

Symposia

Symposium 1: The Epigenome ? Role in Carcinogenesis

S01-01

Living with and without the maintenance cytosine methyltransferase, Dnmt1

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Epigenetic regulatory mechanisms involve heritable alterations in chromatin structure that govern the transcription profile and genome stability of a cell during animal development or in different disease states. The key molecular events underlying epigenetic changes in the nucleus as a result of genotoxic or non-genotoxic chemical exposure include alterations in the patterns of DNA methylation and modification of histones leading to stable changes in the transcription profile. Together, DNA methyltransferase and histone modifying enzymes can alter the chromatin environment resulting in new epigenetic (expression) states that promote the formation of altered foci and predispose to the development of cancer. We have identified a cellular pathway that activates apoptosis in response to changes in the levels of the maintenance cytosine methyltransferase, Dnmt1. We propose that suppression of this pathway is a necessary step in the development of cancer resulting from exposure to genotoxic or non-genotoxic chemicals.

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S01-02

Colorectal cancer epigenetics: Causes, consequences and clinical applications

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Aberrant promoter methylation is involved in transcriptional silencing of tumour suppressor- and DNA repair genes in colorectal cancer. However, the cause of altered epigenetic control of gene expression has not been elucidated yet.

Here I will present genome-wide epigenetic screens and to identify novel tumour suppressor genes in colorectal cancer. In addition, I will present data on the etiology of altered promoter methylation in colorectal cancer. Finally I will discuss the applications of promoter hypermethylation in clinical practice.

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S01-03

Global DNA promoter methylation in cancer

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Two general mechanisms have been identified that are involved in the silencing of cancer related genes. Genetic alterations, includ-

ing mutations and deletions, have been known to be involved in tumor suppression for many years. More recently, DNA methylation has been identified as an additional mechanism to silence genes. Aberrant DNA methylation is an early event in tumorigenesis and a major contributor in the development of solid tumors as well as leukemias. As an epigenetic alteration, DNA methylation does not change the sequence of a gene and thus offers the exciting possibility for therapeutic removal of the methylation group by demethylating drugs. Deregulation of mechanisms that control the establishment of normal DNA methylation patterns leads to both extensive aberrant hypo- and hypermethylation and has been described for several human malignancies. Global DNA hypomethylation in human cancers was one of the earliest changes associated with tumor progression. Our group has shown that human malignancies are characterized by extensive promoter CpG island methylation with non-random and tumor-type specific patterns. It is currently unknown how tumors acquire aberrant DNA methylation patterns. In this symposium I will discuss our current understanding of epigenetic alterations using the example of chronic lymphocytic leukemia (CLL). Data will be presented that describes the changes occurring in the epigenetic states in human CLL genomes. Furthermore, we will present data on a mouse model that recapitulates epigenetic alterations and develops CLL.

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S01-04

Histone acetylation and chemical carcinogenesis

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Pharmacological agents that damage DNA result in a number of genomic responses geared to permit repair. One class of eukaryotic responses involves post-translational modification of histones. Histones are the protein components of the disc-shaped deoxyribonucleoprotein complexes called nucleosomes which wrap 146–165 bp of DNA and form the basic building unit of chromosomes. Their modifications is thought to affect genome structure directly, by modulating the physical properties of nucleosomes as well as by forming new binding sites for specific nuclear proteins, such as those involved in DNA repair. One well-known example is phosphorylation of lysine 129 of the H2A histone variant called H2A.X, which recruits double strand DNA break repair factors.

Acetylation on lysine 56 of histone H3 (H3-K56ac) was reported by our lab as well as by several others in 2005. In yeast it was found that accumulation of H3-K56ac is a genome-wide event that occurs during every S-phase and that this modification persists upon exposure to clastogenic agents. H3-K56ac thus appears to play an important role in the DNA damage response in yeast. We will present data and structural models pertaining to the mode of action of H3-K56ac in the response to chemical DNA damage.

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S01-05**Epigenetic changes in cancer as a signature of exposure to environmental and dietary factors**

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It is now recognized that epigenetic mechanisms are critical for understanding the causes of complex diseases such as cancer. Epigenetic events play key roles in the control of key cellular process and their deregulation has been associated with virtually all stages of cancer development and progression. A number of critical processes found in cancer cells, such as silencing of tumour suppressor genes, activation of oncogenes, aberrant cell cycle, and defects in DNA repair, can be caused by aberrant epigenetic states. Epigenetic inheritance include DNA methylation, histone modifications and RNA-mediate silencing all of which are essential mechanisms that allow the stable propagation of gene activity states from one generation of cells to the next. Although the role of epigenetic events is supported by both epidemiological and experimental studies, the precise contribution of epigenetic mechanisms and cellular targets of epigenetic alterations to human cancers are largely unknown. Almost spectacular technological advances in epigenetics and epigenomics now allow powerful screening of large series of samples with unprecedented resolution. Epigenetic profiling using both genome-wide and candidate-gene approaches in normal and tumour tissues as well as bodily fluids will help to elucidate the mechanism underlying tumorigenesis, but also can be exploited to identify specific epigenetic targets, environmental factors, and the critical windows of vulnerability to cancer. Recent advances in epigenetics and epigenomics and ongoing studies aiming to identify specific epigenetic targets, environmental factors, and the critical windows of vulnerability to environmentally induced epigenetic alterations as well as their implication for biomarker discovery, cancer risk assessment and prevention will be discussed.

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Symposium 2: ATP Transporters in Mechanistic Toxicology**S02-01****Roles of the ABC transporter family in toxicology**

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ABCB1 (MDR1) was the first gene of the ABC transporter family cloned by Victor Ling in 1976, from cells resistant to colchicine. Since that time the family has expanded to 48 in 7 sub-families. Polymorphisms in a substantial number of the genes are associated with a variety of Mendelian monogenic disease states including cystic fibrosis and several biliary cholestasies. In liver ABC genes are essential for the transport of molecules and metabolites across the canalicular hepatocyte membrane. Saturation of these transporters is probably responsible for many of the hepatotoxic pathologies that are observed after drug and chemical exposure. Similarly physiologically expression of some transporters in the intestine, testes and blood–brain barrier fulfils a protective role against xenobiotic exposure. Furthermore the ABC transporters are important in the transport of endogenous molecules, for example of porphyrin across the mitochondrial membrane (Schuetz, J.D., et al.,

Nature 2006). Many of the gene family are inducible by xenobiotics at the transcriptional level often with metabolic genes, allowing co-ordination of metabolic conversion with metabolite transport. This physiological response to toxicity is now often referred to as Phase III after metabolic oxidation (Phase I) and conjugation (Phase II). In cancer increased ABC gene expression is associated with resistance to chemotherapeutic drugs; in essence a toxicological phenomenon with the tumor cells utilizing the ABC genes to protect themselves. These conserved mechanisms underscore the importance of this gene family for protection of the cell against toxic stress.

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S02-02**Involvement of ABC transporters in pharmacokinetics and toxicokinetics**

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ABC transporters are highly expressed at important sites of drug and toxin distribution and elimination, including the intestine, liver and kidney. As a result, they play important roles in determining the bioavailability, distribution and clearance of many xenobiotics. P-glycoprotein, encoded by *ABCB1*, is highly expressed in intestinal epithelial cells and can limit the bioavailability of its substrates. Disruption of intestinal P-glycoprotein function could lead to increased drug or toxin exposure. Important liver efflux transporters include MRP2 (*ABCC2*), BSEP (*ABCB11*) and MXR/BCRP (*ABCG2*). These transporters are involved in the biliary secretion of xenobiotics and their conjugates. Variability in the biliary secretion of SN-38 glucuronide has been associated with the rate-limiting gastrointestinal toxicity of irinotecan. In the kidney, MRP2, MRP4 (*ABCC4*) and MXR/BCRP are the major ABC transporters involved in the renal secretion of drugs and other xenobiotics. Transport of antivirals by MRP4 is implicated in the renal toxicity of these drugs. ABC transport at the blood–brain, blood–testis, and maternal–fetal barriers influences the distribution of xenobiotics into protected areas, and disruption of this function can be associated with toxicity. This presentation will provide an overview of the role of ABC transporters in bioavailability, clearance and distribution to peripheral sites, with a focus on how variability in transport contributes to drug toxicity.

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S02-03**Regulation of ABC transporter genes**

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Metabolism and transport are the main determinants of the pharmacokinetic behaviour of drugs. The role metabolic enzymes is quite established. This is, however, much less the case with regard to transporter proteins. It is accepted that transporter proteins are crucial for drug disposition, but the role of transporter gene regulation is still being uncovered. Transport steps include uptake into epithelial cells of liver, intestine and kidney followed by excretion of either the parent compound or metabolites into bile, intestinal lumen or primary urine, respectively. Conversely, metabolites can also be transported from the epithelial cells back into blood. Excretion across apical membranes is mediated by members of the ATP-Binding Cassette family such as ABCB1 (P-glycoprotein), ABCC2 (MRP2) and BCRP (ABCG2). Excretion of drug metabolites back into blood can be mediated by other members of this family in the basolateral membrane of cells such as ABCC3 (MRP3), ABCC4 (MRP4) or ABCC5 (MRP5). In recent years it has become clear that these ABC transporters are strongly regulated by several nuclear receptors, such as PXR, CAR, VDR and FXR, which are activated by binding of a very wide spectrum of xenobiotics. These nuclear receptors similarly regulate CYP genes and therefore represent a major level of drug–drug interaction. In addition, it is becoming clear that these nuclear receptors represent a level of cross talk between drug elimination on the one hand and lipid and drug metabolism on the other hand.

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S02-04**Role of ABC transporters at the blood–brain barrier**

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Several ABC transporters function as efflux transporters at the blood–brain barrier (BBB). Mediating transport into the direction of the capillary lumen, these transporters efficaciously limit brain penetration of a variety of endogenous and exogenous compounds. Thereby, efflux transporters critically contribute to homeostasis of endogenous substrates but also protect the brain tissue from potentially harmful xenobiotics. During the last decade the role of efflux transporters in the pathophysiology and treatment of central nervous system (CNS) diseases has been recognized.

On the other hand, effective extrusion from the brain by transporters is a frequent cause for the pharmaceutical industry to exclude novel compounds from further development of CNS therapeutics. Moreover, high transporter expression levels that are present in individual patients seem to be a major cause of therapeutic failure in a variety of CNS diseases including brain tumors, epilepsy, brain HIV infection, and psychiatric disorder. In some diseases therapy or pathophysiology-associated factors may further increase the expression of efflux transporters thereby worsening the therapeutic situation.

In recent years awareness of the impact of brain efflux transporters on protection of brain tissue and on treatment of CNS

diseases has increased progressively. Future research focusing on both the physiological and pathophysiological functions of brain efflux transporters and their regulation should help to further elucidate the role of such transporters. Gain in knowledge may allow optimizing strategies for modulation of transporter function taking their spectrum of protective functions into consideration.

