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Genotoxic stress:
novel biomarkers and detection methods

Uncovering RNAs role in epigenetics of carcinogenesis

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Abstract

Background: Cancer is a complex disease that is caused by the interplay of multiple genes and diverse environmental factors. As such, there has been continual debate as to which component of this complicated process towards development of neoplasia plays the most important role. The central dogma has, hitherto, been orientated towards DNA damage and its sequel as the major contributor to chemical/environmental carcinogenesis. More recently however, an important role for aberrant RNA structure and function in controlling cellular functions during normal and pathological conditions has emerged.

Objectives: The overall goal of the present investigation was to further characterize the molecular mechanisms and effects of different types of genotoxic stress on the biology and function of cells exposed and cultured *in vitro*. In particular, we were intrigued and interested in studying novel molecular mechanisms underlying the relative carcinogenicity potential of bulky DNA adduct-forming compounds.

Methodology: In this multi-dimensional investigation we have applied a broad spectrum of methods within fields of biochemistry, molecular and cellular biology, transcriptomics, biostatistics and bioinformatics. Genotoxic compounds used include pro-oxidants (H_2O_2, $KBrO_3$, diamide) and diol epoxides (DEs), which are reactive metabolites of PAHs (the bay-region BPDE, the fjord-region DBPDE).

Results: Data from experiments where cells were exposed to diamide or H_2O_2 indicated that levels of induced protein S-glutathionylation [**Paper I**] correlate well to selective stress gene expression, many of which were related to the induction of nucleic acid damage recognition/repair. Moreover this adaptation was translated to a functional phenotype in form of increased resistance in subsequent exposures to heat shock or oxidative stress. Based on the dramatic alternations (Affymetrix) in steady-state level of mRNAs in cells exposed to the most carcinogenic compound (DBPDE) we hypothesise that RNA and their modifications may function as central components of the epigenome [**Paper IV**]. A number of novel gene targets were identified, the functions of which have not previously been associated with response to genotoxic stress. These results support the notion that the analysis of alternations in gene expression patterns may provide a useful surrogate biomarker in identification of genotoxic agents with high carcinogenic potential. In parallel, we were interested to examine the relative propensity of RNA and DNA to sustain damage in cells undergoing oxidative stress. In **Paper III** we clearly show that RNA was far more sensitive in sustaining oxidative damage on guanine, than DNA. Due to the fact that detection of oxidised DNA/RNA in Paper III requires rather complicated and expensive mass spectrometric methods, we have also attempted to develop simpler methodologies for the differential detection/visualisation of modified RNA and DNA pools in intact cells [**Paper V**]. The data clearly show that combinations of avidin or neutravidin staining, combined with or without RNase or DNase treatments, can be used to visualise oxidative modification to DNA (nuclear and mitochondrial) and RNA in cells undergoing oxidative stress. Small differences in chemical structure of studied DEs have been shown to have pronounced effects on their conformation, target binding preferences and removal efficiency from DNA [**Paper II**] which, in turn, mark distinctive biochemical and biological effects on cellular biology [**Papers II, IV and VI**]. DEs and $KBrO_3$ showed distinct, chromatin-based responses in DNA damage signalling pathways, as measure of induction of H2AX or H2B phosphorylation [**Paper VI**].

Conclusion: The perception of DNA and DNA-driven carcinogenesis must be updated by "modern" epigenetics, which includes the changes in structure, function and distribution of RNAs. And these re-discovered carriers, executors and directors of genetic information have to be recognised as independent components of epigenome and placed more centrally in efforts to understand mechanisms of chemically-derived and perhaps spontaneously derived carcinogenesis. It is hoped that the potential biomarkers, analytical methods and mechanistic results presented in this thesis provide some stimulus to further study.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which in the text will be referred to by their roman numerals:

I. Dandrea T., Bajak E., Wärngård L. and Cotgreave I.A. (2002). Protein S-glutathionylation correlates to selective stress gene expression and cytoprotection. Archives of Biochemistry and Biophysics *406*, 241-52.

II. Dreij K., Bajak E., Sundberg K., Seidel A., Gusnanto A., Cotgreave I., Jernström B. (2004). DNA adducts of benzo[*a*]pyrene- and dibenzo[*a,l*]pyrene-diol epoxides in human lung epithelial cells: Kinetics of adduct-removal, effects on cell cycle check points and gene expression. Polycyclic Aromatic Compounds *24*, 549-566.

III. Hofer T., Badouard C.,# Bajak E.,# Ravanat J-L., Mattsson Å., and Cotgreave I.A. (2005). Hydrogen peroxide causes greater oxidation in cellular RNA than DNA. Biological Chemistry *386*, 333-337.

IV. Bajak E., Dreij K.,# Gusnanto A.,# Stockling K., Jernström B. and Cotgreave I.A. (2005). The highly carcinogenic potential of dibenzo[*a,l*]pyrene maybe partially mediated by disruption of epigenome, including mRNAs structure and homeostasis. *Manuscript*

V. Bajak E., Mattsson Å., Jernström B. and Cotgreave I.A. (2005). An *in situ* organelle specific method for the detection of oxidatively modified DNA and RNA in cells. *Manuscript*

VI. Bajak E., Mattsson Å., Cotgreave I.A. and Jernström B. (2005). Chromatin-dependent responses of A549 lung carcinoma cells to diol epoxides derived from benzo[*a*]pyrene and dibenzo[*a,l*]pyrene. *Manuscript*

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CONTENTS

LIST OF ABBREVIATIONS

ATM	Ataxia-telangiectasia mutated
ATR	ATM-Rad3-related
BER	Base excision repair
BP	Benzo[*a*]pyrene
dA	Deoxyadenosine
dG	Deoxyguanosine
DBP	Dibenzo[*a,l*]pyrene
DDB	Damage-specific DNA binding protein
DE	Diol epoxide
DETs	Differentially expressed targets
DSBs	DNA double strand breaks
EH	Epoxide hydrolase
FA	Fanconi anemia protein
GADD45	Growth arrest and DNA-damage-inducible protein
GGR	Global genomic repair
GSH	Glutathione
GSSG	Glutathione disulfide
HR	Homologous recombination
miRNAs	Micro RNAs
ncRNAs	RNAs non-coding (for protein)
MMR	Mismatch repair
MRI	Magnetic resonance imaging
NHEJ	Non-homologous end-joining
NER	Nucleotide excision repair
NMD	Nonsense-mediated decoy
PAHs	Polycyclic aromatic hydrocarbons
PCNA	Proliferating cell nuclear antigen
PET	Positron emission tomography
PTEN	Phosphatase and tensin homolog protein
RNAi	RNA interference
RNome	RNAs total pool (within the cell)
ROS	Reactive oxygen species
RPA	Replication protein A
TCR	Transcription coupled repair
XRCC	X-ray repair cross-complementary protein

INTRODUCTION

Carcinogenesis

Cancer can be conceived as a complex phenotype of cells which have acquired unlimited replicative potential, achieved independence from growth signals with parallel resistance to growth-inhibitory signaling, evasion of the programmed cell death, sustained angiogenesis, as well as ability of tissue invasion and metastasis [**Hanahn and Weinberg, 2000**]. The process which leads toward malignancy is a result of multiple genetic and epigenetic events which occur through a prolonged period of time. Development of cancer may take several years from its initiation, followed by promotion, clonal expansion and tumor establishment. As a multi-step process, there are broad spectra of genetic changes which may contribute to malignant phenotype. The genetic events associated with tumorigenesis can occur with gain and loss of entire chromosomes, as well as specific chromosomal translocations, gene amplifications, deletions or mutations. The most common pathological events leading toward cancer development are related to altered functions of tumor-suppressor genes and oncogenes [**Cory, 1986**]. In general, as a result of unique combination of genetic changes, pathological behaviors of different types of cancer are reflected in their convergent gene expression pattern profiles [**DeRisi *et al.*, 1996; Sorlie *et al.*, 2001**].

Cancers poses another interesting characteristic, that of genomic instability. Tumor cells lose (to some extent, depending on the type of tumor) their intrinsic capacity to maintain the integrity of their genome. This, in turn, results in increased rates of *de novo* acquired mutations and chromosomal abbreviations, in contrast to normal cells [**Loeb, 1991**]. Genetic instability has become recognized as one more hallmark of the vast majority of solid tumors which is quite often manifested at the chromosome level. This high instability serves as a driving force for tumor progression, as well as the basis of its heterogeneity [**Cahill *et al.*, 1999**].

The multiple mechanisms by which both natural (spontaneously-derived) and exogenous (environmentally-derived) mutations arise in cells and, hence, can trigger noeplasia, are becoming more clear. Evidence from epidemiological studies supports the idea that environmental exposure makes a substantial contribution to development of human cancers [**Doll and Peto, 1981; Kolonel *et al.*, 2004**]. This is the combination of genetic make-up of an individual and their occupational exposures, diet, alcohol consumption, smoking habits, as well as opportunistic infections, which all together constitute a risk associated with cancer development. Thus, environmental factors play a predominant role in carcinogenesis.

Uncovering and understanding the profile of molecular changes (signposts) of the physiological states of normal cell in the chain of events through highly complex pathological transformation(s), will be useful in bio-monitoring, identification of subjects at risk of developing

pathological conditions and early diagnosis of diseases, including cancer. Biomarkers have been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic responses to a therapeutic intervention". Lately, a valid biomarker has been defined as biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results [**Lesko *et al.*, 2003**].

