order to determine the mechanism of these modifications, A2E was chemically modified by: (1) esterification, (2) reactions with specific aldehydes and (3) allowed to spontaneously auto-oxidize. The reactions with aldehydes yielded nearly identical products as found *in vivo* while esterification yielded very different structures as determined mass spectrometrically.

Conclusions: Aging of RPE lipofuscin results from the auto and/or photooxidation of A2E to form aldehydes which then further react with A2E to give a series of polymers that are much more hydrophobic than A2E. This increases log *P* (hydrophobicity) and induces the sequestering of these derivatives into granules with a concomitant diminution in reactivity.

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W10-03

Preclinical photosensitization testing: Models and challenges

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This paper focuses on aspects of preclinical assessment of photosensitizing properties (phototoxic and photoallergic potential) of drugs. As defined by regulatory guidance, drugs with a light absorption range between 290 nm and 700 nm apply for photo-safety evaluation. In the *in vitro* area, one validated test exists for phototoxicity testing (3T3 PT NRU assay), which received formal endorsement by the OECD (OECD TG 432). *In vivo* test methods using guinea pigs were developed in the past and depend largely on the testing scheme applied in contact sensitization testing (e.g. Bühler test). In the last decade murine models for topical and oral photosensitization testing have been described in the literature, which are again mostly based on murine contact sensitization models (e.g. UV-Local Lymph Node Assay (UV-LLNA, IMDS)). Although the underlying contact sensitization models are validated and considered to be reliable because of a long-standing experience, the *in vivo* photosensitization models were never subjected to a formal validation procedure and therefore lack a clear endorsement by drug regulating agencies. Challenges in photosensitization testing arise from the use of the oral route, which requires distribution of the test drug into skin and eyes of test animals. Involvement of the eye as endpoint of photosensitization testing has to consider the light absorption/transmission features in eyes of test animals and humans. Furthermore, due to some deficiencies in the integrating interpretation of preclinical and clinical photosensitization data, preclinically identified photosensitizing hazards are difficult to translate into human risk assessment. This paper will conclude with a strategy about the employment of *in vitro* and *in vivo* photosensitization test methods in drug development whilst recognizing the limitations in human risk assessment.

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W10-04

Clinical photosensitisation: Phototoxicity testing in man

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Such is the variety of presentations of drug-induced phototoxicity, a careful clinical test system that will pick up phototoxicity of an immediate and delayed type with particular attention to detection of the wavelength dependency and degree of abnormality is needed.

Follow-up study with skin biopsy as appropriate is required to monitor the duration of sensitisation following drug cessation, as well as the duration, degree and nature of any pigmentary response. Also important is the symptomatology and duration of urticarial responses known to be a feature of some phototoxic drugs.

When designing a study, blinding, as well as provision of a positive and negative control, with group sizes adequate to pick up a factor of 1.5–2 difference between the drug under study and placebo is required.

Phototesting is usually conducted with narrow wavebands of ultraviolet and visible light covering the terrestrial exposure. In addition, a solar simulator can be employed, although too broad

wavebands may not reveal the wavelength dependency which can be critical when evaluating the clinical relevance of a phototoxic pattern of results.

Although retinal phototoxicity is a particular concern, lens, iris and corneal effects are all possibilities. Fortunately, the eye seems well equipped with antioxidant safety systems. Nevertheless, we know surprisingly little regarding the phototoxicity of the anterior part of the eye. Melanin binding may offer some protection but some phototoxic drugs may augment the phototoxic effect.

This presentation will look into some of the possible scenarios that can arise with phototoxicity testing in man.

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Workshop 11: Methods in In Vitro Embryotoxicity Testing

W11-01

State of the art: Development of methods

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The techniques employed in embryotoxicity testing *in vitro* have been in existence for many years. Indeed, most were available when this field was first a workshop topic 30 years ago. A major advance was the ECVAM-sponsored validation of three techniques: whole embryo culture (WEC), micromass (MM) and the embryonic stem cell test (EST), the results of which were published between 2002 and 2004, and which are embodied in INVITTOX protocols 123, 122/114 and 113, respectively. At the time these methods were selected for formal validation a forth technique, FETAX, based on the *Xenopus* embryo, was being evaluated independently. In the past five years, there have been a number of developments, and these will be the subjects of this presentation. Micromass cell culture approaches have not advanced markedly, whereas there have been a number of technical refinements of EST, most recently a molecular FACS based approach. There have been improvements in the evaluation of WEC data, in particular in benchmark dose approaches to quantitative extrapolation. Both EST and WEC techniques have been adopted and modified by the pharmaceutical and chemical industries, and there has been consideration of how these can be integrated into decision-tree testing, in particular in relation to REACH. The zebrafish has clearly supplanted *Xenopus* as a leading non-mammalian approach, with several interesting applications in pharmacology and in safety assessment. Very recent advances in establishing human induced pluripotent cells (iPS) with the elimination of exogenous reprogramming factors, raises the prospect of a human EST without need of human embryos.