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S02-05**Role in ABC transporters in liver functions and disease**

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Recent studies in ABC transporter knockout models have demonstrated that “drug transporting” ABC transporters have both roles in normal liver function and in pathological conditions. I will describe several ABC transporter knockout models to illustrate their roles in liver biology. The basolateral transporter, Mrp4, normally expressed at low levels in the liver, but is strongly upregulated by cholestasis. This led to the hypothesis that Mrp4 might play a role in protecting the liver from bile acid overload. The Mrp4 knockout mouse model subsequently confirmed that Mrp4 protected mice from cholestatic injury. It is notable that human MRP4 is upregulated in liver cholestasis. Mrp3 is a basolateral transporter that is also upregulated by cholestasis. Mrp3 and Mrp4 appear functionally redundant because in vitro assays showed both transported bile acids. However, careful analysis of bile duct ligated Mrp3 knockout vs. wildtype mice demonstrated comparable liver damage and similar accumulation of bile acids, thus indicating its primary role was not protection from liver bile acid accumulation. These studies indicated that Mrp3 transported glucuronides, most notably bilirubin glucuronide. Mrp2 is a canalicular transporter that has also been demonstrated to transport bile acids in vitro. However, this is likely a minor role under normal conditions as the Mrp2 knockout is strongly impaired in GSH transport into bile. Recent studies have suggested Mdr1 has an adaptive role in the protection against cholestasis. In total, the development of ABC transporter knockout mice has fostered an understanding of their physiological roles apart from their roles as drug transporters.

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Symposium 3: Molecular Mechanisms of Toxicity for Bacterial Toxins**S03-01****Rho-inhibiting toxins**

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The monomeric Rho GTP-binding proteins are regulators of actin cytoskeleton, gene expression, cell cycle progression and cell death. As broad spectrum regulators of cellular functions Rho proteins are preferred targets of bacterial protein toxins. Such toxins are subdivided into modulating and (covalently) modifying toxins. Modulating toxins transiently mimic Rho activating or inactivating regulators. In contrast, modifying toxins harbour an inherent

enzyme activity to covalently attach a functional group such as an ADP-ribose (*Clostridium botulinum* C3-like transferases), a glucose (clostridial glucosylating toxins like *Clostridium difficile* toxin A) or an AMP moiety (*Vibrio parahaemolyticus* VopS) onto their substrate proteins. Covalent modification takes place at pivotal amino acid residues (threonine-37 and asparagine-41 in Rho), which reside in the effector region of Rho essential for down-stream signalling.

Exemplarily the C3-like toxins are presented in detail. The C3 toxin from *C. botulinum* is the prototype of Rho-ADP-ribosylating toxins, which specifically modify RhoA, B and C at asparagine-41. The ADP-ribose moiety alters the activation–inactivation cycle of Rho, thereby preventing activation (GTP-binding) and down-stream signalling of Rho. The specificity of C3 to selectively modify the 3 homologous Rho isoforms out of about 150 monomeric GTP-binding proteins is the basis for its use as tool in cell biology. To this end, it is applied in a cell-permeable form (fusion toxin or fusion to Tat-like delivery-competent peptides) to allow rapid and efficient uptake into intact cells. Based on the cell-type specific functions of Rho, cell-permeable C3 is now approved as locally acting drug to treat spinal cord injury.

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S03-02

Binary actin-ADP-ribosylating toxins: New insights into the interaction between bacterial toxins and host cell chaperones during internalization into mammalian cells

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Binary clostridial actin-ADP-ribosylating toxins comprise *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin and the toxins from *C. difficile* and *C. spiroforme*. These toxins consist of a binding/translocation component and a separate enzyme component, which mono-ADP-ribosylates actin in eukaryotic cells leading to depolymerization of actin filaments and cell death. The sophisticated mechanism by which these unique toxins are taken up into cells was investigated in detail for C2 toxin. The heptameric binding/translocation component C2IIa (420 kDa) binds to a carbohydrate receptor on the cell surface and assembles with the enzyme component C2I (49 kDa). After endocytosis of the toxin complex, C2IIa forms pores in the membranes of early acidified endosomes and unfolded C2I translocates through the pores into the cytosol. We discovered that translocation of C2I requires the activities of the heat shock protein Hsp90 and cyclophilin A, a peptidyl-prolyl cis/trans isomerase (PPIase). Pharmacological inhibition of cyclophilin prevented intoxication of cells with C2 toxin and blocked translocation of C2I into the cytosol of intact cells as well as across endosomal membranes *in vitro*. Moreover, cyclophilin A interacts with C2I in the cytosol of intact cells. The internalization of iota toxin also depends on Hsp90 and cyclophilin thus these chaperones/PPIases might be involved in membrane translocation of all members of this toxin family. This first observation that PPIases are involved in the internalization of bacterial protein toxins contributes to a better understanding of the mode of action of these important virulence factors and could be a rationale for novel therapeutic strategies.

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S03-03

Clostridial glucosylating toxins

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The toxin family comprises *Clostridium difficile* toxins A and B, *C. sordellii* haemorrhaging and lethal toxin and *C. novyi* α -toxin. The toxins are important virulence factors, which act on eukaryotic target cells by modification of low molecular mass GTPases. Most important are *C. difficile* toxins A and B, which cause antibiotic-associated diarrhea and pseudomembranous colitis.

The toxins bind to cell membrane receptors of target cells, which are not well characterized. After endocytosis the low pH of endosomes causes conformational changes of the toxins, which allow insertion of the toxins into membranes and subsequent translocation of the enzyme domain into the cytosol. Only the catalytic domain of the clostridial glucosylating toxins is translocated into the cytosol, where, depending on the toxin type, Rho and Ras GTPases are inactivated by glucosylation.

A multimodular structure reflects the mode of action of the toxins. They are structured according to the ABCD model and consist of the biological active N-terminal glucosyltransferase domain (A), the C-terminal receptor binding domain (B), a cysteine protease domain (C), and a middle part (D), which is involved in delivery of the catalytic domain into the cytosol. Recent data on structure and processing of the toxins will be discussed.

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S03-04

The botulinum neurotoxins between toxicology and pharmacology

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Botulism is caused by any of seven botulinum neurotoxins (BoNT from A to G), which are made of two polypeptide chains (L, 50 kDa and H, 100 kDa, consisting of two 50 kDa domains H_N and H_C). H_C binds rather specifically to peripheral cholinergic nerve terminals and L enters the terminals blocking the release of acetyl choline. Consequently, a flaccid paralysis and autonomic impairment develop; however, if the patient survive the respiratory failure, he/she will recover completely. Owing to the great improvements in food preparation, this disease is rather rare among humans, whilst it strikes heavily in the wild and in intensive bird and fish farms. BoNTs are the most potent known poisons, with mouse LD₅₀ between 1 and 5 ng/kg. Such extreme toxicity is due to their strict neurospecificity and to their enzymatic activity. BoNTs bind to a polysialoganglioside and to other lipids, and to the luminal domain of a synaptic vesicle protein; they are thus endocytosed inside a synaptic vesicle which is used as a “Trojan horse” to enter the nerve terminal. Here, novel acquisitions on the mode of BoNT membrane binding will be presented.

The low pH of the vesicle lumen causes a conformational change that allow H_N to form a transmembrane channel that mediates the transfer of the L chain into the cytosol. The L chain is a metalloprotease specific for one of the three SNARE proteins: VAMP/syntaxin, a membrane protein of synaptic vesicles

SNAP-25 and syntaxin, two proteins of the cytosolic face of the presynaptic membrane. They form a heterotrimeric coil-coiled complex which brings the vesicle close to the membrane ready to fuse. BoNT/B, D, F and G cleave VAMP at single different peptide bonds, whilst BoNT/A, C and E cleave SNAP-25 at different positions of the C-terminus and C also cleaves syntaxin (1). These results show that VAMP, SNAP-25 and syntaxin are key components of the neuroexocytosis apparatus and describe the molecular pathogenesis of botulism.

A remarkable aspect of the action of BoNTs is their duration of action which depends on: (a) animal species, (b) type of toxin and (c) type of nerve terminal. BoNT/A and C block skeletal nerve terminals for 3–4 months and the autonomic ones for >1 year, whilst the other types cause a weeks/months blockade. Here, possible explanations of the different duration of action and novel results on the assembly of the SNARE complex which are of general importance will be presented.

Another remarkable BoNT property is that the toxin “stays” very much at the site of intramuscular injection. Recently, we have set up a very reliable and sensitive assay of BoNT diffusion in vivo and have shown that the three formulations of BoNT available in Europe have a very limited and similar spreading. This result will be discussed during my presentation.

The high neurospecificity, the very limited diffusion and their reversibility of action makes BoNT injection the therapy of choice in all those syndromes caused by hyperfunction of peripheral cholinergic nerve terminals. BoNT/A is almost invariably used in humans. Some of the therapeutic uses of BoNT/A will be illustrated together with recent results that show that BoNT/C is equivalent to BoNT/A in this respect and could be a valid substitute for A when a patient becomes immune to BoNT/A.

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S03-05

Entry of Shiga toxin into cells

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Shiga toxin acts on cells by first binding to the neutral glycosphingolipid Gb3 at the cell surface. Binding is associated with stimulation of Syk, formation of a Syk-clathrin complex and clathrin phosphorylation (Walchli et al., 2009; Lauvrak et al., 2006). The toxin is then endocytosed both by clathrin-dependent and clathrin-independent mechanisms (Sandvig et al., 2008), and it is transported retrogradely to the Golgi apparatus and the ER before the enzymatically active part is translocated to the cytosol and inactivates the ribosomes. As a result, protein synthesis is inhibited, and apoptosis may occur. Sorting of Shiga toxin from endosomes to the Golgi is a requirement for intoxication, and one is beginning to understand the complexity of this process. Not only are components of the retromer (an endosomal protein complex containing sorting nexins) involved (Utscarpen et al., 2007; Bujny et al., 2007; Popoff et al., 2007), but also transport to the Golgi apparatus is also regulated by kinases. For instance, Vps34 (Skånland et al., 2007), PKC delta (Torgersen et al., 2007) and p38a (Walchli et al., 2008) modified by arrestins (Skånland et al., 2009) can be involved, and also the lipid composition of the cellular membrane plays a role (Raa et al., in press). This strict regulation is not observed for transport of the plant toxin ricin, which binds to both lipids and proteins with terminal galactose, and which is also transported retrogradely.

Protein complexes involved in endosome to Golgi transport pathway(s) are being characterized and will be described.

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Symposium 4: DNA Damage-induced Signaling and Cell Death

S04-01

Interplay of DNA repair and damage signaling in genotoxin-induced apoptosis and necrosis

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Damage to DNA is usually considered to be harmful for the cell. However, not every type of DNA damage is mutagenic and not all of them are cytotoxic. Perhaps best understood are alkylating agents, which are powerful mutagens and carcinogens and, moreover, are being used in the therapy of gliomas and malignant melanomas. These agents induce a dozen of DNA lesions, some of them have been identified to be carcinogenic, genotoxic and cytotoxic. DNA alkylation lesions are repaired by ABH proteins, base excision repair and MGMT. In MGMT lacking cells the minor lesions O6-methylguanine (O6MeG) and O6-chloroethylguanine (O6ChlG) pose the major trigger of genotoxicity and apoptosis. O6MeG requires MSH2/MSH6 dependent mismatch repair and excessive cell proliferation in order to become toxic. O6MeG triggered cell death is regulated in a cell type specific manner, utilizing both the mitochondrial and the death receptor (Fas/CD95/Apo-1) pathway. O6MeG triggered apoptosis is preceded by DNA double-strand break (DSB) formation, H2AX phosphorylation and ATM/ATR/Chk activation which occurs, as shown in synchronized cells, in the 2nd post-treatment replication cycle. In p53 wt cells the death receptor pathway becomes activated, whereas in p53 mutated cells Bcl-2 declines, which is followed by caspase-9, -7 and -3 activation. The efficiency of O6MeG in triggering the p53 dependent Fas pathway is much higher than the p53 independent endogenous mitochondrial pathway, which explains why p53 wt glioma cells are more sensitive to methylating agents (temozolomide) than p53 mutated cells. Interestingly, p53 wt glioma cells are more resistant than p53 mutant glioma cells to chloroethylating agents, such as ACNU, indicating p53 to protect against O6ChlG-induced apoptosis and necrosis. This is due to upregulation of repair genes (ddb2 and xpc). O6MeG and O6ChlG are also able to induce apoptosis in malignant melanoma cells, which is preceded by the formation

of DSBs. Cells defective in XRCC2 or BRCA-2 are hypersensitive to O6MeG triggered cell death and chromosomal aberrations while DNA-PKCS mutated cells display only slightly enhanced sensitivity. The data supports a role for DSBs as most critical alkylation-induced downstream apoptosis-triggering lesions. Furthermore, they show that DSB repair by homologous recombination plays a key role in defence and, therefore, is a new determinant of cellular resistance to monofunctional alkylating agents. Data will also be presented on apoptosis induction by the bifunctional alkylating agent cyclophosphamide, and the role of replication and transcription inhibition in triggering apoptosis will be discussed.