Mentioned above signposts can be divided into prognostic and predictive biomarkers. To the first group belong: cellular, histopathological, and imaging biomarkers, which are biological alternations occurring during neoplastic progression (causally related to cancer) and drug effect/pharmacodynamic markers, which are biological effects produced by a drug that may or may not be directly related to neoplastic process. The second group of biomarkers includes: risk markers which are describing risks of cancer occurrence or cancer progression, including for example genetic predispositions, environmental and lifestyle, and biological progression markers which give a measure associated with tumour appearance, progression and burden [**Kelloff and Sigman, 2005**]. In molecular epidemiology, biomarkers have to cover the assessment of the biologic basis for an association, by using biological signposts from exposure to disease development/progression, which measure exposure, internal dose, biologically effective dose, early biologic effect, altered structure/function, invasive cancer diagnosis, tumour metastasis and progression [**Schulte, 1993; Chen and Hunter, 2005**].

New technologies and specific assays directed toward novel biomarker identification, detection and validation are under constant development. The promising tools in this rapidly evolving field are based on high throughput analysis (genomics, proteomics, metabonomics), as well as by applying visualization techniques (microscopy, MRI, PET, X-ray), [**Verma and Srivastava, 2003; Lewin and Weiner, 2004**]. It has to be stressed that each novel biomarker has to be verified in order to be applicable in risk assessment, epidemiological or pre-clinical studies [**Floyd and McShane, 2004**].

Genotoxic stress

Genotoxic stress may be described in general and traditional way, as cellular responsiveness to the consequences of extra- or/and intracellular damage to DNA.

Oxidative stress results from intracellular imbalance between pro-oxidants and antioxidants. Living organisms are continuously exposed to potentially dangerous free radical species, whose origin may be endogenous or exogenous. At moderate concentrations, nitric oxide

(NO) and reactive oxygen species (ROS) play important roles as regulatory mediators in normal physiological signaling processes. These include regulation of vascular tone, monitoring of oxygen tension and signal transduction from membrane receptors, as well as in oxidative stress responses that ensure the maintenance of redox homeostasis [**Dröge, 2002**]. ROS are capable of modifying a broad spectrum of bio-molecules. Cellular targets for oxidative modification by them include DNA, lipids, proteins and RNA [**Djordjevic, 2004**]. The order and preference for modification depends upon several factors, such as location of ROS generation, the relative ability for the target molecule to become oxidized, and availability of metal ions. The members of ROS include the highly reactive hydroxyl radical ($^{\bullet}OH$), superoxide radical anion ($O_2^{\bullet -}$), and the non-radical hydrogen peroxide (H_2O_2). They are potential carcinogens as they facilitate mutagenesis, tumor promotion and progression [**Nakamura *et al.*, 1988; Salim, 1993; Hussain *et al.*, 1994**].

The oxidation of DNA has received the largest proportion of attention among those molecules subjected to this type of modification. A broad spectrum of base- and sugar-derived DNA lesions, as well as DNA-protein cross-links, have been identified [**Evans *et al.*, 2004**]. Most of studies have been focussed upon the guanine modification in form of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG), which serves as a biomarker for oxidative DNA damage [**Dizdaroglu, 1994**]. Although DNA damage is one consequence of ROS production, other cellular molecules can be affected by their activity, including RNAs [**Fiala *et al.*, 1989; Nunomura *et al.*, 1999**]**,** emphasising the fact that genotoxic stress should be extended by cellular responsiveness at the level of the "RNome" [**Bellacosa and Moss, 2003**].

Another form of genotoxic stress may be induced by alkylating agents. The most common environmental pollutants are the polycyclic aromatic hydrocarbons (PAHs), a family of highly lipophilic organic compounds. They are typically charcterised by two or more condensed benzo rings, of a wide range of molecular sizes and structural complexicities. PAHs are found in cigarette smoke, ambient air contaminated with automobile exhausts and industrial effluents, as well as in a broad range of food [**Boström *et al.*, 2002; Nunn *et al.*, 1996; IPCS, 1998**]. In order to exhibit their carcinogenicity they require metabolic activation to electrophilic intermediates and subsequent covalent binding (formation of adduct) to critical macromolecules within the cell, including DNA, RNA and proteins [**Ivanovic, *et al.*, 1978; Gräslund and Jernström, 1989; Szeliga and Dipple, 1998**]. The initial step during conversion of organic xenobiotics into hydrophilic and excretable deriverates is mainly catalized by cytochrome P_{450}-dependent monooxigenases (CYPs). Another enzyme family involved in PAH metabolism are the epoxide hydrolases (EHs). Taken together, PAHs are metabolised to a diverse range of metabolites with diverse biological activities [**Grover, 1986; Jerina and Dalay, 1974**]. Hitherto, most research has been focused on the PAH-DNA interactions and their role in mutation initiation and cancer induction [**Brookes and**

Lawley, 1964; Dipple, 1995]. The classical members of the PAH-derived "ultimate carcinogenic" DNA-binding metabolites are the fjord-region carcinogen dibenzo[*a,l*]pyrene diol epoxide [(-)-*anti*-DBPDE] and the bay-region benzo[*a*]pyrene diol epoxide [(+)-*anti*-BPDE]. Although chemically related, the bay- and fjord-region PAH diol epoxides (DEs) are DNA-alkylating compounds possessing different structural and functional features [Jerina *et al.*, 1986; Glatt *et al.*, 1991], (Figure 1). Available data shows that the fjord-region DEs, in general, are more biologically active than the corresponding bay-region DEs. The most carcinogenic PAH tested so far is the fjord-region DBP and its metabolite, DBPDE [Cavalieri *et al.*, 1991; Luch *et al.*, 1994]. The results from several studies have begun to indicate that these types of extremely carcinogenic DNA-DE-adduct escape recognition/repair mechanisms protecting cellular DNA [Dreij *et al.*, 2005; Binkova *et al.*, 2000].

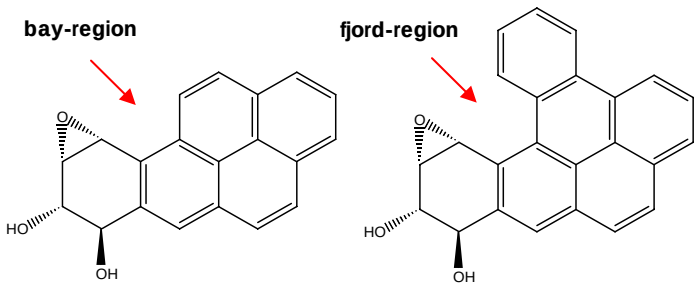

Figure 1. Structure of the bay- and fjord-region PAH DE, BPDE (left) and DBPDE (right)

Cellular response to stress

Mammalian cells have evolved biochemical defense systems in order to protect the cellular micro-environment against deleterious effects of endogenous and exogenous stress. The term *redox signaling* is used to describe a regulatory process in which the signal is delivered through redox chemistry, with the aim to induce protective responses against oxidative damage and to reset the original redox homeostasis after exposure to elevated levels of ROS, for example. In order to combat the attack from them, or from other free radicals which induce the oxidative stress, living cells have acquired several strategies of defense. The first line of protection consists of low molecular weight compounds, endogenous antioxidants such as α-keto acids, bilirubin, urate, coenzyme Q. Additionally, the vitamins C and E, carotenoids and phenols are all delivered to cells from the diet. Another low molecular weight antioxidant with great importance is glutathione (GSH) which is synthesized inside cells in large amounts [Halliwell and Gutteridge, 1999]. Upon oxidation, GSH forms glutathione disulfide (GSSG), or mixed disulphides with proteins in a process called reversible S-

glutathionylation [**Brigelius *et al.*, 1983; Schuppe-Koistinen *et al.*, 1992**]. Reduced glutathione is regenerated from GSSG by glutathione reductase (GR). It is worth noting here that cells can utilize high-level, low-efficiency antioxidants in form of free amino acids, peptides and proteins as quantitatively important ROS scavengers. Other more complex approaches to combating oxidative stress involve enzymes, such as superoxide dismutases (SODs), catalase (CAT) and the glutathione peroxidases (GPxs), [**Dröge, 2001; Evans *et al.*, 2004**].

The highly reactive electrophylic compounds are readily conjugated with GSH. This process is enzymatically catalised by glutathione-S-transerases (GSTs). This is the most important enzymatic pathway for inactivation of PAH diol epoxides [**Robertson *et al.*, 1986; Robertson and Jernström, 1986; Jernström *et al.*, 1996**]. Another way of inactivation of PAHs metabolites includes spontaneous hydrolysis to less harmful tetraols, and further epoxidation by CYP_{450} to triol epoxides and pentaols [**Dock *et al.*, 1986; Grover, 1986**].

Signal transduction and gene expression are vital elements of cellular responses to changes in endo- and exogenously-induced stress. ROS and antioxidants are known to influence the expression of large number of redox-sensitive genes, signal transduction pathways and act as messengers for certain growth factors or cytokines [**Allen and Tresini, 2000**]. For example, H_2O_2 has a relatively long half-life and can cross membranes, therefore its effect on the redox status can be multiplyed and transferred to other cells in the vicinity of the cell where initiating signals have been generated/received. The most studied examples of ROS-mediated signaling cascades include protein tyrosine kinases, the activities of which can be enhanced by inhibitory effect of oxidative stress on corresponding protein tyrosine phosphatases. Here, catalytically essential cysteine residue(s) in the enzyme's active center may undergo(es) oxidation to cysteine sulfenic acid (Cys-SOH) or/and convertion into a mixed disulfide with concomitant loss of catalytic activity [**Barrett *et al.*, 1999**]. Other examples of signaling pathways involved in oxidative signaling and regulation of gene expression are JNK, p38 MAPK, and the transcription factors AP-1 and NF-κB [**Allen and Tresini, 2000**]. Apart from the above, changes in ROS levels influence intracellular calcium homeostasis which in turn, can modulate the activity of calcium-dependent protein kinases (PKC-a), as well as transcriptional induction of the AP-1 proteins c-Fos and c-Jun [**Larsson and Cerutti, 1989; Maki *et al.*, 1992**].