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W11-02

Molecular and cellular endpoints of embryotoxicity in the embryonic stem cell test (EST)

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Compounds of the pharmaceutical and chemical industry need to be tested for their toxicological potential to humans and the environment according to international guidelines. The U.S. High Production Volume Challenge Program and the European chemical legislation program REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) require extensive animal experimentation to assess the toxicologic profile of chemicals. This includes tests for potential teratogenicity and effects on embryo-fetal development as this is a major consideration in drug development.

The embryonic stem cell test (EST) was originally developed and fully validated to potentially reduce the number of animal experiments and to be used as an early screening model for preliminary embryotoxicity profiling of compounds in the chemical and pharmaceutical industry. The basis of the classic EST is the differentiation of murine embryonic stem (ES) cells (permanent cell line D3) into cardiomyocytes (Spielmann et al. 1997). Three toxicological endpoints are assessed in the EST: (1) the inhibition of the differentiation of ES cells into contracting cardiomyocytes (microscopic evaluation) is compared with (2) cytotoxic effects on ES cells and (3) cytotoxic effects on 3T3 fibroblasts. Determined parameters are then included into a biostatistical model for the subsequent classification of the embryotoxic potential of the test compounds into non-, weakly or highly embryotoxic.

Recently, further efforts were undertaken to improve the classic EST. Protocols were developed to differentiate murine ES cells into other cell types, e.g. neural cells, chondrocytes, osteoblasts or endothelial cells and to detect them by specific molecular markers at the RNA and protein-expression level. Further improvements could include using human embryonic stem (hES) cells in embryotoxicity testing, which could relieve the difficulties of hazard identification due to inter-species variations, and the integration of a metabolizing system into the EST may be a chance to further improve the predictivity of the assay. Consequently, there is a need to comprehensively characterize selected molecular and functional endpoints of both the mouse and the human embryonic stem cell tests.

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W11-03

Relevance of WEC as additional test to animal based testing

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Rodent WEC is the only *in vitro* test permitting the treatment, the manipulation and the analysis of mammalian embryos maintaining the integrity of both the embryonic tissues and the extraembryonic membranes.

The highly controlled culture conditions allow to select the medium composition, the embryonic stages, the time and the length of exposure. Moreover, it is possible to perform a contemporaneous or subsequent exposure to different molecules or factors.

The limit of WEC consists of the fact that the embryos can be cultured just during a limited period, even if the main organogenetic phases organizing the embryo at its phylotypic stage take place during the culture.

Considered as a time-saving, not highly expensive, and well controlled method, WEC has been proposed for the comparative screening of chemicals, when a large number of molecules of the same chemical family has to be tested. However, the WEC predictability is not certain, due to the absence of the maternal compartment (with its toxicokinetic and toxicodynamic parameters), instructing also on the limit doses that are reasonably to be tested. WEC is not considered acceptable by agencies as a substitutive method for *in vivo* tests used to register new chemicals.

However, the possibility to study in the isolated embryo compartment the mechanism and the pathogenic pathways on the basis of malformation is unique for WEC and often not performable by using *in vivo* methods.

The present lecture will show some example concerning the study of the mechanisms and the pathogenic pathways by using WEC and will illustrate how WEC results can allow the interpretation of data collected by *in vivo* experiments.

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W11-04

Human neurospheres as a potential tool for developmental neurotoxicity testing

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Current developmental neurotoxicity (DNT) testing in rodents *in vivo* possesses two major obstacles: the requirement of large amounts of animals and the uncertainty of extrapolating the data to humans. To reduce animal consumption and tackle species specific differences, we have established a human *in vitro* model for DNT based on normal human neural progenitor cells (hNPCs) and their murine correlates (mNPCs, E16) which are both cultured as proliferating neurospheres.