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S04-02

DNA damage response: Mechanisms and role in human cancer

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Recent work in the field of DNA damage recognition, signaling and repair identified multiple protein modifications that operate in concert with the ‘classical’ phosphorylation/dephosphorylation network governed by the ATM/ATR-regulated DNA damage response (DDR). This lecture will summarize our recently published and unpublished data documenting the biological and pathophysiological role of DDR, with emphasis on the emerging ubiquitylation/deubiquitylation cascade, including the RNF8, RNF168 and BRCA1 ubiquitin ligases, USP7 and other deubiquitylation enzymes and additional components, in DNA damage signaling and repair in human cells. The data will include results from pan-genomic RNAi-based screens for novel DDR components, live-cell imaging of human cells to analyze the spatiotemporal orchestration of the key DDR pathways, and mechanistic insights into the cooperation between phosphorylation, ubiquitylation and protein-protein interactions in response to DNA double strand breaks. Secondly, recent results extending our concept of DDR as a tumorigenesis barrier in early human cancer development, and exploitation of DDR defects in tumors as predictive markers to guide individualized chemotherapy, will be presented.

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S04-03

DNA damage-induced mitotic catastrophe, necrosis and apoptosis

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In dividing cells DNA damage caused by genotoxic insults results in the activation of cell cycle checkpoints followed by DNA repair to ensure the integrity of the transcribed genome. p53, being a guardian of genome is activated by stress and, depending on the severity of damage, it triggers either G1 or G2 arrest or cell death. DNA damage-induced apoptotic pathway includes caspase-2, which is activated within the PIDDosome complex, and causes cytochrome c release and caspase activation. Hence, PIDDosome-

mediated caspase-2 activation might be an important link between DNA damage and the engagement of the mitochondria-mediated apoptotic pathway. In addition to PIDDosome, caspase-2 is able to use the CD95 DISC as a platform and the recruitment of caspase-8 to this complex is required for activation of both enzymes. Investigation of the contribution of p53 and caspase-2 to apoptosis and mitotic catastrophe (MC) induced by DNA damage in carcinoma cells revealed that both functional p53 and caspase-2 are required for the apoptotic response, which was preceded by translocation of caspase-2 to the cytoplasm. In the absence of functional p53, DNA damage resulted in caspase-2-independent MC followed by necrosis. In these cells apoptotic functions could be restored by transient expression of wt-p53. Hence, in this experimental model p53 appeared to act as a switch between apoptosis and MC followed by necrosis-like lysis. It seems that the final mode of cell death triggered by DNA damage in cancer cells is determined by the profile of proteins involved in the regulation of the cell cycle.

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S04-04

Interplay of EGF receptor and DNA damage pathways

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EGFR-dependent signal transduction through the cytoplasmic as well as the nuclear pathways is a major component of cellular proliferation survival of normal and tumor cells. Many human tumor entities present overexpressed or mutated EGFR as well as other erbB receptors, which trigger result in enhanced signaling leading to resistance to both chemo- and radiotherapy. Moreover, the EGFR receptor and potentially other erbB family members can be activated ligand-independently by exposure to radiation and thereby stimulating cell proliferation and survival pathways, mainly PI3K-AKT signaling. Most interestingly, however, EGFR signaling has also been demonstrated to be involved in the regulation of DNA repair processes removing radiation-induced DNA damages. Both the EGFR-dependent cytoplasmic, especially the PI3K-AKT signaling, as well as EGFR nuclear signaling could be identified as specific regulators of DNA double-strand break repair through the non-homologous end-joining mechanism. EGFR-dependent stimulation of DNA repair via cytoplasmic PI3K-AKT cascade is achieved through direct interaction of AKT and DNA-PK the major enzyme of the non-homologous end-joining repair mechanism. DNA repair stimulation through EGFR nuclear signaling, however, seems to be triggered through nuclear translocation of EGFR and direct interaction of EGFR with DNA-PK in the nucleus. Consequently, antagonistic approaches to either block cytoplasmic or nuclear EGFR signaling affect the DNA repair capacity of irradiated cells resulting in a significant radiosensitization. Thus, these new insights into erbB receptor-mediated control of DNA damage repair not only provide a mechanistic explanation for the antitumor activity of EGFR antagonists, but also additionally offer new options for molecular targeting strategies in oncology and especially radiation oncology by using multitargeted approaches.

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S04-05

Ultraviolet light induced DNA damage that triggers apoptosis pathways

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Ultraviolet (UV) light causes severe damages in the DNA molecule that are efficient obstacles to RNA transcription and DNA replication. Both processes may trigger signals that lead to cell death, mainly by apoptosis, in human cells. We have investigated the damaging effects of UVC and UVB in primary human fibroblasts deficient in DNA repair. Confluent cells were analyzed and the data demonstrated that apoptosis is reduced in this condition, although transcription blockage is affected as in proliferating cells, and, curiously, p53 is activated. On the other hand, synchronized cells were investigated after UVB irradiation at G1-phase of cell cycle. Doses employed were either high enough to trigger apoptosis or sufficiently low to induce only low frequency of apoptotic cells. Even in cells with different DNA repair abilities, one common feature observed was that low UVB doses caused only delays in cell cycle progression, while higher UVB doses, which triggered apoptosis, caused consistent cell blockage, in G1 or early S-phase. These doses varied clearly with the DNA repair ability of these cells, and p53 induction corresponded to the levels of apoptosis induction. Curiously, however, the induction of MDM2 protein was similar in DNA repair deficient (XP-C) or proficient cells. This protein acts as a negative regulator of p53, but these results indicate that MDM2 induction is not the main factor determining cell evasion from UVB-induced apoptosis. Moreover, the data suggest that blockage of S-phase progression is a crucial step in apoptosis induction by UVB DNA lesions in primary human cells.

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Symposium 5: Nitrate and Oxidative Stress in Toxicology and Disease

S05-01

Peroxynitrite: Biochemistry, toxicology and development of therapeutics

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Peroxynitrite, the product of the diffusion-controlled reaction of nitric oxide with superoxide, is a short-lived oxidant species that is a potent inducer of apoptosis and necrosis and is an upregulator of certain pro-inflammatory pathways. Conditions in which the reaction products of peroxynitrite have been detected and in which its pharmacological neutralization has been shown to be of benefit include vascular diseases, reperfusion injury, circulatory shock, inflammation and neurodegeneration. Peroxynitrite formation has also been demonstrated in response to exposure to various environ-

mental toxins. Several drugs used in medicine and agriculture exert their toxic side effects through mechanisms involving the formation of peroxynitrite, via redox cycling, uncoupling of nitric oxide synthase, stimulation of the endogenous formation of nitric oxide and superoxide or lowering of the antioxidant defenses. The role of peroxynitrite has been demonstrated to play an important role in the toxicity of doxorubicin, paraquat, acetaminophen and MPTP. Peroxynitrite-mediated toxicity can be ameliorated by decreasing the levels of the precursor radicals (i.e. using NOS inhibitors or SOD mimetics) or reducing the levels of peroxynitrite itself (peroxynitrite scavengers or decomposition catalysts). These approaches can serve to attenuate or neutralize some of the drug-induced toxicities. There are a number of endogenous compounds (vitamins, antioxidants) that can mitigate some of the deleterious effects of peroxynitrite. In this lecture, we first review the biochemistry and pathophysiology of peroxynitrite and then focus on pharmacological strategies to attenuate the toxic effects of peroxynitrite (e.g. catalytic reduction to nitrite and its isomerization to nitrate by metalloporphyrins).

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S05-02

Nitrative stress and glial-neuronal interactions in the pathogenesis of Parkinson's disease

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The etiology of Parkinson's disease (PD) remains controversial but increasing evidences indicate that persistent neuroinflammation influences disease progression. Activation of both microglia and astrocytes is associated with long-term neuroinflammation and sustained expression of inflammatory genes, such as inducible nitric oxide (NOS2) and tumor necrosis factor- α , that are damaging to neurons. Expression of NOS2, in particular, is closely associated with enhanced nitration of proteins in midbrain neurons from PD patients. Astrocytes have diverse and critical functions in the central nervous system that include providing energetic, antioxidant, and other trophic support essential for the survival and function of neurons but their deleterious role in neuroinflammatory injury is under increasing scrutiny. Activated astrocytes are noted clinically in PD and have been associated with increased aggregation of alpha-synuclein and loss of dopaminergic neurons. Diverse inflammatory cytokines and stress signals converge on the nuclear factor kappa beta (NF κ B) signaling pathway to induce expression of NOS2 and other inflammatory genes in astrocytes and activation of NF κ B stimulates astrogliosis in vivo and in vitro, resulting in increased neuronal protein nitration and apoptosis. Transcriptional activation of NOS2 by NF κ B requires chromatin remodeling and removal of transcriptional repressor proteins, which is influenced by endogenous and exogenous factors that regulated NF κ B. Modulation of these signaling pathways may offer new therapeutic approaches for limiting neuroinflammation in PD and related disorders.

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S05-03**Oxidative and nitrosative stress-induced neurotoxicity in primary cultured rat cerebellar granule neurons**

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Glutamate is the major excitatory neurotransmitter in the mammalian CNS, acting on both ionotropic (NMDA, AMPA, kainate) and metabotropic receptors. However, excessive stimulation, especially of the *N*-methyl-D-aspartate (NMDA) receptor, can generate the production of reactive oxygen species and/or nitric oxide causing oxidative or nitrosative stress, ultimately leading to neuronal damage and death. Glutamate toxicity is known to underlie many neurological and neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases and stroke (Fatokun et al., 2008a,b). Elucidating the mechanisms triggered by NMDA receptor over-stimulation is pivotal in the development of strategies in ameliorating these disorders.

Our work has focused on the effects of oxidative stressors – including glutamate, NMDA, hydrogen peroxide, neurotoxic metabolites of the kynurenine pathway, and the xanthine/xanthine oxidase system for generating free radicals – on primary cultured rat cerebellar granule neurons. Means of attenuating the damage caused by these have also been investigated. We have also studied the effects of the nitric oxide donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), the nitrosative stress from which ultimately causes cell death. In most cases, the death pathways triggered by such insults were seen to involve a mixed profile of both apoptosis and necrosis. Application of adenosine receptor ligands protected neuronal cultures against glutamate (Fatokun et al., 2008c). Similarly preconditioning with exposure to sublethal doses of NMDA was also effective in protecting against subsequent lethal challenges (Smith et al., 2008).