The genotoxic stress induced by exposure of cells to PAHs or by their metabolites evokes biological responses of varying degrees depending on how severe the incoming insult is, on how long it lasts or which type of the cell is targeted. Constituting one of the most fundamental initial events, p53 mediated cell cycle arrest will be triggered in order to allow activation and mobilization of DNA surveillance/repair machinery. However, molecular decisions on the fait of the affected cell must be

continually reviewed during the stress response. There are several options to be chosen but the primary choice lies between growth inhibition *versus* cell death. Growth inhibition could be temporary where, during cell-cycle arrest, cells have time to repair damage and re-enter cell division. When the damage is severe, but still at the level of tolerance, cells enter a stage of senescence. This proliferative barrier protects the organism from the loss of cells. However, it may put itself into jeopardy of developing neoplastic transformation [**Gire, 2005**]. Senescence occurs following telomere shortening or after exposure to both acute or chronic physiological/environmental stress signals. Telomere mediated senescence triggers focal recruitment of protein sensors of the double strand breaks (DSBs), leading to activation of the DNA damage checkpoint responses. The tumor suppressor gene product p53 is activated and the cell cycle progression inhibited *via* p21/WAF1. When loss of p53 and pRb takes place, cells can re-gain capacity to divide, increasing telomere dysfunction and leading towards immortalization, which is an essential prerequisite for the formation of a tumor cell. However, it has to be noted that senescence can be induced in a telomere-independent manner too [**Stampfer and Yaswen, 2003**]. Furthermore, over-expression of oncogenes, modification of chromatin towards more decondensed structure, different types of radiation, exposure to compounds inducing formation of DSBs and oxidative stress, all may induce senescence-like growth arrest events that are stress-dependent and telomere-independent (premature senescence or stress-induced premature senescence: SIPS), [**Toussaint *et al.*, 2000; von Zglinicki *et al.*, 2005**].

When cells become exposed to severe stress resulting in irreversible DNA damage, the programmed cell death (apoptosis) signaling pathways take over the cell cycle arrest/repair signaling, in order to ensure elimination of cells with deleteriously damaged DNA. In addition to DNA-damage *per se*, apoptosis can be triggered by aberrant patterns of transcription, as well as by changes in chromatin structure in the absence of any DNA lesions [**Berardi *et al.*, 2004**]. Apart from being a universal response of cells to internal or external injury, apoptosis plays very important roles in development of multi-cellular organisms and is essential for the regulation of their tissue homeostasis. The elimination of cells by apoptosis is tightly controlled. When this process becomes deregulated accelerated cell death results in acute and/or chronic conditions like, for example, immuno-supression, whilst abolished responsiveness towards apoptotic stimuli/signaling/execution promotes development of cancer [**Danial and Korsmeyer, 2004**]. Furthermore, cross-talk occurs between apoptotic pathways and the machinery which controls cell proliferation and DNA damage signaling/repair control. Here, the balance between expression of oncogenes and tumor suppressor genes, and their post-translational modifications, play very important roles in coordinating and dictating at which level of cellular injury, the affected cell(s) should become senescent or be destroyed.

The PI3-K (phosphatidylinositol 3-kinase)/AKT pathway is one of the critical pathways that generally suppresses apoptosis [**Franke *et al.*, 2003**], whilst NF-kappaB (NFκB) can acts both as pro- or anti-apoptitic regulator of programmed cell death [**Kucharczak *et al.*, 2003**].

The capacity of DNA damaging agents to induce apoptosis is mediated by a complex network of pro- and anti-apoptotic signaling pathways. The p53 dependent pathway promotes apoptosis mostly *via* its transactivation of pro-apoptotic members of the Bcl-2 family (Bax, Bid, Bad and Bim), PTEN phosphatase or GADD45 [**Fridman and Lowe, 2003**]. Some of the targets of p53 and this protein by itself are involved in regulation not only of the apoptosis but also of cell cycle checkpoints and DNA repair [**Sheikh *et al.*, 2000**]. For example, the breast cancer 1, early onset (BRCA1) which expression is regulated by p53 [**Arizti *et al.*, 2000**], forms a complex referred to as the BRCA1-Associated Surveillance Complex (BASC), which consists of at least 15 subunits, such as the ATM, BLM, MSH2, MSH6, MLH1 and MRN protein complex [**Wang *et al.*, 2000**]. Many proteins in BASC are tumor supressors, indicating that loss of integrity of this complex may play a very important role in mechanisms of tumor development. Thus, BRCA1 activities represent one of many links bridging the DNA damage/repair surveillance with cell cycle and genomic stability control, apoptosis and cancer development [**Jhanwar-Uniyal, 2003**].

There are two major apoptotic pathways in mammals. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered by the members of tumor necrosis factor (TNF) receptor family, such as Fas, TNF1 and the TRAIL receptors DR4 and DR5 [**Zapata *et al.*, 2001; Pan *et al.*, 1997**]. The second is the intrinsic or mitochondrial pathway that, when stimulated, leads to the release of cytochrome *c*, Apoptosis Inducing Factor (AIF), Smac/DIABLO, endonuclease G and Omi/HtrA2 from the mitochondria [**van Gurp *et al.*, 2003**]. Both pathways converge to a final common pathway involving the activation of cascade of proteases called caspases that cleave regulatory and structural molecules. As a result of the above, cells which undergo apoptosis shrink, their chromatin becomes condensed, the intact membrane forms small extrusions and finally, the so-called apoptotic bodies are formed. Affected cells and apoptotic bodies are then disposed of by macrophages. Apoptosis usually does not induce immune responses. On another hand, necrotic cell death evokes inflammation which, in turn, may accelerate primary tissue damage [**Savill *et al.*, 2002; Dumitriu *et al.*, 2005**]. A broad range of reactive metabolites, membrane-damaging xenobiotics and chemicals may cause acute lethal cell injury leading to necrosis. This type of cell death is a passive process and does not depend on ATP.

Nucleic acids damage surveillance/repair systems

In order to survive genotoxic insult and maintain genomic integrity, cells have evolved sufficient mechanisms which detect DNA damage. These

include damage sensor machinery and DNA damage repair systems. When the levels of damage overwhelm the repair capacity of the cell, the DNA damage tolerance can trigger replicative senescence or intolerance, therefore inducing programmed cell death or necrosis. Both the efficiency and kinetics of DNA repair and DNA damage tolerance/intolerance are influenced by regulatory responses (see the previous section).

DNA damage recognition signaling networks alert cells to endogenous/exogenous genotoxic stress. The PI3-K signal cascade and the ADP-ribosylation of proteins both play very important roles in sensing the DNA damage and integrating the repair response. The members of PI3-K-like kinases (PIKKs) are Ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), DNA-dependent protein kinase (DNA-PK), suppressor of morphogenesis in genitalia-1 (SMG-1) and mammalian target of rapamycin/FKB12-rapamycin binding (FRAP). These kinases respond to a broad range of DNA damage and regulate cell cycle checkpoints and DNA repair. ATR phosphorylates proteins such as Brca1, Chk1, p53 and Rad17. These phosphorylated substrates, in turn, mediate inhibition of DNA replication and promote DNA repair [**Friedberg *et al.*, 2004; Abraham, 2001**]. ATM-dependent targets for phosphorylation include 53BP1, c-Abl and Mre 11, and some of its substrates are shared between several members of the PIKKs like p53 or H2AX [**Kurz and Lees-Miller, 2004**]. Most recently [**Denning *et al.*, 2001; Brumbaugh *et al.*, 2004; Abraham, 2004**], the involvement of stress-responsive ATM-related kinase, hSMG-1 was shown to bridge between the genome and the RNA surveillance pathway termed nonsense-mediated decay (NMD). As with DNA, RNA can be directly damaged, as well as incorrectly copied.

How then can the cell sense RNA damage or check that RNA is correctly synthesized? Similarly, how will the cellular apparatus deal with incorrectly transcribed/spliced/folded RNAs? These are the subject of intense investigations today, and are concepts of high relevance to the present thesis. One way of "sensing" that the DNA template may be damaged, which increases the possibility of incorrect RNA transcription, is the fact that both RNA- and DNA-dependent RNA polymerases posses a 3'- 5' nuclease activity, which is augmented by cleavage-stimulatory factors [**Fish and Kane, 2002**]. This characteristically makes RNA polymerases able to negotiate barriers to elongation. It seems that, upon arrest of the RNA polymerase at some barrier to elongation, cleavage-stimulatory factors interact with RNA polymerase, causing it to back-up and hydrolyze the nascent transcript. Elongation is then reinitiated. Additionally, cells could deal with RNA damage by the action of RNA chaperones, which recognize and sequester "dangerous" RNA species. These are YB-1, LSM1, A18 hnRNP or HPC2/ELAC2, just to name a few [**Bellacosa and Moss, 2003**]. A further very important mRNA surveillance pathway that eliminates aberrant mRNAs which harbor premature translation termination codons (PTCs) is, as mentioned earlier, the NMD [**Conti and Izaurralde, 2005**]. Only most recently,

it has been discovered that the presence of PTCs in mRNA can induce silencing of transcription of its cognate gene through chromatin remodeling [**Bühler *et al.*, 2005**].

Repair machinery exists for both damaged DNA and RNA. During evolution, several repair systems have evolved. For DNA repair there are four main pathways: The nucleotide excision repair (NER) system, which repairs the majority of DNA damage; the mismatch repair (MMR) system, which repairs mispairs caused by errors in DNA replication; the base excision repair (BER) pathway, which deals with certain types of alkylation and oxidative damage; and repair of DSB, the most toxic of all DNA lesions, which are induced by genotoxic insults, as well as during normal physiological processes including, for example, meiotic recombination, immunoglobulin gene rearrangements or DNA replication [**Hales, 2005**].

