

Exploring co-genotoxicity of
chemicals using traditional
experimental methods and
microarray systems

by Ville Erling Sipinen

Master thesis in Toxicology
Department of Toxicology and Ecophysiology
Institute of Biology
University of Oslo

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Forord

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Summary

Due to the use of a great number of existing chemicals and many new being produced, there is a strong need for efficient and cost-effective methods, in order to classify and regulate the use and release of these potential environmental pollutants. Effects on the environment and on human health have been described for many important toxicants; there is however a great lack of studies addressing the effects of co-exposures from such harmful agents. Toxicogenomics is a scientific field under continuous improvement and represents a hallmark of future toxicological research. Toxicogenomics has a great potential in relation to classification and risk assessments of environmental contaminants.

The aim of this study was to elucidate the complex nature of co-exposures to toxicants by combining traditional toxicological tests with modern microarray technology. We investigated the effects of two well documented toxic agents; the heavy metal cadmium, and UVC irradiation (< 280 nm). Cadmium elicits many genotoxic effects, including inhibition of Nucleotide Excision Repair (NER). UVC induces DNA-lesions, mainly cis-syn cyclobutane pyrimidine dimers (CPDs) which are repaired by NER. NER plays a key role in the removal of DNA-damage caused by various toxicants. Different modes of action of cadmium and UVC make them good models for our investigations. Human testicular cancer cells (833K) were exposed to cadmium ($\text{CdCl}_2 \cdot 2,5\text{H}_2\text{O}$) and UVC radiation; relevant doses were determined by measuring cell viability, and cell-cycle responses to treatments were estimated by flow cytometric analysis. DNA-repair efficiency of cells pre-incubated with $5 \mu\text{M Cd}^{2+}$ and exposed to UVC (1 J/m^2), was assessed by measuring DNA single strand breaks (SSBs) using the alkaline elution assay. Global gene expression profiles were estimated from microarrays, whereas induction of specific proteins was measured by Western analysis. We found that cadmium treatment led to significant attenuation of DNA-repair of UVC induced lesions. Microarray analysis demonstrated differences in gene-expression profiles in response to both cadmium and UVC treatments, whereas Western analysis displayed differences in induction of specific proteins involved in DNA repair, cell cycle and apoptosis. In accordance with other studies we have found cadmium to elicit important co-genotoxic effects, especially affecting the removal of DNA damage performed by NER.

We conclude that microarrays and traditional experimental toxicology, as used in the present investigations, represent adequate means to study the combined effects of toxicants.

1. Abbreviations

8-oxo-dG	8-oxo-2'-Deoxyguanosine
β -ME	β -Mercaptoethanol
6-4PPs	Pyrimidine (6-4) Pyrimidone Photoproducts
8-oxo-dGTPase	8-oxo-2'-deoxyguanosine5'-triphosphate pyrophosphohydrolase
AP site	(Abasic) apurinic/aprimidinic site
Ape1	Mammalian AP Endonuclease 1
AraC	Cytosine Arabinoside
AraCTP	Cytosine Arabinoside Triphosphate
AS	Australian Calf Serum
BER	Base Excision Repair
BPB	Bromo Phenol Blue
BSA	Bovine Serum Albumin
PTWI	Provisional Tolerable Weekly Intake
CAT	Catalase
cDNA	Complementary DNA
CPDs	<i>cis-syn</i> Cyclobutane Pyrimidine Dimers
CSA	Cockayne Syndrome Factor A
CSB	Cockayne Syndrome Factor B
CTD	Comparative Toxicology Database
Cy3	Cyanine 3
Cy5	Cyanine 5
DDB2	UV-DNA Damage Binding Protein, subunit 2
DNA MeTase	DNA (5-cytosine) Methyltransferase
EBI	European Bioinformatics Institute
eGOn	Explore Gene Ontology
ERCC1	Excision Repair Cross Complementing Group 1
ERK1	Extracellular Signal-Regulated Kinase 1
ERK2	Extracellular Signal-Regulated Kinase 2
FEN1	Flap-structure Specific Endonuclease 1
Fpg	Formamido-Pyrimidine-DNA Glycosylase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEO	Gene Expression Omnibus
GEPAS	Gene Expression Pattern Analysis