The potential relevance of our findings to the development of improved therapeutic intervention for the management of neurodegenerative conditions is stressed.

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S05-04**Oxidative and nitrate stress: Role in the response to liver toxicants**

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The liver is a target organ for many toxicants since it is in the front line of defence. Persistent inflammation plays a pivotal role

in the response of liver to both chemical and viral damage, ranging from transient liver injury and repair through to hepatocarcinogenesis. Accumulating evidence implicates the dedicated hepatic macrophage, the Kupffer cell, as the primary target for chemical and viral insult since Kupffer cells release cytokines and reactive oxygen and nitrogen species. Thus, the Kupffer cell orchestrates the hepatic response often via perturbation of hepatocytes proliferation and death via apoptosis and necrosis.

The mechanisms by which Kupffer cells detect and respond to stress are unknown, but it is clear there is a role for stress signalling pathways and stress activated transcription factors such as NFκB. Additionally, there is complex interplay between ligand-activated receptors such as the PPARs which appear to play different roles in the Kupffer cell versus the hepatocytes. For example, PPARα is expressed in hepatocytes where it mediates the response to the peroxisome proliferators class of nongenotoxic liver carcinogens. Conversely, PPARα is absent in Kupffer cells that instead express PPARγ. Recent data also suggest a role for epigenetic regulation of the oxidative response to hepatic toxicants that may specify downstream choices between adaptation and damage.

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S05-05**Oxidative and nitrate stress in multi-stage skin carcinogenesis**

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The murine model of carcinogenesis is a powerful tool to characterize the stepwise alterations that occur during development of squamous cell carcinomas. The most well defined stage of murine skin carcinogenesis is tumor promotion, which is induced by application of 12-*O*-tetradecanoylphorbol acetate (TPA) or ultraviolet light to the dorsal epidermis of genetically susceptible mice. The process of tumor promotion during which pre-neoplastic papillomas develop has been commonly associated with both a rapid and persistent infiltration of inflammatory leukocytes as well as epidermal hyperplasia. Although we demonstrated that oxygen free radicals are produced during tumor promotion that are sufficient in amount to induce mutagenic oxidative DNA adducts, significantly less was known about the role of nitric oxide in multi-stage carcinogenesis. Our laboratory was the first to demonstrate that gene expression of the inducible form of nitric oxide (NOS2) was compartmentalized to only dermal infiltration leukocytes. The lack of NOS2 within hyperplastic epidermis is consistent with the down-regulatory role of NOS2 on keratinocyte proliferation. In contrast to the presence of NOS2, the expression of NOS3 was elevated within the dorsal epidermal at early times of cutaneous inflammation and increased vascular permeability and at later times during papilloma development, during which there is robust angiogenesis. Increased NOS3 gene expression within papillomas was associated with increased gene expression of vascular endothelial growth factor A (VEGF A). Interestingly, NOS3 gene expression was associated with production of a novel splice variant of VEGF A, defined as VEGF₂₀₅ which was present only in papillomas and carcinomas. These studies suggest that nitric oxide and tumor angiogenesis are interlinked and are an integral part of the process of multi-stage skin carcinogenesis.

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Symposium 6: Best Practice in Biologically Based Toxicokinetic Modelling for Risk Assessment

S06-01

Principles of characterizing and applying physiologically based pharmacokinetic models—An introduction

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Physiologically based pharmacokinetic (PBPK) models are part of a broader continuum of increasingly data-informed approaches to dose response analysis ranging from default based on external dose to more biologically realistic models. By facilitating the incorporation of dose measures of relevance to the mode of action of chemicals, and quantitative physiological scaling taking into account relevant chemical-specific physical chemical properties and biological constants, PBPK models provide a representation of biologically effective dose as a basis for conducting more informed extrapolations across studies, species, routes, and dose levels. Resultingly, they increase accuracy and reduce uncertainty in risk estimates.

Despite the availability of PBPK models for a number of chemicals incorporating significant additional biological data over default and the potential of such models to contribute more broadly to the development of additionally informative testing strategies, their adoption in regulatory risk assessment has been limited. This limited uptake is being addressed in a project undertaken as part of the World Health Organization/International Programme on Chemical Safety project on harmonization. The initiative includes preparation of guidance and case studies on the characterization, documentation, evaluation and communication of PBPK models for risk assessment. Aspects being addressed include the need for early and continuing communication between risk assessors and modelers, greater consistency in consideration of mode of action as a basis for relevant PBPK models and sufficiently transparent documentation of model development to support potential application. More consistent and transparent consideration of the basis for and output of PBPK models relative to default approaches in risk assessment is also being addressed.

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S06-02

Model development and characterization—The biological part

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Physiologically based *pharmacokinetic* models have been constructed in order to describe processes in the mammalian body and its organs. The model consists of structural aspects taking into account the given anatomical structure, physiological properties such as weights of the organs, blood flow through the organs which are common for all chemicals. Chemical specific data are implemented such as partition coefficients, parameters characterizing metabolism and excretion. The models are used to describe and simulate the kinetics of substances, assuming exposure/dose as the driving force and diffusion and convection as the main mechanisms for the uptake into the body and into the organs and tissues. From this basic framework additional

complexities, as saturable transport or degradation processes, binding processes, and heterogeneous tissue composition can be added.

The processes are characterised by mathematical expressions based on known or hypothesized mechanism of behaviour of a biological system. The parameters correspond to physical or conceptual entities in the subject-matter domain of the model. The parameters are in accordance with kinetic, physicochemical, biophysical, physiological and patho-physiological principles, and have direct identifiable biological or biophysical interpretation.

¹Retired.

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S06-03

The rapid generation of PBPK models: A tool for good modelling practice

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A major obstacle to the acceptance of PBPK models in chemical risk assessment is the ability of regulators to evaluate them. Whereas this will always be a problem for regulators without the required mathematical and programming background, there are tools, which with further development, can assist in the evaluation process to a considerable degree. A PBPK model equation generator called, MEGen is one such tool. MEGen provides a capability to generate models rapidly and to shift the balance away from the need for mathematical and programming expertise to the biology underlying disease and quantitative chemical risk assessment. MEGen enables a user to describe physiology, anatomy and toxicology in order to output a set of mathematical equations that emulate the information supplied by the user and constitute a PBPK model. During this process, the software interrogates a built-in database, supplying pertinent data for use within the model. The resulting output is translated into mathematically consistent script, free from typing and syntactical errors that can be imported into a number of commercial modelling packages where it may be visualised and exercised. A very important feature of MEGen is the provision of a model building process that is transparent, auditable and documented. MEGen provides a structured description of the conceptual model, free of mathematical equations and confusing syntax, a schematic diagram and a corresponding table listing the value, units, source, origin and reference for each parameter specified. The diagrams and tables can be exported directly into documents prepared in standard word processors.

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S06-04

Special aspects: Species differences, different life stages, exposure routes

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Implicit in any application of pharmacokinetics in risk assessment is the assumption that the effects of a chemical in a particular tissue

can be related in some way to the concentration time-course of an active form of the substance in that tissue. In the absence of evidence for differences in the nature or extent of the tissue response (susceptibility), it can be expected that similar responses will be produced at equivalent tissue exposures regardless of species, life-stage, or exposure route. Due to their physiological structure, PBPK models are particularly suited to perform the extrapolations across exposure routes and species that may be necessary for estimating human risk on the basis of animal toxicology studies. The physiological structure of PBPK models is also useful for examining the effects of changing physiology on target tissue dosimetry, as in the case of early life exposure. However, for these models to be useful and trustworthy in performing the necessary extrapolations, they must be thoughtfully constructed in accordance with known biology and pharmacokinetics, documented in a form that is transparent to risk assessors, and shown to be robust using diverse and appropriate data. This presentation describes the application of PBPK models to support risk assessment extrapolations and highlights issues related to the identification of model structure and parameters, model evaluation, and consideration of uncertainty.

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S06-05

Special aspects: Local kinetics[☆]

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Toxicokinetics and -dynamics are linked at the target site. Response is determined by the concentration of the substance at target and its mechanism of action. However, concentration at target cannot be easily measured *in vivo*; surrogates, e.g. plasma concentration are used instead. Emergence of *in vitro/ex vivo* approaches to study primary TK/TD processes in simple experimental setups provides ways to estimate the concentration at target, which can be used in reliable extrapolation and prediction of *in vivo* behaviour and action of the substance. Novel techniques, e.g. imaging, provide more direct measurements of *in vivo* concentrations in tissues, which provide useful information for validation of *in vitro* approaches. In this presentation, ADME processes, affecting concentration of the substance at the active site are described, analytical possibilities to measure directly or estimate indirectly concentration at active site are covered, and some examples on *in vitro*–*in vivo* systems are presented. On this basis, following claims will be scrutinized: (1) *in vitro* systems should be characterized in a more detailed way than has been done so far, especially with respect to 'biologically relevant' concentrations; (2) kinetics of the compound in the *in vitro* system should be more thoroughly characterized; (3) local models encompassing the *in vitro* systems should be developed and used; (4) PB-TK/TD models should incorporate these local models.

[☆] Based partially on the review article in Crit Rev Toxicol ('Local Kinetics and Dynamics of Xenobiotics', 2008; 38: 697–720) by Pelkonen, Kapitulnik, Gundert-Remy, Boobis and Stockis (COST Action B25).

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Symposium 7: Chemical Sensitization: From Immunobiology to Quantitative Risk Assessment

S07-01

Dendritic cell biology: Current state of the art

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Dendritic cells (DC) play important roles in the orchestration of immune responses. Their responsibilities include antigen processing, presentation of antigen to T lymphocytes and influencing the development and characteristics of adaptive immune responses. It is clear that DC of various types are similarly pivotal to the initiation of allergic responses to chemicals. This presentation will outline the basic biology of the various DC subsets, including their anatomical localisation, functional plasticity and cellular and molecular markers, and describe the cellular machinery that allows them to perform their multiple functions. In addition, the contribution of DC subsets to the development of the two main forms of chemical allergy (allergic contact dermatitis or contact sensitisation and sensitisation of the respiratory tract) will be considered. The former is a cell-mediated hypersensitivity reaction resulting from the activation of T helper (Th) 1 and T cytotoxic (Tc) 1 cells, whereas the latter is associated with the preferential stimulation of Th2 type cells and in some cases the production of IgE antibody. Evidence deriving from the use of transgenic mouse models in which different DC subsets can be ablated in order to investigate the role of cutaneous DC (including epidermal Langerhans' cells and dermal DC) in allergic responses will be discussed. Finally, the potential contribution of DC subsets (DC1 and DC2) that polarize towards type 1 or type 2 immune responses to the development of divergent forms of chemical allergy will be considered.