Bulky DNA lesions, such as those resulting from PAH DEs, are subjected to the NER pathway, the efficiency of which greatly depends on the stereochemistry and the conformation of the lesion induced [**Hess *et al.*, 1997**]. The NER is an extremely active process which is orchestrated by more than 30 different proteins. These complexes consist of, for example, CSA/CSB, DDB1/DDB2, XPs, replication protein A (RPA), DNA polymerases δ/ε and ligase IV. Mutations in NER genes are responsible for the majority of human DNA repair genetic disorders, including xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) [**Lehmann, 2003**]. NER encompasses the repair of both transcriptionally-silent (termed as global genomic repair (GGR)) and transcriptionally-active regions of the genome (termed as transcription-coupled repair (TCR)). The TCR is far more effective in lesion recognition/removal than the GGR.

BER is responsible for removing damaged, potentially highly mutagenic, bases, such as 8-oxodG. The components of BER machinery include DNA glycosylases, DNA polymerase β, apurinic or apyrimidinic endonuclease (Apex or APE/REF-1), DNA ligase I or III and Flap endonuclease. In the case of long-patch (2-6-nucleotides long) nucleotide replacement BER, this DNA repair pathway is proliferating cell nuclear antigen (PCNA)-dependent. Here, apart from the components involved in single nucleotide replacement BER, the replication factor C (RFC), DNA polymerase δ/ε are involved. In addition to BER, direct removal of 8-oxodG is mediated by the glycosylase OGG1, as well as by the MTH1 and MYH proteins [**Chung *et al.*, 1991; Nakabeppu, 2001**].

Post-replicative MMR increases the fidelity of DNA replication by correcting errors of DNA polymerases that escape their 3' → 5' exonucleolytic proof-reading activity. In mammals, these errors, mismatches, are predominantly recognized by the heterodimers MSH2-

MSH6, referred to sometimes as MutSα [Stojic *et al.*, 2004]. Other forms of DNA structures targeted by MMR are short insertion-deletion loops (IDLs), which are recognized and bound by another heterodimer, composed of MSH2 and MSH3 (MutSβ),[Allen *et al.*, 1997]. MutS proteins also interact with other DNA lesions, including subtle alternations such as O^6-methylguanine, 8-oxoguanine and thymine glycol, and major UV light photoproducts such as Cyclobutane pyrimidine dimmers (CPDs) and 1,2 intrastrand G-G cross-links produced by cysplatin [Christman and Kaina, 2000]. In addition, the MMR machinery includes the MSH1-PMS2 heterodimers, PCNA, exonuclease EXO1, RPA, RFC, DNA polymerase δ and DNA ligase I [Stojic *et al.*, 2004]. Most recently the DNA methyltransferase 1(Dnm1) was identified as a member of MMR system [Guo *et al.*, 2004; Kim *et al.*, 2004].

The DSB is one of the most severe forms of DNA damage, serving as a potent trigger of cell cycle arrest and apoptosis [Valerie and Povirk, 2003]. During DSB repair by homologous recombination (HR), (**Figure 2**), the damaged chromosome enters into physical contact with an undamaged DNA molecule, which serves as a template for error-free repair. This takes place more often in late S/G2 whilst the non-homologous end-joining (NHEJ) DSB repair occurs predominantly during entry into G1/GO and in early S phase.

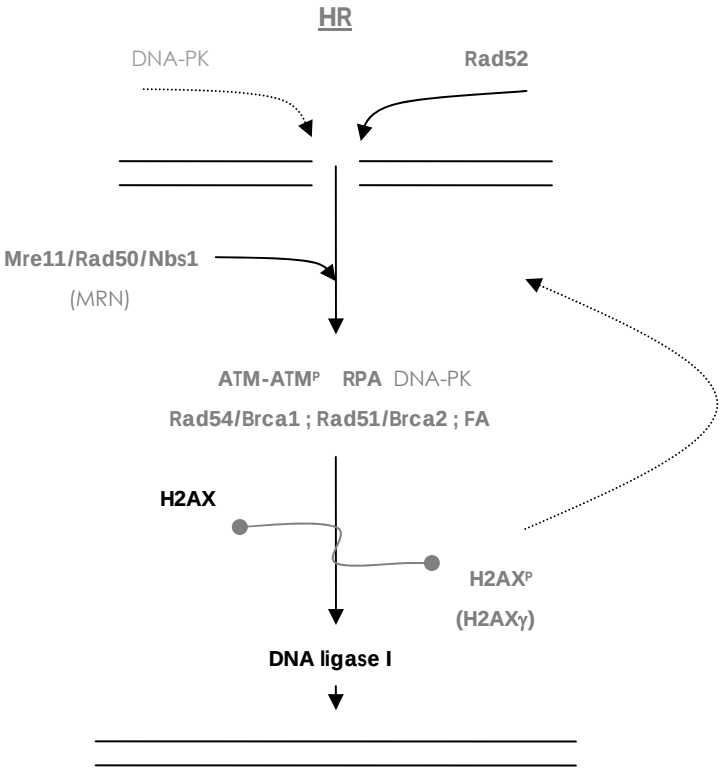

Figure 2. Major components of the HR repair.

NHEJ (**Figure 3**) is error-prone as the two ends of damaged DNA are joined without the requirement of sequence homology between the DNA ends.

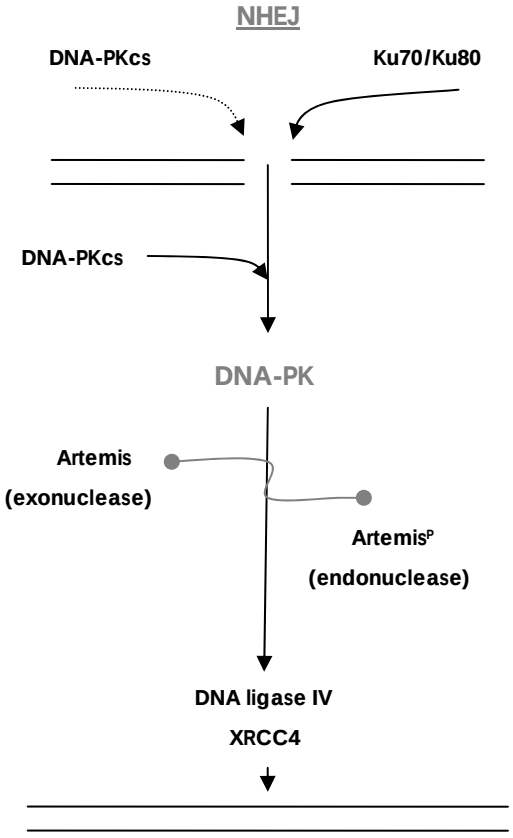

Figure 3. Major components of the NHEJ repair.

One of the hallmarks of DSB recognition is the formation of gamma-foci (γ-foci) at the site of DNA lesion. The PI3-K signaling cascade evokes phosphorylation of histone H2AX (H2AXγ) which, in turn, forms large protein agglomerates visible as γ-foci (**Figure 4**). Other proteins are also co-localized with H2AXγ such as, for example, the MRE11-Rad50-NBS1 (MRN) complex. Very important components of the DSB repair, include Artemis, DNA-PK, Brca1, DNA ligase IV, Xrcc1/4 [**Mashous *et al.*, 2001; Ma *et al.*, 2002; Friedberg *et al.*, 2004**]. The p53 tumor suppressor protein is active downstream of PI3-K, playing a pivotal role in cell cycle arrest, DNA repair and apoptosis. Additionally, it co-localizes with other proteins involved in HR of DNA damage, thus modulating NER and BER activities [**Offer *et al.*, 1999; McKay *et al.*, 1999; Ljungman and Lane, 2004**].

DNA repair can be also achieved by the reversal of base damage. DNA lesions, in form of 1-methyladenine and 3-mathylcytosine, may be

repaired by AlkB protein, by reversing this alkylative DNA damage *via* its deoxygenase activity [**Trewick *et al.*, 2002; Falnes *et al.*, 2002; Aas *et al.*, 2003**].

For RNA there are so far identified, apart from the earlier described proof reading by RNA polymerases, the following forms of RNA repair mechanisms: tRNA repair [**Reichert and Mörl, 2000**], restricted to mammalian mitochondria; oxidative demethylation of RNAs [**Aas *et al.*, 2003 ; Ougland *et al.*, 2004**]. Thus, RNA repair may be considered as a specific form of RNA editing [**Poole and Logan, 2005**], so it seems reasonable to assume that other forms of repairing an altered RNA sequence/structure will be soon "re-discovered" among these already known RNA editing processes.

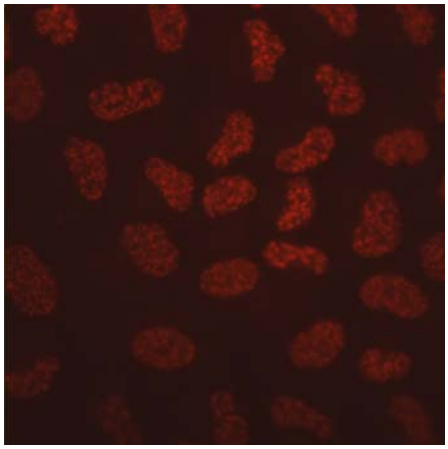

Figure 4. H2AXγ foci formation in response to genotoxic stress induced by treatment of A549 cells with 25 mM $KBrO_3$ (time point: 3h after exposure).

Epigenetics and cancer

Classical descriptions of epigenetics refer to modifications of the genome heritable during cell division that do not involve changed DNA sequence. The common characteristics of epigenetic changes are: (i) Potential reversibility; (ii) positional effects, i.e., effects on genes at some distance, that can decline with distance from the affected gene from the epigenetic mark, or end abruptly; (iii) effects on groups of genes that are juxtapositioned to one another in the genome; (iv) present at a surprisingly high frequency of alteration, orders of magnitude greater than that of mutation; (v) can be modified by the environment [**Feinberg, 2004**]. A mechanism unifying all characteristics described above requires the involvement of chromatin changes, including post-translational modification of histones, the arrangement of nucleosomes into higher order structures, as well as methylation of DNA.