On poly-D lysine/laminine coated surfaces, NPCs migrate radially out of the sphere and thereby differentiate into the major neural cell types of the brain. Thus, cell proliferation, migration and differentiation, which are key steps in brain development, can be quantified. Moreover, hNPCs react with Ca^{2+} signaling to diverse stimuli. Comparing these endpoints between the different species, we found that murine neurospheres proliferate faster, migrate initially at a higher speed but over a much shorter period of time and mature more rapidly during differentiation than human spheres. Furthermore, they have an overall shorter lifespan than human cultures. These data suggest that the *in vitro* cultures mirror the gross developmental differences of mice and humans. First testing of positive and negative test compounds which are known to interfere or are not associated with brain development indicate that e.g., IC_{50} values for inhibiting proliferation, migration and differentiation for methylmercury are in the nanomolar and for paracetamol, an agent known to exert liver toxicity, in the millimolar range.

More test compounds are investigated and will give us information on the validity of this promising system.

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Workshop 12: What Does It Tell Us About Dioxin Toxicity?

W12-01

Mechanisms of AH receptor action

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Modern embryonic stem (ES) cell technology is helping us to understand the mechanism of action of toxic chemicals. In this talk, the use of gene targeting in murine ES cells will be discussed. Strategies will be described where the generation of null conditional and hypomorphic alleles are produced from a single manipulation of the genome *in vitro*. The application of these various alleles will then be described with respect to understanding the toxicity of the environmental pollutant and potent toxicant, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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W12-02

The structure of the AH receptor transactivation domain as a determinant of dioxin sensitivity

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There exists an exceptionally wide variation in sensitivity to the acute toxicity of TCDD among, and even within, species. We have long exploited as an animal model a 1000-fold difference between two rat strains, Long-Evans (*Turku/AB*) and Han/Wistar (*Kuopio*), and shown that this difference mainly emanates from a splicing alteration in the AH receptor (AHR) of TCDD-resistant H/W rats resulting in the presence of two AHR proteins, a deletion (DEL) and an insertion (INS) variant (the predominant form), both of which are restructured at the C-terminal transactivation domain of the AHR. To compare the function of rat wild-type (rWT) and variant AHRs on a genetically homogeneous background, we generated transgenic mouse lines which globally express these isoforms. They proved to have no major phenotypic changes apart from increased testis size (associated with high expression of the AHR), and their AHRs were shown to be functional both physiologically and as mediators for TCDD. The lines exhibited clear differences in sensitivity to TCDD lethality with rWT mice being highly sensitive, DEL mice moderately resistant and INS mice highly resistant. Short-term dose-response and time-course studies revealed that INS mice failed to exhibit almost any of the biochemical or morphological effects of TCDD while rWT mice were highly responsive. Interestingly, TCDD treatment induced degradation of the AHR protein and to a larger degree in DEL and INS than in rWT mice, which may contribute to the line differences. Thus, AHR transactivation domain structure is critical to TCDD sensitivity, probably through interactions with co-regulatory proteins.

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W12-03**The aryl hydrocarbon receptor and cellular plasticity**

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The cellular response to the influx of potentially toxic xenobiotics is initiated by xenobiotics receptors. We have focused on the dioxin receptor (Arylhydrocarbon Receptor, AhR). Functional genomics studies allowed us to identify three different gene sets which are altered by exposure to dioxin or related toxicants. The first set includes genes involved in specific adaptation to xenobiotics exposure, i.e. metabolic enzymes and transporters. The second set includes genes involved in cell cycle control or apoptosis which are modulated during a variety of cellular stress conditions. The third set includes genes that do not clearly exhibit adaptive functions to xenobiotics exposure. Their regulation could reflect endogenous functions of the AhR or conserved ancestral functions and may reveal novel toxic mechanisms of AhR activation.

Addition of dioxin to the hepatoblastoma HepG2 cells induces a set of genes that encode proteins involved in cellular plasticity and migration. We have characterized the induction of the Hef-1 gene which is implicated in integrin signaling as well as other cellular pathways. To confirm the relevance of these observations, we have shown that dioxin alters cellular plasticity of the MCF-7 and HepG2 cells. It also increases cell scattering, cell spreading and cell migration. An engineered constitutively active AhR receptor mimics these effects. Cellular plasticity is involved in epithelio-mesenchymal transition, a process that is critical for normal development, immune function and cancer progression. These studies could shed light on the mechanisms of dioxin and PAH toxicity and on their putative effects on cancer progression and fetal development.