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S07-02

Use of dendritic cells for the identification and characterization of chemical allergens

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Dendritic cells were first identified in the epidermis in 1868 and were named Langerhans cells. Their presence in other tissues was identified in 1973 and DCs are now recognized as an integral part of the immune system. During *in vivo* immune responses, the role of antigen presenting cells (APC) is played primarily by DCs acting as initiators, stimulators and regulators of Ag-specific T lymphocytes. Thus, DCs form a sentinel network able to detect, capture, and process antigens such as invading bacteria, viruses, tissue damage and haptens. In peripheral tissues such as the skin, the DC can be found in contact with keratinocytes in an immature state with a high capacity for antigen uptake and processing but unable to stimulate T-cells. Upon antigen capture, stimulation by microbial products but also in response to inflammatory cytokines the DC undergo a maturation process leading to the upregulation of co-stimulatory molecules (CD86, CD80, CD40), MHC class II molecules, the CD83 protein and cytokine production (IL-12, TNF- α , IL-18). These maturing DCs acquire the ability to migrate through expression of chemokines and chemokine receptors and

down-regulation of molecules such as E-cadherin. Indeed, upon maturation DCs express a new chemokine receptor, CCR7, enabling them to migrate in response to gradients of chemokines (CCL21 and then CCL19). Expression of CCR7 is particularly important, as CCR7-deficient mice show an impaired migration of activated LCs into draining lymph nodes after skin painting with FITC and consequently, lack any contact hypersensitivity. Knowledge of DC physiology has progressed considerably because of the discovery of culture techniques, in the early 1990s, that support the *in vitro* generation of large numbers of DCs from hematopoietic progenitors. Two main protocols to generate DCs, from either monocytes or CD34+ hematopoietic cell precursors (HPC), have been described. Application of low-molecular-weight chemicals (haptens) on the skin may result in allergic contact hypersensitivity. *In vivo* painting of murine skin with chemicals, such as FITC, provokes an influx of epidermal dendritic cells (DCs) in the draining lymph nodes 24 h after hapten application. These epidermal DCs also named Langerhans cells (LCs) express major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules such as CD86. Thus, it seems that considering similarities between immunity to simple chemicals and that to infectious agents, it is reasonable to speculate that hapten itself stimulates DC maturation. The establishment of human *in vitro* models of DCs offered the possibility to demonstrate that haptens were able to directly activate cultured DCs derived from peripheral blood monocytes or from CD34+ HPCs. Several studies confirmed these observations showing the upregulation of maturation markers (CD83, CD80, CD86, CD40, MHCII) on human DCs. Haptenized DCs also cause T-cell proliferation in allogeneic mixed lymphocyte reaction but not in the autologous MLR. The expression of *ccr7* mRNA in human DCs stimulated by haptens and the migration of these cells in response to CCL19 has been also observed. Mitogen-activated protein kinase (MAPK) have been described to play a major role in DC maturation induced by LPS through Toll-like receptors or by inflammatory cytokines through binding to specific receptors. Haptens such as nickel (NiCl₂ and NiSO₄), 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) induce the phosphorylation of p38MAPK and c-Jun N-terminal kinase (JNK). Inhibition of p38MAPK is correlated with alteration in phenotypic indicators of maturation such as CD86, HLA-DR, CCR7 or CD83 in DCs derived from monocytes or from CD34+ HPCs stimulated with nickel or DNCB suggesting again similarities between DC activation by danger signals or haptens.

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S07-03

Cell-based *in vitro* alternatives to predict the contact and respiratory sensitizing potential of chemicals

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An *in vitro* model for testing sensitization potency of low-molecular weight chemicals remains a major challenge. For *in vitro* identification of contact and respiratory sensitizing compounds, different cell models and biomarkers relating to the underlying biological mechanisms are being used. Many of the currently used biomarkers, however, have been selected on the basis of fragmentary knowledge on the sensitization process, obtained using classical biochemical or molecular techniques, and often lack specificity and sensitivity.

Therefore, identification of new biomarkers is urgently needed to promote alternative test development.

Toxicogenomics is considered as a promising tool that may provide more sensitive, mechanism-based biomarkers. Expression profiling of thousands of genes simultaneously in relevant cell or tissue models, combined with biostatistical methods and pathway analysis tools became recently available. They allow to reveal targeted biological pathways and networks, rather than individual molecules, that are affected by exposure to sensitizing chemicals. Using signature expression profiles in dendritic cells, specific biomarkers were identified which are able to classify chemicals according to their skin (non-)sensitizing potential. A similar approach was used in lung cell lines to identify genetic biomarkers specific for respiratory sensitizing chemicals.

Priority needs and possible strategies to develop the field further will be discussed.

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S07-04

Potency of skin sensitization—LLNA data used for a more reliable classification, labeling and risk assessment

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The evaluation of skin sensitisation potential is an important requirement for existing and new chemicals in particular for those chemicals intended to come in contact with skin. With the advent of REACH skin sensitisation assessments assumes even greater importance for the development of effective risk management strategies. The local lymph node assay (LLNA) is most often the first choice to obtain reliable data.

Currently, the classification of a chemical as a skin sensitizer is based on a yes/no approach, but industry and also regulators are becoming more and more interested in individual potency. The establishment of subcategories to suit this interest is widely accepted and discussed under the Globally Harmonised System (GHS).

An ECETOC task force of industry scientists determined whether an EC3 potency value derived from the LLNA could be used to provide a cut-off criterion for the classification and labelling of substances and preparations, according to GHS and Directives 67/548/EEC and 99/45/EEC.

Proliferation of lymph node cells is related causally and quantitatively to the extent to which skin sensitisation is acquired and therefore directly defines potency. With the definition of four potency classes, the strategy to manage the use of skin sensitising chemicals becomes more effective, both in the case of traditional benchmarking approaches, as well as with newly developed quantitative risk assessment approaches. This approach effectively moves the LLNA from the realm of hazard identification to a key component of the development of accurate risk assessments, which can be used as a sound scientific basis for classification and labelling.

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S07-05**Potency and trends of contact sensitisation in humans with regard to consumer products**

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Contact sensitisation is not an all-or-none phenomenon. Dose response characteristics for both induction and elicitation of contact sensitisation depend on the allergen, the product matrix as well as on the individual. Low doses of an allergen may cause sub-clinical sensitization. Higher doses lead to increased frequency of sensitisation. Patients with strong positive patch test reactions tend to get more severe dermatitis upon subsequent exposure to the allergen in question. On the other hand, there is a threshold dose below which clinical contact dermatitis does not develop following controlled exposure tests in allergic individuals. The route and mode of allergen exposure is also a decisive factor. Frequent use of rinse-off products may elicit allergic contact dermatitis, and a low concentration of an allergen in the product may be quite as harmful as a higher concentration if the product is used more frequently on a daily basis. Some people seem to be more susceptible to develop contact allergies, especially those with multiple contact allergies. Serial dilution testing and use tests as the Repeated Open Application Test (ROAT) on normal as well as on dermatitis skin are important tools in the relevance evaluation process challenging the dermatologist, the toxicologist and the patient. Clinical and clinical experimental examples with preservatives, fragrance chemicals, and both stay on and rinse off products will document the varying reaction patterns.

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Symposium 8: Biomarkers of Exposure and Metabolism at Low Concentrations of Carcinogen**S08-01****The application of DNA adduct measurements in population studies**

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The general purpose of measuring DNA adducts is to assess the biologically effective dose of carcinogenic exposures and to identify groups of individuals at enhanced cancer risk. The application of DNA adduct measurements in human populations depends on the access of suitable surrogate tissues that preferably reflect levels in target-tissues.

Methods: In a number of studies, PAH related DNA adduct levels have been determined in bronchoalveolar lavage cells (BAL cells), cells derived from induced sputum, buccal mucosa and white blood cells.

Results: Generally, DNA adduct levels are higher in exposed individuals than in non-exposed, but saturation could be observed at high exposures. Furthermore, DNA adduct levels varied according to changes in exposure and seasonal variations of air-pollution. Intra-individual variation during continuous exposure was low over a short period of time (weeks), but varied significantly when longer time periods (months) were investigated. Genetic variants in genes

involved in activation/detoxification of carcinogens and DNA repair explain part of the inter-individual variation in adduct levels.

Conclusion: DNA adduct measurements may have advantages over traditional exposure assessment. First, they can smooth the variability in environmental exposures and may integrate exposure over a longer period of time. Secondly, biological monitoring of DNA adducts accounts for all exposure routes. Thirdly, DNA adducts may account for inter-individual differences in uptake, elimination, distribution, metabolism and repair amongst exposed individuals. At present, there is sufficient evidence to justify the application of DNA adduct measurements as biomarkers in exposure assessment and intervention studies. Their use in risk-assessment, however, requires further investigation.

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S08-02**DNA adduct accumulation during the course of human cervical cancer development, and during and after radiotherapy**Ramesh Gupta^{1,*}, Radha Munagala¹, Manicka Vadhanam¹, Bala Nagarajan², Srivani Ravoori¹¹ *University of Louisville, Pharmacology & Toxicology, Louisville, United States*, ² *Cancer Institute, Chennai, India*

Human papillomavirus (HPV) is considered a causative factor for human cervical cancer. However, HPV infection alone is insufficient to induce transformation and tumor progression and emphasizes the role of co-factors like cigarette smoking, inflammation and immune suppression. Human cervix provides a suitable system to determine progressive changes in DNA damage in target tissue. The ³²P-postlabeling with new TLC solvents revealed a wide array of novel DNA adducts in human tissues, including cervix. We investigated modulation of DNA adducts during cervical cancer development by analyzing uterine cervix samples from normal, inflammation, dysplasia and invasive cancer. We also measured effect of radiotherapy on DNA adduction in cervix samples collected before, during and post-treatment. Six subgroups of DNA adducts were detected with varying polarities, of which 8-oxodG predominated. Adduct levels increased significantly from normal cervix to cervix with inflammation and dysplasia; adduct burden in cervical tumors was also elevated. Patients undergoing radiotherapy showed the same DNA adduct profile in specimens collected before, during and post-treatment, although the levels varied. The radiation treatment resulted in 5- to 6-fold elevated adduct levels, but 6 weeks after the cessation, adduct burden either declined, remained unaltered, or increased compared with initial levels, indicating interindividual variability in the adduct removal capacity. Inability to repair the damage post-treatment by a group of patients may be relevant to recurrence and secondary cancers.

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S08-03**TSNA adducts in smokers, nonsmokers and snuffers**

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The tobacco-specific nitrosamines (TSNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine, classified by IARC as human carcinogens, require metabolic activation. A common reactive intermediate leads to pyridyloxobutylation (POB) of proteins and DNA releasing 4-hydroxy-1-(3-pyridyl)-1-butanone upon hydrolysis. These adducts have been thought to be specific for TSNA exposure. However, first results with POB hemoglobin adducts from smokers and nonsmokers showed a less than three-fold difference in adduct levels. In lung cancer patients, sevenfold higher POB-DNA adduct levels were present in tumor-free lung tissue of smokers compared to nonsmokers. This could not be reproduced in tissues from tumor-free sudden death victims showing only 1.5-fold higher levels in lung tissues from smokers compared to nonsmokers and no smoking-dependent differences in mucosal tissue from esophagus and cardia. Using an improved analytical method allowing determination of adducts in biopsies from esophageal mucosa, even higher adduct levels were obtained compared to those in larger samples taken from sudden death victims. Results with esophageal biopsies confirm the lack of smoking-dependent differences but show a tendency to increased levels in patients with chronic reflux and in alcohol drinkers. High POB-DNA adduct levels were also observed in brushings of oral mucosa with highest values in Swedish snuffers followed by smokers, eightfold and fivefold higher than in nonsmokers, respectively. In summary, HPB-releasing adducts are weak biomarkers of tobacco smoke exposure and do not reflect tumor risk in users of smokeless tobacco. The minor tobacco alkaloid myosmine occurring in a wide variety of foodstuffs is suggested as a major source of the high background in nonsmokers.

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S08-04**Metabolic activation of nitroaromatics and arylamines**

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The outline in question: Metabolic activation of nitroaromatics and arylamines such as environmental and industrial pollutants and carcinogens 3-nitrobenzanthrone (3-NBA), 3-aminobenzanthrone (3-ABA), *o*-nitroanisole and *o*-anisidine and a plant alkaloid, aristolochic acid (AA), is responsible for their carcinogenicity. The aim of our studies was to resolve the metabolic pathway leading to reactive species of these chemicals binding to DNA and enzymes responsible for such reactions.