The most prevalent molecular mechanisms underlying eukaryotic gene regulation include a broad range of post-translational histone modifications [**Margueron *et al.*, 2005**]. The formation of nucleosomes and

their arrangement on the DNA is regulated by ATP-dependent chromatin remodeling machines [**Cairns, 2005**]. Catalytic reactions either drive the movement of histones to new locations on DNA, or disassemble their octamers altogether [**Flaus and Owen-Hughes, 2004**]. These changes are also very important in the regulation of histone variant deposition [**Henikoff and Dalal, 2005**].

Alteration in DNA methylation status is another epigenetic marker of changed cellular gene expression. Aberrant promoter hyper-methylation is a prevalent phenomenon in human cancers [**Herman and Baylin, 2003; Li *et al.*, 2005**], and tumor suppressor genes are often silenced in this manner. In contrast, many oncogenes become activated by hypo-methylation. The CpG dinucleotides (with the cytosine as a target for this covalent modification) are the most frequent targets for methylation. As a result of this, the methyl group protrudes from cytosine nucleotide into the major groove of the DNA, leading to displacement of transcription factors that normally bind to the DNA [**Hark *et al.*, 2000; Kim *et al.*, 2003**] and to recruitment of methyl-binding proteins, followed by their interaction with protein-complexes which are involved in chromatin re-modeling [**Bird and Wolffe, 1999**]

At early stages of tumorigenesis, these so-called non-genotoxic effects are reversible and require continuous presence of compound. The long-term exposure to low doses of genotoxic carcinogens may also contribute to promoting the outgrowth of transformed cell clones *in vivo* [**Luch, 2005**].

Emerging concepts and discoveries in the field of biology and biogenesis of non-coding RNAs have opened a new perspective with which epigenetically-mediated gene regulation has begun to be viewed and perceived. The concept of modern epigenetics acknowledges the role of small RNAs, microRNAs (miRNAs) and other RNA molecules in effecting the cellular ribotype which is "translated" into the phenotype of a given cell, at a given time and within its endo- and exo-environment [**Herbert and Rich, 1999; Pasquinelli *et al.*, 2005**]. One of many newly discovered epigenetic ways of changing/regulating the gene expression, without altering the DNA coding sequence is, for example, by RNA interference (RNAi). Here, the very short RNA species are able to match antisense RNA target molecules and mark them towards directed mRNA degradation [**Fire *et al.*, 1998**]. Another RNA-mediated gene silencing mechanism at the chromatin structure level has been described. In this phenomenon, the RNA molecules direct DNA and/or histone methylation [**Wassengger, 2000; Zilberman *et al.*, 2003**].

Recent evidence suggests that gene expression which has been altered via several diverse epigenetic mechanisms can lead to permanent or reversible changes in cellular behavior. As the role which epigenetic events play in carcinogenesis attracts more attention, a better understanding of this area will give opportunities for development of new therapies against, not only tumors, but also other diseases where,

besides gene therapy, novel "ribo" therapeutic approaches may come to age [Felsher and Bishop, 1999; Hamar *et al.*, 2004].

The RNA world we live in

Evolution of life and how it started has long perplexed the scientific community. The term "the RNA world" [Gilbert, 1986] gave a name to the hypothesis that the RNA molecule was the first main biological catalyst and genetic material, which appeared long before the DNA and proteins evolved [Woese, 1967; Crick, 1968; Orgel, 1968]. The discovery of RNAs with catalytic activities [Kruger *et al.*, 1982; Guerrier-Takada *et al.*, 1983] and RNA functioning as the genetic material in some viruses [Fraenkel-Conrat, 1956; Gierer and Schramm, 1956], or both, in the case of plant viroids [Diener, 1971; Flores *et al.*, 2004], strengthens the theory that at a very early stage of biological evolution, it was most likely that RNA served as the primeval genome. The most recent progress made in re-discovering the biogenesis and biochemical functions of RNAs which do not code for protein (non-coding RNAs or ncRNAs) but which code for very important co-regulatory RNA species, in parallel with the proof of existence of mRNA proof-reading and repair, has uncovered new evidence that, indeed, it is most likely that the last universal common ancestor to the present plethora of life on the planet possessed an RNA genome [Poole and Logan, 2005].

According to the central genetic dogma [Crick, 1970], the flow of information is as follows: Genetic information which is encoded in form of DNA (genes) is transcribed into mRNA, and this message (mRNA) is then translated into a final product, the protein. Growing evidence suggests that, besides the paradigm described above, the direction of flow of genetic information has many different routes. Interestingly, the experimental data describes a novel phenomenon of miRNAs generation from a pool of functional mRNAs [Cai *et al.*, 2004]. It seems that the RNAs are not just passive elements of gene expression, but that they are also evoluntary forces, which have only during the last decade started to become recognized us such. Apart from well established functions of mRNAs, transport RNAs (tRNAs), small ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs), there is a newly discovered subset of RNA species, large in numbers and structural diversity, which actively coordinate [Herbert, 2004] and regulate the read-out of genetic information flow (coRNAs). The terms "hard-wired" prokaryotes and "soft-wired" eukaryotes describe very well the way life on Earth has evolved and that it is still evolving as we speak [Herbert and Rich, 1999].

The prokaryotes have their genome in a very impacted form, and the generation of diversity is primarily achieved by mutation processes. In higher organisms, the genetic information read-out can affect both the expression of genotype and its rybotype, which finally influences the phenotype of an organism. The generation of diversity occurs *via* the regulation of gene expression rather than by mutation sustained over a few generations of cells. Additionally, RNA processing by itself is much

faster than is gene transcription. The changes in mRNA stability can be triggered by endogenous or/and exogenous signals. As a consequence, the relative amount of encoded protein product varies with the time, which permits the temporal coordination of many disparate processes and cross talk between them [**Kren and Steer, 1996**].

It becomes clear that post-transcriptional gene silencing (PGTS), which includes RNA interference (RNAi) and inhibition of translation, is a common phenomenon shared amongst eukaryotes. Here, the double stranded RNAs (dsRNAs) play an essential role in RNAi, as they are cleaved by the enzyme Dicer into small interfering RNAs (siRNAs). When pairing of siRNAs with complementary sequence on target mRNA occurs, the latter undergoes cleavage/degradation, mediated by set of specific proteins called RISC (RNA-induced Silencing Complex). The microRNAs (miRNAs) are also part of RNAi and are involved in the inhibition of translation [**Pasqueinelli *et al.*, 2005**]. Recent studies have shown that miRNAs play important roles in genetic pathways regulating embryogenesis, hematopoiesis, neuronal differentiation and Hox-mediated developments [**Suh *et al.*, 2004; Chen *et al.*, 2004; Nelson *et al.*, 2004; Yekta *et al.*, 2004**]. In addition, siRNAs have been shown to be involved in direct gene silencing at the chromatin level [**Zamore, 2002; Westhof and Filipowicz, 2005**]. Connections between the RNAi machinery and DNA methylation, chromatin silencing and centromere function had already been found in plants and vertebrates.

RNAs can re-direct the flow of genetic information from themselves toward DNA, *via* the reverse transcriptase activity present in the cells [**Jones and Foulkes, 1989; Jordan et al., 2003; Feldser *et al.*, 2003**]. Moreover, it has been shown that small RNAs can regulate enzymatic activity of several proteins involved in transcription [**Lanz *et al.*, 2002; Reichman and Mathews, 2003; Yang *et al.*, 2001**]. Interestingly, mitochondrial 12S and 16S rRNAs can act as "molecular chaperones" by folding chemically denaturated proteins and reactivate heat-induced aggregated proteins *in vitro* in ATP-independent manner [**Sulijoadikusumo *et al.*, 2001**]. The processes in which RNAs play important roles are not limited to these presented earlier as RNA-directed/mediated events [**Finnegan and Matzke, 2003**], but are still growing, and will certainly involve many more.

Endogenous and exogenous toxic compounds affect not only DNA, proteins or lipids, but also RNA [**Ivanovic *et al.*, 1978; Sotomayor *et al.*, 2003; Usha *et al.*, 2005**]. As described above, the role of RNA species in epigenetic control of gene expression and generation/inheritance of phenotypes has been only recently placed under more detailed investigation. Results from the literature, including our own data presented in this thesis, point out that mRNAs have to be recognized as an autonomous unit of inheritance, and both as the mediator of and direct target for regulation of gene expression. The ribotype, in parallel with genotype, but sometimes in completely independently from it, defines the

characteristics of cellular phenotype. Therefore, when altered by endogenous or exogenously-derived insult, the changes in RNA homeostasis and biology may give immediate deleterious effects on the biology of the cell, facilitating pathological process towards malfunction. This, in turn, may prime the affected cell(s) and facilitate development of disease, including carcinogenesis.

In view of the centrally active role of RNA in physiology, there are very serious grounds for toxicologists to consider these molecules as fundamental targets for generating toxicity in living systems.

"The RNA world does exist and is calling right now for recognition of its importance in the past, present and future" E.Z.B.

THE PRESENT INVESTIGATION

Aims

The overall goal of the present investigation was to further characterize the molecular mechanisms and effects of different type of genotoxic stress on the biology and function of cells exposed and cultured *in vitro*. In particular, we were intrigued and interested in investigating novel molecular mechanisms underlying the relative carcinogenicity potential of bulky DNA adduct-forming compounds.

The specific aims included:

- To access the relationship between the biochemical nature/structure of genotoxic agents and their potential to induce DNA lesions and further down stream signaling, leading to their recognition and removal.