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W12-04**Cell specific action and toxicity of the AhR: Focus immune system**

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Cell specificity is an outstanding feature of aryl hydrocarbon receptor (AhR) toxicity. Persistent overactivation of the AhR with halogenated aromatic hydrocarbons like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) leads to primary and secondary gene activation. This in turn results in pleiotropic effects, which become evident as changes in cellular differentiation programmes, physiological functions, and on the organismic level as adverse or toxic symptoms. Like most transcription factors, the AhR is not a simple molecular switch, but subject to several tiers of control; other proteins of the transcription machinery, chaperones, co-factors, accessibility of a locus. Last, but not least, cell type, cell stage and cell differentiation events affect AhR action. We found that in promoters of AhR-targeted genes NFκB-Rel, HRE, PPARγ, GR, PAX-4, and estrogen receptor binding sites were over-represented. Co-treatment experiments with TCDD and CoCl₂, to mimic hypoxia, or TCDD and 17-β-estradiol (E2) verified a crosstalk between AhR and HIF or ER, in agreement with other experimental models.

Recent findings about physiological AhR ligands highlighted effective ligand differences, and much of the current research focuses on the physiological role of the AhR, and the link between AhR and adaptive responses to the chemical environment. Adverse immune reactions are a hallmark of TCDD and AhR-mediated toxicity, affecting almost all parts of the peripheral immune system. In recent studies we have added findings on the barrier immune organs—skin and gut. Contact hypersensitivity was suppressed in TCDD-exposed mice, and fewer Langerhans cells (i.e. the antigen-presenting cells in the skin) and significantly fewer dermal dendritic cells DC reached the lymph node after antigen-exposure. This presumably results in a lower T cell activation capacity. At the same time, the major cell type in skin, keratinocytes, are targets of TCDD, and AhR is involved in constitutive cytokine-production of GMSCF and other cytokines. Oral tolerance is broken by TCDD, a finding which opens up many new questions as to the known AhR agonists in normal diets.

TCDD activation of AhR should be differentiated from physiological activation with quickly degraded ligands such as FICZ, a UVB generated photoproduct of tryptophan. Not all tissues and cell types express AhR. We identified by Western Blot high expression levels of AhR in Langerhans cells, and dendritic cells. Other groups found AhR exclusive in Th17 CD4 T cells, but not in Th1 and Th2 CD4+ T cells - which are arbiters of TCDD-mediated immunosuppression. Beyond the canonical pathway, also other AhR signalling modes may be important in the immune system. We noted that stem cell populations express high levels of AhR, but very little of its canonical partner ARNT. We suggest, that the AhR system is physiologically relevant in immune cells, and particularly relevant for immune cell differentiation, and responses to small chemicals. The cell-specific action of AhR and its tight control must be considered for a correct understanding of dioxin toxicity.

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W12-05**Investigating partial agonism of the aryl hydrocarbon receptor**

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The ability of a compound to bind to a cellular receptor is not sufficient to explain the biological action of the receptor. According to current theory, the binding of a ligand to a receptor can result in activation of the receptor (agonism), in inactivation of the receptor (antagonism), or a ligand can result in a mixed response, where a proportion of receptors are activated, and a proportion inactivated; this latter state is known as partial agonism. The ability of a compound to be a partial agonist has important consequences for risk assessment, as the effect of adding a partial agonist will not necessarily be dose-additive to the effect of an agonist, and so the established concept of dose-additivity may fail.

We have studied a series of benzothiazoles, and shown that the ability of these compounds to bind the rat AhR (affinity) does not predict their ability (potency) to induce an AhR-target gene, CYP1A1, in the rat cell line H4IIEC3. Moreover, by means of a competitive assay, we have shown that a benzothiazole (5F203) with high affinity, yet low potency, is able to compete with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) in activating the induction of CYP1A1 RNA. By contrast, a benzothiazole with high affinity and high potency is unable to compete the ability of TCDD to induce

CYP1A1 RNA. We were further able to quantitate the antagonistic effects of 5F203 by means of a Schild assay, and show that the antagonism of this compound is competitive, and that the antagonistic activity (the K_B) of 5F203 is comparable to its affinity for binding the AhR (Bazzi et al., 2009).

These data show that a model compound acts as a partial agonist for the AhR. We are currently setting this assay system up to systematically investigate other AhR ligands, to determine if they have partial agonist activity, and to determine whether the notion of partial agonism is of general importance in risk assessment of dietary dioxins and furans. We will also investigate novel food con-

taminants, to determine if they are AhR ligands which may be of toxicological interest.

Reference

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