Methods: HPLC with mass- and NMR spectrometry was employed to characterize activation and detoxication metabolites of these carcinogens and the ³²P-postlabeling assay for detection of DNA adducts.

Results: Reductive and oxidative activation of nitroaromatics, 3-NBA and *o*-nitroanisole, and arylamines, 3-ABA and *o*-anisidine

leads to the formation of their *N*-hydroxyderivatives producing nitrenium and carbenium ions generating adducts with purine bases in DNA. Cytosolic nitroreductases, NAD(P)H:quinone oxidoreductase and xanthine oxidase are the major enzymes activating 3-NBA, *o*-nitroanisole and AA, while microsomal NADPH:cytochrome P450 (CYP) reductase plays a minor role. The CYP enzymes of a 1A subfamily (CYP1A1/2) and CYP2E1 are predominantly responsible for activation of 3-ABA and *o*-anisidine. Cytosolic *N,O*-acetyltransferase (NAT), NAT2, followed by NAT1, and sulfotransferases 1A1/2 are the phase II enzymes activating *N*-hydroxyderivatives of several studied carcinogens. The activities of these enzymes in human individuals are, therefore, crucial for initiation of genotoxic changes leading to carcinogenic processes.

Conclusions: The results indicate that the nitrenium and carbenium ions of studied carcinogens and enzymes participating in their formation are the critical determinants of their carcinogenic potential.

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S08-05**Competing roles of reductases in the detoxification of carcinogens**

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Identification of genetic, environmental and nutritional factors that affect lung-cancer risk upon smoking might help to explain why some smokers are more likely to develop lung cancer than others. Recent data suggest that the balance between metabolic activation and detoxification is critical in determining the susceptibility to lung cancer upon exposure to the tobacco-specific nitrosamine 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Activation of NNK occurs by cytochrome P-450 mediated oxidation, whereas detoxification in man is initiated by carbonyl reduction of NNK to its corresponding alcohol NNAL which is glucuronosylated and excreted into urine or bile. Six different enzymes mediating NNK carbonyl reduction in man have been identified, from which microsomal 11b-hydroxysteroid dehydrogenase type 1 (11b-HSD1) appears to be the most important. 11b-HSD1 physiologically catalyzes the interconversion of active cortisol to inactive cortisone, but evidence is emerging that 11b-HSD1 fulfills an additional role in the detoxification of non-steroidal carbonyl compounds. We could demonstrate that 11b-HSD1 is active as a dimeric enzyme which exhibits cooperativity with cortisone. Accordingly, this enzyme dynamically adapts to low as well as to high substrate concentrations, thereby providing the fine tuning required as a consequence of great variations in circadian plasma glucocorticoid levels. Due to this kinetic peculiarity, 11b-HSD1 is also able to even metabolize nanomolar concentrations of NNK, a fact which is important in view of the relatively low levels of this carcinogen observed in smokers. Finally, 11b-HSD1 is potently (in nM concentrations) inhibited by glycyrrhetic acid, the main constituent of licorice. Hence, licorice exposure may affect NNK detoxification by inhibition of 11b-HSD1, a condition which may advance lung-cancer incidence, especially in smokers expressing low levels of this enzyme. Collectively, the extent of expression and activity of 11b-HSD1 strongly influences

the tissue selectivity and interindividual susceptibility to NNK-mediated cancer.

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S08-06

Epoxide hydrolases: Structures, functions, mechanisms and toxicological implications

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Epoxide hydrolases (EH) comprise a family of enzymes that metabolize epoxides to the generally less active corresponding diols. Because many epoxides are chemically reactive and possess mutagenic and carcinogenic potential, EH were traditionally viewed as detoxifying enzymes. However, the majority of mammalian epoxide hydrolases identified to date apparently hydrolyse endogenous epoxides that serve signalling functions. Well documented is the role of soluble EH (sEH), whose physiological substrates are epoxyeicosanoids (EETs), signalling molecules involved in the regulation of many (patho)physiological processes, including blood pressure regulation, nociception, inflammation and angiogenesis. sEH is therefore considered a new drug target as respective inhibitors promise therapeutic potential in the treatment of hypertension and possibly other diseases.

The most important xenobiotic-metabolizing isoenzyme is the microsomal epoxide hydrolase (mEH). It has a rather broad substrate specificity that is surprisingly combined with a high affinity (low K_m) for structurally very different substrates. An explanation for this apparent contradiction is offered by a closer look at its catalytic mechanism: the enzyme traps its substrate in the form of a covalent ester intermediate. Because mEH concentration is particularly high in mammalian liver, the major site of xenobiotic-derived epoxide formation, we conclude that the enzyme is usually in excess of its substrates and can therefore act like a molecular sponge, as long as the epoxide formation rate does not exceed its capacity. This hypothesis is supported by computer simulation as well as by experimental results.

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Symposium 9: Emerging Pesticide Issues Related to Human Health

S09-01

Biomonitoring of pesticides in the industrial world and in developing countries

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New pesticides are substituting the class of organophosphates and carbamates in industrially developed countries while in the developing world the organochlorinated compounds are still in use. Biomonitoring of pesticides is usually performed by analyzing blood and urine for recent exposure or by examining hair or fat tissue to assess chronic exposure. Recently hair has been used to assess chronic exposure to organophosphates and to withdrawn organochlorine substances like DDTs, PCBs and HCHs

which are currently detected as environmental pollutants. Many of the examined samples were found positive for HCHs and DDTs despite the fact the use of DDT is banned for over 30 years in the developed world. A low incidence of positive hair samples was found for few organophosphorus pesticides and several pyrethrins. This was attributed to their fast and effective metabolism in the human organism and to the low levels of exposure of the studied population. Lately GC–MS methods have been developed for the quantification of non-specific metabolites of organophosphates the dialkylphosphates (DAPs) in hair, meconium, amniotic fluid and urine. Differences in the hair levels of the DAPs tested were observed between the non-exposed and exposed groups.

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S09-02

Genetic polymorphisms of pesticide-metabolizing enzymes as potential biomarkers of susceptibility to pesticide toxicity

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Chronic pesticide toxicity can be greatly affected by genetic differences in pesticide-metabolising enzymes such as CYP450, esterases (paraoxonase-PON1-, cholinesterase-BChE-) and transferases (GST). These polymorphisms would render an individual more or less susceptible to the adverse effects of pesticides, so that they can be regarded as biomarkers of susceptibility. This presentation is aimed to address whether the combined effects of a number of genetic polymorphisms involved in pesticide metabolism (PON1, BChE, GSTM1 and GSTT1) put individuals at an increased risk of early changes in certain biochemical targets, such as erythrocyte enzymes and liver function parameters, and clinical outcomes. PON1-192QQ genotype was an independent predictor of pesticide-related ill health and was significantly more frequent in patients with acute pesticide poisoning than in controls. In turn, carriers of the PON1-192R allele exhibited lower acetylcholinesterase (AChE) activity than non-carriers, supporting genotype-dependent inverse relationships between AChE and PON1 activities involving exposure to low doses of pesticides. Subjects with null genotype for both GSTM1 and GSTT1 were also more prone to develop pesticide-related toxicity. Regarding liver enzymes, carriers of unusual BChE phenotypes had higher levels of ALT, AST and GGT and lower levels of alkaline phosphatase. In turn, subjects with null GSTT1 had significantly higher levels of ALT and GGT than those with functional GSTT1. Therefore, unusual BChE phenotypes and null GSTT1 increased the risk of hepatotoxicity upon exposure to pesticides. Knowing the genotype/phenotype for key genes that metabolize pesticides will allow for a better prediction of exposure responses and will decrease uncertainty factors.

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S09-03**The diversity of molecular targets of pesticides—New developments**

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Modern pesticides attack a bewildering variety of targets in order to minimise cross-resistance. This makes it impossible to consider even the common divisions: insecticides fungicides and herbicides, as single classes, and also makes it difficult to assess adverse health effects of pesticides. Although it is sometimes possible to study a single pesticide, e.g. in manufacturing workers (Albers et al., 2007), most exposures are to a wide range of pesticides when assessed over several years (Kamel et al., 2005). Thus the significance of epidemiological associations between pesticides and Parkinson's disease (Brown et al., 2006) still remain unclear due to their diversity of targets. Even within single pesticide classes: e.g. pyrethroids or organophosphates, there are a variety of target molecules. These will be summarised and potential common factors emphasised.

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S09-04**Pesticide exposure and risk of Parkinson's disease**

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Parkinson's disease results from either genetic or environmental factors, or a combination of both. Genetic Parkinson's disease accounts for less than 5% of all cases and has led to the search for environmental factors that may be involved. Amongst these, pesticides have been implicated from epidemiological studies and the plausibility of this hypothesis has been supported by studies in experimental animals which show that some pesticides cause neuronal loss in the *Substantia nigra, Pars compacta*. These studies have mostly used the C₅₇B₆ mouse.

Although exposure to pesticides may be a risk factor for Parkinson's disease there is no evidence of a causal relationship. Also, the loss of neurons from the mouse brain has usually been demonstrated with routes of exposure, in doses unrealistic for humans. Also, the diversity of chemical structures that cause neuronal loss is indicative of a more general mode of toxicity, rather than a specific biochemical mechanism. This is further complicated by the concern that multiple chemicals may be involved in damaging the *S. nigra*.

A significant difficulty with assessing the association or causality of pesticides in the development of Parkinson's disease or Parkinsonism is the measurement of exposure. A variety of indirect measures of exposure have been used which limits the certainty of the conclusions.

Continued recycling and representation of inadequate data, or development of ecologic measures of exposure are unlikely to improve the risk assessment of pesticides on this important issue.

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S09-05**Current issues in pesticide exposure and health risk—Risk assessment of multiple residues and endocrine disrupting pesticides**

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A prominent current issue regards the assessment of potential risks of combined exposure to multiple pesticide residues within the diet, addressing the concern that mixtures of substances displaying a common toxic mode of action or targeting the same organ may lead to cumulative effects. The European Food Safety Authority (EFSA) has recommended a tiered approach for both toxicological evaluation and intake estimation. Grouping compounds in cumulative assessment groups is suggested, based on criteria such as chemical structure, mechanism of pesticidal action, common target organ or toxic mode of action. Methods for cumulative risk assessment include the hazard index, the reference point index, the relative potency factor method and physiologically based toxicokinetic modelling. Acute and chronic exposure scenarios are to be taken into account for cumulative exposure assessment, considering data on pesticide residues likely to occur in food and data on food consumption.

A further topical issue concerns assessment of substances with endocrine disrupting potential. According to new EU regulation, active substances, safeners or synergists intended for use in pesticides shall only be approved if they are not considered to have endocrine disrupting properties that may cause adverse effects in humans, unless exposure under realistically proposed conditions of use is negligible. To provide a basis for assessment of pesticides with endocrine disrupting potential, the BfR is suggesting a framework, central aspects of which include dose-dependency, selectivity of effects on the endocrine system, establishment of a mode of action, and qualitative and quantitative comparison of key mechanistic events between experimental animals and humans.

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Symposium 10: Omics: Value and Application for Research and Regulatory Toxicologists**S10-01****Genomics and its surrounding technologies. Looking to the future of toxicogenomics**

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Toxicogenomics has been applied to find early indicators of toxicity, the identification of new safety biomarkers and the development of predictive screening assays.