- To perform a large scale screening of alterations in mRNA steady-state expression profiles induced by genotoxic compounds using microarray technology.

- To validate the accuracy of the microarray-based analysis of altered gene expression patterns to oxidizing and alkylating DNA damage using model *in vitro* systems (A549 and ECV 304 cells).

- To identify novel DNA-damage "type-specific" or "general" biomarkers, at the level of individual and patterns of mRNA and protein expression, as well as at the level of chromatin modifications.

- To improve existing and to develop novel approaches for the direct detection and/or quantification of nucleic acids damage/modifications in biological samples.

- To test and demonstrate the importance of RNA molecules and their modification/damage in controlling the cellular fate in response to genotoxic insult.

Methodologies

More detailed descriptions of the applied methods are presented in each of the separate papers, in the respective Materials and Methods sections. Here, only a brief overview of methodologies is given.

Cell culture and exposures

All experiments were performed *in vitro* using various cell types in a conventional culture system. The **A549** human epithelial lung carcinoma and **ECV 304** human endothelial-like cell lines were subjected to treatments with: DNA-adduct forming compounds BPDE or DBPDE [**Papers II, IV and VI**], pro-oxidants H_2O_2 [**Papers I and III**], diamide [**Paper I**], $KBrO_3$ [**Papers I, II, IV, V and VI**] or heat shock [**Paper I**]. In addition, the rat osteosarcoma cell line **UMR 106-01** was used [**Paper IV**] as a positive control for detection of vitamin D receptor (VDR) gene at the protein level by western blotting. In the same paper, nuclear extract from HeLa human cervical cell line was used as a positive control for histone deacetylase (HDAC) activity.

Glucose oxidase (GOD) from *Aspergillus niger* was used in order to induce endogenous production of H_2O_2 in A549 or ECV 304 cells [**Paper I**]. **Hydrogen peroxide** (H_2O_2) is a strong oxidant. It shows high reactivity towards biomolecules, under catalysis by redox-cycling transition metals (Fe^{2+}, Cu^{+}), using "Fenton chemistry". We were also employing the addition of [^{18}O]H_2O_2 directly to cells [**Paper III**]. **Potassium bromate** ($KBrO_3$) is a very potent pro-oxidising agent with a pleiotrophic mode of action. The mechanisms underlying its oxidative activities are still not clear [**Papers II, IV, V and VI**]. However, $KBrO_3$ has been shown to deplete glutathione (GSH) and to induce oxidation of DNA, as well as the formation of double strand breaks on DNA molecules. **Diamide** is known as a thiol oxidising agent, altering the redox status of intracellular gluthathione [**Paper I**]. Following exposures to diamide or GOD (H_2O_2) the cellular GSH or GSH-protein mixed disulphide formation was determined [**Paper I**] by the method of **Cotgreave and Moldeus (1986)**.

Diol epoxides (DEs), polycyclic aromatic hydrocarbon (PAH) metabolites, were used as examples of very potent model carcinogens [**Papers II, IV and VI**]. These compounds are very potent inducers of bulky DNA-adduct formation (alkylation). Here, the structurally different bay-region benzo[*a*]pyrene DE *versus* fjord-region dibenzo[*a*,*l*]pyrene DE were compared with each other [**Papers II and IV**] or with $KBrO_3$ [**Papers II, IV and VI**].

Finally, physical stress, in the form of **heat shock** (45°C), was applied to cells [**Paper I**]. Prolonged exposure of cells to temperatures above 37°C

leads to broad range of biological effects which may include, for example, protein miss-folding, altered gene expression, DNA damage, cell cycle arrest or apoptosis.

Cell number and viability

The trypan-blue exclusion method was used to estimate the viability of cells after exposure to genotoxic stress [**Papers I, III and IV**]. After exposures, cells were harvested by standard trypsinisation and fixed. In the next step an aliquot of single cell suspension was diluted in trypan-blue solution and cells were counted in a Bürker chamber under the microscope. The percentages of viable (clear) and dead (blue) cells were determined. In some cases, Fluorescence Activated Cell Sorting (FACS) was performed by running the samples on FACScan instrument followed by data analysis with Cell Quest Pro software [**Paper IV**]. For this method, the cells were fixed and stained with propidium iodide (PI). Reduction of PI staining was used as an estimate of the level of cellular death (subG1) in cells treated with DEs *versus* control populations.

In situ affinity and cytoimmunochemical staining

Cells were grown on glass cover slides. After exposures and fixation, cellular preparations were subjected to avidin-FITC or neutravidin-FITC [**Paper V**] staining/visualisation of oxidised DNA/RNA. In immunocytochemical-based target detection of biotin [**Paper V**], phosporylated histones H2AX or H2B [**Papers V and VI**] were targeted with antigen-specific antibodies and visualised with fluorochrome-conjugated secondary antibodies.

Western blotting

Detection and visualisation of protein levels in cellular extracts was performed using standard protein separation by electrophoresis on SDS-polyacrylamide gels, followed by transfer to a nitrocellulose membrane and immunodetection with specific antibodies directed toward the protein of interest. The membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, which were subsequently detected using enhanced chemiluminescence [**Papers I, IV and VI**].

HPLC and HPLC-MS/SM

High performance liquid chromatography (HPLC)-based analysis of DEs derived DNA-adducts formation and removal [**Papers II and IV**] was performed as previously described [**Dreij *et al.*, 2005**]. After exposures, cells were harvested and cellular DNA isolated. In the following steps, the DNA-adduct preparations underwent enzymatic digestions.

Subsequently, adducts were analyzed by established HPLC methods [**Papers II and IV**]. The levels of oxidised DNA and RNA [**Paper III**] were analysed using HPLC electrospray ionization tandem mass spectrometry (HPLC-MS/MS), as previously described [**Ravanat *et al.*, 1998; 2002**]. A549 cells were exposed to [^{18}O]H_2O_2, followed by harvesting and parallel isolation of DNA and RNA. Levels of isotopically-labelled 8-oxodG (biomarker of DNA oxidation) and 8-oxoG (biomarker of RNA oxidation) were measured with HPLC-MS/MS.

HDAC assay

Histone deacetylase (HDAC) activity was determined by using the HDAC colorimetric substrate, which comprises an acetylated lysine side chain (BioVision, USA). Deacetylation of the substrate (mediated by HDAC enzymatic activity present in cellular protein extracts) sensitizes the substrate, in a way that, in the following step of assay, after addition of developer, a chromophore is produced which, in turn, is detected with a spectrophotometer at 400 or 405 nm [**Paper IV**]. A HeLa nuclear extract served as positive control for HDAC activity.

Microarray technology

For studying alternations in gene expression in A549 or ECV 304 cells exposed to genotoxic stress we have applied a screening method collectively termed microarray technology, pioneered by **Shena and colleagues (1995)**.

The **Clontech ATLAS™** arrays are nylon membrane-based arrays with a few hundred genes represented on them. We have used the cDNA Humans Stress Gene Array, containing 234 gene targets [**Paper I**]. After treatment, cells were harvested and mRNA was isolated. For this microarray platform radioactively (^{32}P) labelled cDNAs were synthesized and subsequently allowed to hybridise with the gene-specific targets immobilised on the array. Hybridisation signals are visualised on X-ray films. In data analysis of ATLAS™ microarrays we were comparing the size and intensities of radioactive signals emitted by the hybridisation events, which were captured on X-ray film. The method was intuitive, semi-quantitative and did not include application of any statistical algorithm.

The **Affymetrix GeneChips** are high density glass slide arrays with short DNA oligos directly synthesized on the array surface using special photolithographic methods. The Human Focus™ array, with 8500 gene targets on it, was used by us [**Paper IV**]. In steps following total RNA isolation, the RNA is converted into biotin-labelled cRNAs *via* cDNA synthesis. Hybridization signals are detected with streptavidin-phycoerythrin conjugate and visualized with an array scanner. For the Affymetrix platform, in order to obtain the expression level of each gene

from probe level data, we used the methodology developed by Irizarry and colleagues [**Irizarry *et al.*, 1996 and 2003**]. This methodology has been implemented in the Affymetrix package in R statistical language. The data analysis is achieved using an empirical Bayes method [**Efron *et al.*, 2001**].

PCR

The polymerase chain reaction (PCR) method amplifies fragments of DNA [**Mullis and Faloona, 1987**]. It uses the ability of a thermo-stable DNA polymerase enzyme to extend short, single-stranded synthetic, sequence-specific oligonucleotides (primers) during repeated cycles of heat denaturation, primer annealing and primer extension. Primers are designed to bind a desired sequence on the DNA fragment to be amplified. At each cycle, more DNA is synthesized as the PCR proceeds in an exponential manner, doubling the amount of target each cycle, until it reaches a plateau. In **semi-quantitative PCR** [**Paper I**], we have used total RNA as the template for complementary DNA (cDNA), using reverse transcriptase (RT). Ribosomal protein S9 (RPS9), which was not affected by treatments served as an internal standard. Products of DNA amplification were run on agarose gels, stained with ethidium bromide and visualized under UV light. The estimate of the ratio of target mRNA to the internal standard was obtained.

Real Time quantitative PCR [**Paper IV**] is a method that has been introduced relatively recently. The technique combines the PCR-based DNA amplification with detection of fluorescence emitted by products of that amplification in a single tube. Current methods are based on detection of changes in light emission proportional to the increase in product. Fluorescence is monitored during each PCR cycle (in real time), to provide an amplification plot for each amplified gene target. For cDNA synthesis we have used total RNA. Here, the 18S rRNA served as internal standard in estimating the relative gene expression levels for selected targets.