The technologies available for studying global gene expression profiling are well accepted, and are now being used for new, innovative purposes. These include gene expression from formalin fixed paraffin embedded (FFPE) tissues, blood and bone marrow, miRNA analysis and digital gene expression.

Although RNA extracted from FFPE tissue, which have been accumulated in toxicology archives, is significantly degraded, novel technologies allow their use for gene expression analysis. The DASL™ assay from Illumina, based on 512 liver toxicity genes, and the WT-Ovation™ FFPE-System from NuGEN (whole genome) were applied to evaluate the effect of formalin fixation time and RNA extraction techniques. Both systems showed that longer fixation times resulted in increased alterations of signal intensities correlating to increased degradation of mRNA. However, using FFPE tissues from studies with model toxicants revealed reliable gene expression data which confirmed data from fresh frozen samples and histopathology. Therefore, retrospective analysis without the need for new animal studies could be enabled by using FFPE samples.

miRNAs are interesting in toxicological studies since these small regulatory RNA molecules play an important role in the response to xenobiotics. Therefore, new analysis platforms, including microarray platforms, are now being employed. Furthermore, to measure different kinds of non-coding RNA molecules digital gene expression will gain more recognition by enabling gene expression studies in any organism.

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S10-02

Proteomic surrogate biomarkers for *in vitro* testing of embryotoxicity: Quantitative differential investigation of ESC models

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One of the major challenges of proteomic profiling of complex biological material is the huge dynamic range of possible concentrations and the ever-increasing number of protein species due to posttranslational modifications. In particular the chemical diversity of the latter imposes a necessity of improved resolution of separation technologies, because otherwise the crucial quantitative information is lost in pools of poorly resolved peptides.

Here we present and analyze one example of successful development of protein biomarkers for embryo toxicity. This includes a detailed discussion of requirements regarding resolution of initial separation techniques, linear dynamic range and statistics of differential quantification. The results from a European integrated project (www.reprotect.eu) are discussed in the frame of challenges for hazard and risk assessment in the chemical industry with regard to REACH legislation in Europe and related activities in the US and Japan. In particular the investigation of developmental toxicity so far is regulated by guide lines including *in vivo* assessments of brain morphology, behaviour, development of young animals, measurements of biomarkers for gliosis and cytotoxicity and more, requiring huge numbers of test animals. Here protein lysates from mouse embryonic stem cells according to the validated embryonic stem cell test (EST) protocol and related ES models were used in a differential quantitative proteomic study to identify novel surrogate protein biomarkers for embryo toxicity.

Data set was generated using the EST and a set of model substances assigned to four categories of embryotoxicity *strong*,

moderate, *mild*, or *non-embryotoxic* based on *in vivo* data (selected by independent experts for the Reprotect consortium). Substance-dependent cardiomyocyte protein extracts were subjected to systematic differential proteomic profiling. Dual radioisotope labelling of proteins provided the rigorous quantitative pattern control necessary to obtain statistical significance. Moreover human and mouse embryonic stem cell models for neuronal differentiation and further substances were included to investigate the general significance of the results, which could provide molecular content for novel and fast *in vitro* strategies for safety tests for developmental toxicity, and have the potential to substantially reduce animal experiments according to the “3R” concept (Reduce/Refine/Replace).

Further validation and development strategies are discussed.

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S10-03

Metabolite profiling—A new tool for the identification of toxicological effects of chemicals

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Metabolite profiling is a technique analysing endogenous metabolites in biological matrices in order to identify changes of metabolites under insults like toxicity or disease. This methodology has already been applied in several areas and has, compared to other ‘omics technologies, the advantage that metabolite changes are determined as a direct consequence of biological effects’.

The purpose of the project is to establish defined relationships between metabolite profiles in plasma of rats and known toxicological modes of action. Therewith, it is possible to generate a comprehensive database for the detection of toxicological modes of action by test substances-based metabolic profiles. Such a database (MetaMap®Tox) containing the metabolite profiles of up to now more than 400 chemicals, agrochemicals and pharmaceuticals tested in rat studies has been established. Additional studies were performed in order to address reproducibility, the animal’s nutrition status or other confounding factors. In the in-life part of the studies, test substances are administered to five male and five female Wistar rats at two different dose levels for 28 days. Blood samples are taken on days 7, 14 and 28. The metabolite profiles in these blood samples are determined using multiple mass spectrometry-based technologies (LC/GC coupled with MS). For metabolite profiling more than 1000 analytes are detectable of which about 300 are known in rat plasma. These metabolites can be assigned to carbohydrates, lipids, fatty acids, amino acids, nucleobases, steroid-hormones and catecholamines, signal substances, intermediates of energy metabolism and others. The levels of endogenous metabolites of treated rats are compared to the levels of untreated controls applying univariate and multivariate statistical analyses.

The toxicity of a new test compounds could be predicted by the comparison with metabolite profiles of reference compounds with known toxicity profile. These predictions can be used for several applications, e.g., for the development of drugs, agrochemicals and

other compounds, mechanistic research in toxicology as well as for grouping and read across strategies within the REACH framework.

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S10-04

Cross-omics comparison in toxicological research

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Incorporation of new concepts and technologies as a basis for the development of more sensitive safety biomarkers and a better understanding of key mechanisms of toxicity may reduce attrition rates and assist regulatory decision making in the Pharmaceutical Industry. Thus, several collaborative projects have been initiated with the aim of delivering tools for improved prediction of toxicity.

The FP6-PredTox project, a collaborative effort by 15 groups from pharmaceutical companies, 2 from SMEs and 3 universities, set out to assess the value of combining omics technologies with conventional toxicology methods to better understand and predict liver and kidney toxicity. Comprehensive data sets were collected from short-term *in vivo* experiments in which rats were treated with a model drug or 1 of 12 proprietary compounds that previously failed during drug development. Integration of high-throughput transcriptomics, proteomics, and metabolomics data proved instrumental for expanding the mechanistic understanding of drug induced toxicities and identification of novel safety biomarkers. However, due to different levels of standardization and robustness, availability of biologically relevant annotation and analyte identifications, the contribution of individual omics technologies varied considerably. Transcriptomics as a stable, standardized and well-established technology with fast data generation and intrinsic availability of biological annotation delivered mechanistic hypothesis, which were supported by proteomics (2D-DIGE, SELDI) and metabolomics (¹H NMR, LC-MS) data. These technologies provided potential (non-invasive) biomarkers and useful mechanistic information but would not have identified the mechanism of toxicity if used alone due to the limited numbers of proteins and metabolites identified. However, it was recognized that further maturation of proteomics and metabolomics techniques combined with the development of advanced computational models for cross-omic/integrated analysis may considerably increase their value in the future.

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S10-05

Towards improvement in understanding hepatotoxicity using Omics

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Although animal testing identifies most toxicants thus preventing human exposure to such deleterious agents, surprisingly there are many unexpected adverse events that arise in the clinic. Stud-

ies have reported that 30% of adverse events seen in humans are not observed in animal species. This lack of concordance was even higher for hepatotoxicity and is reflected in post-marketing occurrence of drug-induced liver injury (DILI) being a leading cause of regulatory action. Unfortunately, this problem is not limited to a few compounds as approximately 1000 drugs have been associated with human hepatotoxicity and it has been estimated that 1% of patients admitted to a medical service develop DILI during hospitalization. Clearly, new biomarkers of deleterious liver effects are needed to prioritize compounds during development and to improve patient care. Omics promises to provide new inroads into our understanding of hepatotoxicity. This presentation will describe efforts to identify biomarkers of hepatotoxicity through active research and *in silico* data mining to improve human health.

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S10-06

The study of mechanisms of new drugs: Potential toxicity using genomic, proteomic and metabolomic tools

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X-Omics technologies have been widely used to study the mechanism of action of rather known pharmaceuticals. Reports of their use in the actual drug R&D process are unusual.

Noscira, in the frame of the Spanish Programme INGENIO2010, gained a grant from CDTI for its project MELIUS (2007–2010). The objective of MELIUS is to integrate both standard and X-Omics data into toxicity evaluation, from preclinical development to early clinical trials. Samples obtained from regulatory studies (up to 12 months in non-rodents) and from clinical studies have been studied using either Affimetrix or Agilent platforms for gene expression profiling; 2D gel electrophoresis (GE Healthcare platform), protein detection with fluorescent dyeing (Typhoon Trio) and identification by mass spectrometry (MALDI-ToF) for protein expression; and LC-MS/MS for metabolome characterization. Whenever possible, samples were obtained from pretest animals. Invasive samples were obtained at sacrifice. Solid samples were frozen in liquid nitrogen and grinded; aliquots of each powdered sample were distributed for evaluation. Liquid samples were distributed in frozen aliquots. Each sample was processed following individual SOPs at each laboratory. Data analysis was conducted to investigate specific questions from regulatory studies and to better understand the mechanism of action of the tested chemicals.

Results showed that interpretation is more difficult when evaluating compounds under development, where the available information is limited. Nevertheless, the integration of results from standard regulatory studies (including pathology) together with data obtained from X-Omics technologies gives better knowledge on the compound under development and supports step-wise decisions regarding the advancement in its development.

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Symposium 11: Environmental Risk Assessment for Human Pharmaceuticals

S11-01

Occurrence and fate of pharmaceuticals in the environment

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Approximately 3000 different pharmaceuticals are commonly used in the Europe today, including painkillers, antibiotics, antidiabetics, beta-blockers, contraceptives, lipid regulators, antidepressants, impotence drugs and cytostatic agents. As these compounds are frequently transformed in the body, a combination of nonaltered pharmaceuticals and metabolites are excreted by humans. Human-use pharmaceuticals and hormones enter raw sewage via urine and faeces and by improper disposal of medicinal products. In 2003, 6500 t of synthetic human-use pharmaceuticals are consumed in Germany, resulting in an average consumption of about $79 \text{ g cap}^{-1} \text{ a}^{-1}$. About 2/3 of the applied pharmaceutical are prescribed and about 1/3 are sold over the counter. In recent years several studies in Europe, Asia and North America reported the identification of these emerging compounds in wastewater, surface water, ground water and final drinking water. For most addictive drugs, including opioids, prescription and sale are strongly regulated and controlled in Europe and North America. A large number of these bioactive compounds are entering wastewater and the receiving water bodies without being tested for special environmental effects. All these legal and illicit pharmaceuticals are discharged from private households and from hospitals and eventually reach municipal sewage treatment plants (STPs). Sorption and (bio)degradation pharmaceuticals in wastewater treatment plants as well as in surface water are mainly unknown. Therefore, an approach was developed to identify transformation pathways of selected pharmaceuticals. In total, more than 40 transformation products were identified, enabling the proposal of transformation pathways under aerobic conditions. Several identified TP structures were detected in environmental samples.

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S11-02

Assessing environmental effects of human pharmaceuticals

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Pharmaceuticals are now being widely detected in surface waters, ground waters and soils. As pharmaceuticals are biologically active substances, concerns have been raised over the potential negative effects of pharmaceuticals in the environment on environmental and human health. This paper will therefore synthesise the literature produced over the past few years into the occurrence and potential effects and risks of pharmaceuticals in the natural environment. Amongst other things, the talk will discuss (a) the acute and subtle effects of pharmaceuticals on aquatic and terrestrial organisms; (b) the implications of the presence of pharmaceuticals in the environment in terms of risks to organisms in the environment and to humans; (c) the advantages and limitations of different testing approaches for assessing the environmen-

tal risks of pharmaceuticals; (d) the application of intelligent testing approaches for the risk/hazard assessment of pharmaceuticals; and (e) major knowledge gaps and future research needs.