Summary of presented papers

I. Protein S-glutathionylation correlates to selective stress gene expression and cytoprotection.

During situations of oxidative stress phenotypic adaptation to altered redox state is achieved by changes in expression of selected genes. The mechanisms regulating this may involve reversible S-glutathionylation of cellular proteins. In this study we compared and contrasted changes in gene expression patterns in human type II lung epithelial A549 cells and human endothelial-like ECV 304 cells in correlation to glutathione oxidation and the formation of glutathione-protein mixed disulphides, after exposure to subtoxic levels of hydrogen peroxide or the thiol oxidant diamide. Both the number of specific mRNAs and their levels of induction were grossly correlated to the degree of S-glutathionylation of cellular protein. Thus, diamide induced the expression of a variety of protein and DNA chaperones and transcriptional regulators, particularly in ECV 304 cells. On the other hand, the peroxide failed to induce many of these species, in association with only minimal disturbances to glutathione homeostasis. The induction of the chaperone responses at the level of mRNA was clearly shown to translate into a more resistant morphological phenotype in response to both heat shock and oxidative stress induced by the DNA-damaging pro-oxidant potassium bromate ($KBrO_3$).

II. DNA adducts of benzo[*a*]pyrene- and dibenzo[*a,l*]pyrene-diol epoxides in human lung epithelial cells: kinetics of adduct removal, effects on cell cycle checkpoints, and gene expression.

The fjord-region PAH dibenzo[*a,l*]pyrene (DBP) is considerably more carcinogenic than the bay-region benzo[*a*]pyrene (BP). In order to further substantiate previous findings we have studied DNA adduct formation of anti-DE of DBP [(±)-anti-DBPDE], in A549 cells and monitored the levels of adducts as a function of time. A high-performance liquid chromatography (HPLC) procedure that allows monitoring of all cis- and trans-nucleoside adducts of dA and dG was used. Incubation of cells with 0.1 μM (±)-anti-DBPDE resulted in rapid formation of adducts, followed by a decline. After 6h of incubation, about 20% of adducts were remained. Repeating the experiment with 0.01 μM (±)-anti-DBPDE resulted in a correspondingly lower adduct level initially, but in this case a larger proportion (35%) of the adducts remained after 6h of incubation. Preliminary results from global gene expression analysis demonstrated interesting differences in the stress response elicited in the cells following exposure to the distorted and flexible non-planar DBPDE or the rigid and planar BPDE molecule. As expected some major common induction events were clearly related to the activation of p53-dependent cell cycle checkpoint.

III. Hydrogen peroxide causes greater oxidation in cellular RNA than in DNA.

It is becoming clear that the propensity for reactive intermediates to damage cellular nucleic acid structures relies not only on their primary structures, but also on their higher order organization and their localization in the cells. One clear case where these additional considerations could be collectively dominating arises when considering the fate of DNA and RNA in the same cell undergoing oxidative nucleic acid insult. Human A549 lung epithelial cells were challenged with ^{18}O-labeled hydrogen peroxide ([^{18}O]-H_2O_2), the total RNA and DNA extracted in parallel, and analyzed for ^{18}O-labeled 8-oxo-7,8-dihydroguanosine ([^{18}O]-8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine ([^{18}O]-8-oxodGuo), respectively, using high-performance liquid chromatography, electrospray ionization tandem mass spectrometry (HPLC-MS/MS). [^{18}O]-H_2O_2 exposure resulted in dose-response formation of both [^{18}O]-8-oxoGuo and [^{18}O]-8-oxodGuo and ^{18}O-labeling of guanine in RNA was 14−25 times more common than in DNA. Kinetics of formation and subsequent removal of oxidized nucleic acids adducts were also monitored up to 24h. The A549 showed slow turnover rates of adduct in RNA and DNA giving half-lives of approximately 12.5h for [^{18}O]-8-oxoGuo in RNA and 20.7h for [^{18}O]-8-oxodGuo in DNA, respectively.

IV. The highly carcinogenic potential of dibenzo[*a,l*]pyrene maybe partially mediated by disruption of epigenome, including mRNAs structure and homeostasis.

Products of PAH activation to model "ultimate carcinogenic" DNA-binding metabolites were studied using an *in vitro* exposure system in order to elucidate their effects on host cell gene expression. The A549 cells were treated with either the fjord-region carcinogen dibenzo[*a*,*l*]pyrene diol epoxide [(-)-*anti*-DBPDE], the bay-region benzo[*a*]pyrene diol epoxide [(+)-*anti*-BPDE] or the pro-oxidant potassium bromate ($KBrO_3$), and gene expression studied using Affymetrix Technology. It was demonstrated that, under conditions where initial DNA-adducts were induced at similar levels by both diol epoxides (DEs), after 12h, the highly carcinogenic DBPDE more dramatically affected the steady-state levels of mRNAs within the transcriptome, when compared to the less potent carcinogen BPDE. However, part of the response was common to both of the compounds, particularly with respect to the canonical, p53-dependent genotoxic stress markers involved in DNA damage/repair, cell cycle and apoptosis regulation pathways. Interestingly, DBPDE caused significantly larger changes in gene expression, both quantitatively and qualitatively, when compared to BPDE. This may be due to more extensive alkylation of the mRNA species themselves, thus affecting

their homeostasis and turn-over rates. In the context of type of genotoxic insult (alkylation *versus* oxidation) we were also able to identify shared and distinctive gene expression patterns in A549 cells exposed to DEs and $KBrO_3$, respectively. Novel elements of the stress response were also identified. Several of affected gene products, namely PLK1, ATF3, POLQ, PIM2, BCL2A1 and CDKN2C underwent additional target validations with real time quantitative PCR conforming their induction/repression. Furthermore, the protein levels for a few (PLK1, ATF3, VDR) were monitored by Western blot analysis. Results from this study indicate that structurally-related carcinogens initiate subtly different patterns of gene expressional alteration in the stress response following DNA adduct formation. The data also indicate that, apart from intrinsic DNA-forming capacity, the carcinogenic potential of DEs may be associated with their disruptive effect on biology of RNA, leading to disturbance in the epigenome. This in turn, could prime the cellular environment towards facilitated tumour development or/and progression towards malignancy. Taken together, these data support the notion that the analysis of alternations in gene expression patterns may provide a useful surrogate biomarker of identification of genotoxic agents with high carcinogenic potential.

V. An *in situ* organelle specific method for the detection of oxidatively modified DNA and RNA in cells.

Avidin is traditionally used for detection of biomolecules labelled with biotin. However, strong and rather specific avidin binding to nuclei of oxidative damaged cells and tissues was demonstrated recently, and we were interested to further explore the specificity of this binding. Here we have developed an analytical method for the detection of oxidised nucleic acids in different cellular compartments using *in situ* staining with fluorescein-conjugated avidin (avidin-FITC) and fluorescein-conjugated neutravidin (neutravidin-FITC). During oxidative stress, induced by treatment of A549 and ECV 304 cells with $KBrO_3$, we were able to reveal differences in binding preferences between these two FITC-conjugated proteins. Avidin preferably targets the nucleus, whereas neutravidin targets the mitochondria. Pre-treatment of fixed cells with DNase I or RNase A, prior to detection, resulted in distinct changes in staining intensities/localisation, indicating that both oxidised DNA and RNA represent potential binding targets for avidin/neutravidin in an organelle specific manner. These protocols allow for simple, semi quantitative analysis of the intracellular compartmentalisation of oxidised DNA and RNA in cells.

VI. Chromatin-dependent responses of A549 lung carcinoma cells to diol epoxides derived from benzo[*a*]pyrene and dibenzo[*a,l*]pyrene.

Chromatin is a highly structured and dynamic nuclear component of eukaryotic cells, serving some of their most vital biochemical/biological functions. Early events in the cellular response to DNA damage rely on lesion recognition and activation of proteins involved in maintenance of genomic stability. One important component in this process is the H2AX variant of H2A histone. Here we have studied differences in chromatin-derived stress responses in A549 cells exposed to the ultimate carcinogens, benzo[*a*]pyrene diol epoxide [(+)-*anti*-BPDE] or dibenzo[*a,l*]pyrene diol epoxide [(-)-*anti*-DBPDE]. Visualisation of the extent of phosphorylation of H2AX histone (H2AXγ) in relation to DNA damage/repair revealed that, at similar levels of adducts, the number of cells exhibiting gamma-foci (γ-foci) did not differ significantly between BPDE and DBPDE. However, there was a notable difference between the two treatments in the time-dependent changes in intensity of formed γ-foci. Furthermore, the levels of H2AX phoshorylation, revealed by Western blot analysis, showed distinct and time-dependent patterns of histone modification induced by studied diol epoxides (DEs). Recently it has been shown that the lysine tail of histone H2B proteins undergoes phosphorylation in cells exposed to ionizing radiation. We also investigated how DNA-adduct formation affects signal transduction as reflected in $H2B^{Ser14P}$ modification, which was detectable in $KBrO_3$ treated cells, whilst being less visible in DEs treated ones. Overall, the results demonstrated some commonalities in the chromatin-derived stress response to BPDE and DBPDE in the mobilization of cellular recognition, repair signalling pathways. The H2AX histone phosphorylation kinetics correlated well with the kinetics of DNA-adducts removal at earlier recovery time points. However, this γ-foci formation was far less pronounced in the cells after treatment with DEs relative to treatment with the pro-oxidant $KBrO_3$, indicating that bulky DNA-adducts may represent a more severe threat to the genomic stability of the cell than do oxidiative DNA lesions.

Conclusions

An intrinsic component in the ability of a cell to respond to electrophilic and oxidative stress lies in the ability to react to altered macromolecular structures. In terms of oxidative stress, much effort has been placed on the role of oxidatively modified nucleic acid in initiating response, but there is a strong possibility that post-translational redox modification of proteins may also play a pivotal role in coordinating cellular response. One such mechanism which has been proposed is the reversible interaction of GSH with protein thiols. Results from experiments where cells were exposed to diamide or H_2O_2 indicated that levels of induced protein S-glutathionylation [**Paper I**] correlate well to selective stress gene expression, many of which were related to the induction of nucleic acid damage recognition/repair pathways. Moreover this adaptation is translated to a functional phenotype in form of increased resistance in subsequent exposures to heat shock or oxidative stress.