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S11-03

Pharmaceuticals in the environment: Issues in balancing human health benefits with ecological risks

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Pharmaceuticals have brought enormous benefits to humanity in terms of healthier and longer lives. Increasing amounts are used, especially as the frequency of aged individuals increases in the human population. Inevitably pharmaceuticals escape into the environment where they can have adverse effects on biodiversity and ecosystem processes. People value nature and ecosystems but reducing the risks of pharmaceuticals to the environment may be costly in terms of the cleanup of releases and/or the lost benefit to human health from any restrictions. This paper will address some of these issues and put them in the broader context of cost-benefit analysis and welfare economics.

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S11-04

The CHMP guideline on environmental risk assessment of medicinal products for human use: Three years of regulatory experience

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The European legislation requires an environmental impact assessment of human pharmaceuticals before they enter the market. Following various drafts the adopted guideline on the environmental risk assessment of human pharmaceuticals came into effect in December 2006. The German Federal Environment Agency (UBA) is currently assessing the environmental safety of >150 human pharmaceutical products per year. Since the coming into force of the guideline the dossier quality with full environmental risk assessments (ERA) is improving. The major groups assessed so far are analgesics, anti-infectives, cytostatics, hormones and psychotropics. The ERA according to the EMEA is a tiered approach. An algorithm in Phase I identifies those products whose concentration in surface water exceeds 10 ng/L and thus need an in-depth ERA according to Phase II. Tier A of Phase II requires the submission of a base data set on fate and effects on aquatic organisms and micro-organisms in sewage treatment plants. If a risk is identified at the end of Tier A or if the exposure of the terrestrial compartment or bioaccumulation is expected, further tests need to be performed according to Tier B. In contrast to previous drafts the EMEA guideline does address the continuous release of human pharmaceuticals into the environment and asks to test the effects of pharmaceuticals using long-term tests, only. Available short-term and long-term effects data have now been evaluated and the results underline the necessity of long-term ecotoxicity tests

used in the ERA of pharmaceuticals. In order to further specify the EMEA guideline the identification of however substances and models that utilise therapeutic data and predict biochemical targets in non-target organisms are currently investigated.

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S11-05

Experiences with the Swedish environmental classification system of pharmaceuticals on www.fass.se

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On the initiative of the Swedish Association of the Pharmaceutical Industry, LIF, Sweden has, as the first country in the world, introduced a voluntary system for environmental classification of pharmaceuticals.

The model for presenting the environmental data was designed by LIF in cooperation with the Medical Products Agency, the Swedish Association of Local Authorities and Regions, the Swedish pharmacy chain Apoteket AB and the Stockholm County Council. It is available on the web version of the Physicians desk reference, www.fass.se and aims at presenting environmental information of pharmaceuticals both to the general public and to professionals in the health care system.

The information is given for the Active Pharmaceutical Ingredient (API) of the medicine and is based on the environmental risk, i.e. the ratio between the Predicted Environmental Concentration (PEC) and the Predicted No Effect Concentration (PNEC). In addition, information is given on the degradation and bioaccumulation potential. Finally, all background data for the assessment of the environmental risk, degradability and bioaccumulation, and the details regarding how the assessment was made are available for the readers.

Environmental information for the first groups of medicines was published in 2005 and it is expected that all therapeutic groups should have been reviewed for environmental information by 2010. The presentation will give an understanding of the system and the outcome of the classification so far.

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Symposium 12: Lung Immunotoxicity and Health Effects of Particulate Matter

S12-01

Current concepts of allergic airway disease

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Allergic rhinitis and allergic bronchial asthma are diseases of high prevalence in societies with western lifestyle. In recent years, substantial progress has been made in understanding the underlying mechanisms, and explanations have been developed why the disease prevalence has increased dramatically over the past decades. The most popular explanation is the so-called hygiene hypothesis, postulating that decreased bacterial infection and microbiologi-

cal contact are responsible for an imbalanced immune response leading to an allergic predisposition. However, the physiological and immunological mechanisms in the lung leading to bronchial asthma are still not fully understood. Therefore, animal models of asthma have been established and improved to study the complex cellular and physiological interactions *in vivo*. Murine models that reproduce certain features of asthma have been developed. In standard protocols inbred mice are first sensitized systemically to allergen and then challenged by aerosol. Most of the knowledge regarding the mechanisms of allergic inflammation has gained in these animal models. However, protocols of e.g. segmental allergen provocation in human have demonstrated similar mechanisms of allergic immune response in human. This presentation will focus on the investigation of inflammation and mechanisms of immune reactions in lung tissue by e.g. confocal microscopy. Focus will be the role of dendritic cells and their interaction with other cell types in the lung.

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S12-02

The influence of air pollutants on allergic sensitization in the lung

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Air pollution has long been associated with detrimental health risks in susceptible populations including asthmatics. Experimental evidence in rodents indicates that inhaled or instilled air pollutants such as diesel exhaust particles (DEPs), residual oil fly ash or its constitutive metals, can cause lung injury, inflammation, and potentiate allergic airway responses. These effects which have also been demonstrated with particulates sampled from ambient air are dependent on the underlying chemistry. Genomic analysis of mouse lungs following instillation or inhalation of various particles show broad differences in pathways associated with immune signaling, cell metabolism and oxidative stress. Studies with human airway cell cultures have shown similar alterations in cell signaling and may provide an *in vitro* high throughput screening framework for predicting allergic adjuvant effects. This presentation will describe a range of environmental factors in urban air that have been implicated as having had a potential impact on susceptibility to the development of atopic allergy and asthma. Included among those considered will be diesel exhaust particles, and ambient air samples. The relative importance of such factors, and the mechanistic basis for their action, will be discussed. (This abstract does not reflect EPA policy.)

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S12-03**Toxic and inflammatory effects of particulate matter PM₁₀ sampled during teaching hours in elementary school classrooms**

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Background: Because most individuals spend about 85% of their time indoors, we investigated the health effects of indoor air PM₁₀.

Methods: PM₁₀ was collected in five schools in Munich during teaching hours. Toxicity was assayed in human primary keratinocytes, human lung epithelial A549 cells and Chinese hamster V79 lung fibroblasts at concentrations from 3 ng/ml to 10 µg/ml. Toxicity after metabolic activation was assayed in cells expressing human cytochrome P450 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, 3A4, or 3A5. Additional toxic effects were analyzed with affimetrix genome wide gene expression analysis in BEAS-2B bronchial epithelial cells at 10 µg/ml PM₁₀.

Results: In A549 and V79 cells no toxicity was observed. In human primary keratinocytes PM₁₀ evoked a slight, but significant decrease in vitality. PM₁₀ was also toxic after metabolic activation by CYP1A1 or CYP2C9. PM₁₀ induced in BEAS-2B cells the expression of xenobiotic metabolizing genes (CYP1A1, CYP1B1) and inflammatory cytokines (IL-1A, IL-1B, IL-6, IL-8). Indoor PM₁₀ induced xenobiotic metabolizing genes less but inflammatory cytokines up to 6-fold more than outdoor PM₁₀.

Conclusion: Direct cytotoxicity and toxicity after metabolic activation by cytochrome P450 1A1 and 2C9 were significant at a PM₁₀ concentration of 10 µg/ml (about 100l indoor air/ml), which is more than 10,000 times higher than exposure encountered in classrooms. We therefore expect no toxic effects of these particles in school children. The induction of inflammatory cytokines in indoor PM₁₀ treated cells suggests that classroom particles have a higher inflammatory potential than outdoor air particles.

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S12-04**Ambient air particulate matter: A cause of airway and systemic disease**

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Airborne particulate matter is a mixture of particles that differ in physical properties, chemical composition and microbiology, and they also range over a few orders of magnitude in size. Particles in the submicrometer range are generated mainly from combustion, gas to particle conversion, nucleation or photochemical processes, and contain a mixture of components including soot, acid condensates, sulfates and nitrates, as well as trace metals and other toxins. Coarse particles, resulting mainly from mechanical processes, generally contain earth crustal elements and compounds. In addition, the rapid expansion of nanotechnology has led to the engineering of new types of particles, which are not formed through other natural or anthropogenic processes.

The last 10 years or so have seen an unprecedented increase in scientific interest in relation to airborne particles and the potential health risk they create. A clear turning point for this interest was the publication of findings from the Harvard epidemiological study, which pointed out a more distinct correlation between health effects and exposure to fine particles, as opposed to coarse particles.

The relationship between airborne particle mass concentration (i.e. larger particles) and health outcomes has been extensively investigated. Recent findings show that the most significant health end points include: decreased lung function, increased respiratory symptoms, increased chronic obstructive pulmonary disease, increased cardiovascular and cardiopulmonary diseases, and increased mortality. As a result, this research has led to a better understanding of the complex mechanisms by which the inhalation of particulate matter causes health effects. For example, it has shown that: (i) there is no threshold in response, (ii) the response is linear, (iii) the response is similar over different geographic settings, and (iv) in general, the susceptibility depends on the specific end point, as well as the level and length of exposure.

The potential risks associated with the inhalation of ultrafine particles by humans are different to those associated with the inhalation of larger particles, since ultrafine particles are not readily removed from the airstream of inhaled air in the upper parts of the respiratory tract and therefore, they are inhaled much deeper into the lung. In the small containments of the alveoli region, the particles undergo diffusional deposition onto the epithelium, which has been hypothesised to cause a number of physiological responses in the body, including: airflow resistance in the small airways; lung inflammation in the small airways and alveoli; systemic inflammation; and impaired vascular endothelial function.

There is no doubt that there have been significant advances in knowledge on the impact of airborne particulate matter on health, however there are still many unanswered questions in relation to the toxicology and epidemiological impact of the particles. This presentation will provide a summary of the 'state of the art' in science of airborne particulate matter and its impact on health.

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S12-05**An *in vitro* model of the human epithelial airway barrier to study the toxicity of nanoparticles**

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In addition to the generation of combustion-derived nanoparticles (<0.1 µm in diameter) from industrial or traffic-related combustion processes, there are progressively more engineered nanoparticles released into the air. However, exposure to NP is associated with adverse respiratory health effects. In order to study nanoparticle–cell interactions and possible toxic reactions we have established a triple cell co-culture system composed of epithelial cells, macrophages and dendritic cells which simulates the most important barrier functions of the epithelial airway: the surfactant, a tight epithelium, and cells of the defence system (Rothen-Rutishauser et al., 2008). By applying advanced imaging techniques and stereological approaches the intracellular particle trafficking can be studied (Mühlfeld et al., 2007a,b). Recently, we have shown that dendritic cells and macrophages collaborate as

sentinels against fine particles by building a transepithelial interdigitating network of cell processes (Blank et al., 2007), whereas the nano-sized material has different translocation characteristics (Rothen-Rutishauser et al., 2007).

Lung cell culture models may help to elucidate the mechanisms of how particles and other antigens that are inhaled and deposited on the lung surface can interact with the cells and induce cellular responses. Even though *in vitro* models exhibit a number of limitations, they can be used for high-throughput screening and the screening of large numbers of newly developed particles, in particular nanoparticles, within a short time. However, the presented 3D model of the epithelial airway barrier is a step forward since, including several cell types, it offers the possibility not only to study the reaction of individual cell types but also the interaction of the different cell types with each other. In addition, our model offers a great tool to study particle-lung cell interactions at the nanostructural level.

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