Small differences in chemical structure of studied DEs have been shown to have pronounced effects on their conformation, target binding preferences and removal efficiency from DNA [**Paper II**] which, in turn, mark distinctive biochemical and biological effects on cellular biology [**Papers II, IV and VI**].

DEs and $KBrO_3$ showed distinct, chromatin-based responses in DNA damage signaling pathways, as measure of induction of H2AX or H2B phosphorylation [**Paper VI**]. Interestingly, observed formation of γ-foci, rich in $H2AX^{Ser139P}$, indicates that DEs may induce DNA strand breaks. Assuming that γ-foci are "true markers of DSBs", as described in the literature, we thus may have identified a novel biomarker of DNA damage associated with *in vitro* exposure to DEs. However, we can not rule out the possibility that formation of γ-foci may be in part due to phosphorylation of H2AX mediated by DNA-PK, which becomes activated not only by the presence of DSBs but also by the "distorted structure of DNA" [**Dip and Naegeli, 2005**] induced at sites of bulky DE-adducts.

Data from microarray-based [**Paper IV**] gene expression screening revealed that structurally-related alkylating carcinogens initiate subtle qualitative and quantitative differences in indigenous patterns of steady state mRNA levels following the induction of genotoxicity, when compared with responses to DNA-damaging pro-oxidant treatment. Further, the extent of the biological response to alkylative DNA damage strengthens the view that alkylation may represent a more severe threat to cells than that induced by oxidative stress, and that evolution has equipped cells with multiple mechanisms for dealing with DNA damage derived from different kinds of chemical insult.

A number of novel gene targets were identified in the high density [**Paper IV**] microarray assays employed (ESTs, open reading frames), the functions of which have not previously been associated with response to genotoxic stress. Notably the chromosome 12 open reading frame 5 transcript seems to be a good biomarker of genotoxic stress as it was altered during both alkylating and oxidative stress.

Taken together, results support the notion that the analysis of alternations in gene expression patterns may provide a useful surrogate biomarker in identification of genotoxic agents with high carcinogenic potential. However, several technical issues arise from the use of microarray analyses. The first concerns the intrinsic reproducibility, selectivity and sensitivity of the assay, and the second concerns how well the data predict a functional response at the protein level. In several of our studies we could confirm that changes in gene expression detected with microarrays could be verified with real time PCR, but that such changes were not always correlated to changes in corresponding protein levels [**Papers I and IV**]. This reinforces the need to monitor both RNA and protein, possibly with their activity as well, before deriving conclusions on the functionality of the stress response.

In terms of the alkylative capacity of foreign compounds, most attention has been paid to events around DNA. However, based on the dramatic alternations (mainly a disproportionately large population of suppressions) in steady-state level of mRNAs in cells exposed to the most carcinogenic compound (DBPDE), we hypothesise that RNA and their modification may function as central components of the epigenome [**Paper IV**]. This presents a number of tantalising problems and possibilities for future studies. Firstly, it may reveal that analytical methods based on conversion of mRNA templates may be artefactually disturbed in cells undergoing metabolic bioactivation of foreign compounds. Secondly, modifications of RNA represents an attractive and novel biomarker event for detection of gentoxic effect of presence/ exposure to endogenous/exogenous compounds with unknown or having suspected adverse effect(s) on function and stability of the genome/RNome/epigenome.

We have therefore focussed on experiments studying the relative propensity of RNA and DNA to sustain damage in cells undergoing oxidative stress. In Paper III we clearly show that RNA was far more sensitive in sustaining oxidative damage on guanine, than DNA, and that the repair kinetics varied considerably between these nucleic acid species, reflecting the extent of evolutionary development of repair of oxidatively damage DNA.

Due to the fact that detection of oxidised DNA/RNA in **Paper III** requires rather complicated and expensive mass spectroscopic methods, we have also attempted to develop simpler methodologies for the

differential detection/visualisation of modified RNA and DNA pools in intact cells [**Paper V**]. The data clearly shows that combinations of avidin or neutravidin staining, combined with or without RNAase or DNAase treatments, can be used to visualise oxidative modification to DNA (nuclear and mitochondrial) and RNA in cells undergoing oxidative stress.

Discussion

The contents of this thesis have been generated from the application of a diverse techniques and experimental design in order to study the cellular responses to different genotoxic compounds. In our work, we have attempted to use this multidisciplinary approach towards "systems biology" in addressing the biological consequences and detection of chemically-derived genotoxicity. A combination of molecular biological, cytochemical, biochemical and biophysical methods were variously used to study the contrasting efficiency of bulky DNA adduct formation and removal by BPDE/DBPDE [**Papers II and IV**], as well as to examine the biology of DNA-lesion recognition and repair signalling at the level of chromatin structure [**Paper VI**].

On a broader scale, and with a partial focus on revealing novel biomarkers, we have also centred our efforts on the responses of cells to oxidative and alkylating stress at the level of mRNA and protein expression [**Papers I and IV**], with parallel testing of how this response affects protein expression and cellular adaptation to genotoxic stress. In addition, in focusing our attention on uncovering a more detailed picture of the cellular targets for oxidative damage in particular, we have further developed methods for quantitative investigation of oxidised DNA and RNA [**Paper III**] and established an organelle-specific method for qualitatively detecting these events in cells *in situ* [**Paper V**].

One of the main goals of the majority of research projects [**Papers I, II and IV**] was to study the genetic response of cells to various genotoxicities using transcription profiling with microarray technology. In our hands, these methods have proven to be an excellent tool in obtaining a panoramic view of alternations in gene expression patterns at mRNA levels in cells which underwent genotoxic stress. This gives a "global view" of the extent of the stress response the cell can mount as a result of insult, which may lead to a better understanding of the particular mechanisms underlying biological adaptation to nucleic acid damage. Additionally, these efforts throw up the possibility of defining novel biomarkers, represented by individual or clusters of gene expression alterations. There is a great need to identify novel biomarkers of genotoxicity, particularly from the point of view of human exposure to carcinogens, and subsequent risk assessment. Here it is important that the biomarker event be amenable to relatively facile analysis, and should be well validated with respect to qualitative and quantitative aspects of the exposure.

Our microarray experiments have revealed a number of novel genetic stress responses, which may provide further impetus for their validation as biomarkers of human exposure to DNA damaging compounds. It now remains to be tested if some of these DETs have relevance to human biomonitoring. Therefore, it is of interest that we are involved in efforts to assess the exposure of children to DNA-damaging compounds within the Childrengenotox Network Program of the EU, where several of these candidate genes will be evaluated in a pilot epidemiological study (Teplice Program, Czech Republic). This will, of course, involve assays directed against specific mRNAs and proteins. In the first instance, however, the microarray-based screening of gene expression profiles in children from polluted region (Teplice) *versus* children from control region (Prachatice) is already undergoing and is expected to be completed by the end of June 2005 [**Kleinjans *et al.*, 2005**].

In efforts to validate biomarkers which are screened from the transcriptosome using microarrays, caution must be stressed however. Our data clearly reveal the possibility of a disconnection between genotype, rybotype and protein pool of each cell within the organism. As presented by others [**Chen *et al.*, 2002; Kwong *et al.*, 2005**], and by us [**Papers I and IV**], some DETs correlate very well with the changes in corresponding protein products. This good qualitative, and to some extent even quantitative correlation could be observed quite often. But the question arises is it generic? The answer is clearly not necessarily, as we detected exceptions, even in our rather limited pool of tested DETs. The extent of this exception will, however, depend on the combination of many factors, such as the type of insult the cell is exposed to, its intensity and its duration. Additionally, responses are usually tissue specific, affected by a "sensitivity/time window" when the exposure took place and, collectively, correlations between different levels of gene expression will be revealed as any of the following "weak, absent, reverted or good". This indicates that molecular profiling, performed with any of the –omics techniques, should be combined with other, complementary method(s) in order to elicit the very complex stress response/adaptation pathways.

The results of our microarray studies, particularly those obtained with DBPDE, as well as those observations which we have obtained using the novel approaches to compare and contrast oxidative DNA and RNA damage, reveal how important it is to realign opinions in reactive intermediate-derived genotoxicity to include events other than "primary" DNA damage. From this point of view [**Bajak, 2005**], and in agreement with "the RNA world theory" [**Gilbert, 1986**], DNA serves as a vehicle which has been/is used by the majority of living organisms or perhaps by RNA itself, to store and transport encoded information through time and space. On the other hand, it is the RNA within the epigenome which imparts to cells and organisms much of the flexibility to adapt, interact and sense dynamic changes in surrounding micro- and macro-worlds. Thanks to RNAs the diversity of RNomes are

generated and translated into amazingly mosaical phenotypes [Herbert, 2004]. It can, therefore be argued that what allows us humans, although we are still only primates, "to fly without wings, reach the stars and explore the macro- and micro-cosmos" are actually these very small but structurally/functionally diverse and biochemically very powerful RNA species! Therefore, events at the level of RNA must be more widely considered when studying mechanisms of foreign compound-induced toxicity and responses to constantly changing environments.

Having taken this rather philosophical "wide-angle" stand point, in direct relation to the work in this thesis, the classical perception of chemical carcinogenesis in response to DNA-binding should be updated with the inclusion of epigenetic considerations, centred on changes in the structure, function and distribution of RNAs. Indeed, disturbance and damage to RNA in the epigenome may have wider implications in the biological response to physical agents, viral infections, as well as being operative in fundamental biological processes controlling the life and death of the cell.

Edyta Z. Bajak

Stockholm, 2001- 2005

To be continued A.D.

…

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***

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