GGR	Global Genome Repair
GO	Gene Ontology
GPx	Peroxidase
GSH	Glutathione
HAP1	Human AP-Endonuclease 1
HRP	Horseradish Peroxidase
HU	Hydroxyurea
IARC	International Agency for Research on Cancer
JNK	c-JUN N-terminal Kinase
LPR	Long-Patch Repair
MAPK	Mitogen Activated Protein Kinase
MBD	Minor DNA Binding Domain
MGMT	<i>O</i> ⁶ -Methylguanine-DNA Methyltransferase
MIAME	Minimum Information About a Microarray Experiment
MIAN	Department of Analytical Chemistry
MIKT	Department of Chemical Toxicology
MMR	Mismatch Repair
MT	Metallothionein
NER	Nucleotide Excision Repair
NMC	Norwegian Microarray Consortium
NAAC	Normalised Area Above (the elution) Curve
P/S	Penicillin & Streptomycin
PARP	Poly (ADP-ribosyl)ation
PARPs	Poly(ADP-ribose) Polymerases
PCNA	Proliferating Cell Nuclear Antigen
PI	Propidium Iodide
PKC	Protein Kinase C
Pol β	Polymerase β
RFC	Replication Factor C
RIN	RNA Integrity Number
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RT (enzyme)	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate

SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SPR	Short-Patch Repair
SSBs	DNA Single Strand Breaks
T4-pdg	(bacteriophage)T4-Pyrimidine Dimer Glycosylase
TCR	Transcription-Coupled Repair
TFIIH	Transcription Factor IIIH
TGCT	Testicular Germ Cell Tumour
TTD	Trichothiodystrophy
UV-DDB	UV-DNA Damage Binding Protein
XP, A-G	Xeroderma Pigmentosum -Complementation group A-G
XRCC1	X-ray Cross Complementing Protein 1

*In this study genes are denoted by *italic* names.

2. Introduction

2.1. Environmental exposure

Already from the moment of conception we are continually exposed to a plethora of hazardous chemical and physical agents that can be deleterious to our health. These include food contaminants, ionising radiation, UV-sunlight, industrial and municipal wastes, toxic ion metals, emissions from fossil fuel combustions and refuse incinerations, pesticides and thousands of other manmade chemicals. There is an ever ongoing elaborate research worldwide to map and regulate the use and release of potentially harmful agents, yet their effects on the environment and on human health have only been described for relatively few pollutants. Furthermore, there is a great shortage of information addressing the combined effects of toxicants. Consequently, due to the very large number of chemicals, there is a great need for reliable and time-efficient methods to assess environmental and human health risks. Toxicogenomics is a rapidly growing scientific discipline which is very promising and specifically designed to address these problems.

2.2. Aim of this study

We wished to investigate the effects of co-exposures to environmental pollutants using a toxicogenomic approach, by merging classic *in vitro* test methods with modern microarray technology. In order to understand the complexity behind combined effects of toxicants, it is necessary to first establish a platform from where to commence. We therefore chose to investigate two appropriate model agents, for which the biological effects are well documented. Our choices fell on the ubiquitous environmental pollutant cadmium, and UVC (<280nm) irradiation. Cadmium is a “modern time” contaminant of considerable importance to human health and causing environmental concern. Some studies have shown that cadmium interferes with DNA repair mechanisms, including Nucleotide Excision Repair (NER), and was therefore chosen as a suitable candidate for our study (Fatur *et al.*, 2003). UVC exposure is not relevant from a public health point of view, since essentially all of it is absorbed by atmospheric ozone. However, we chose UVC because of its ability to induce well-defined DNA lesions; the most frequent being cis-syn cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidone photoproducts (6-PPs), reviewed by (Pfeifer *et al.*, 2005). In human cells the UVC-induced lesions are mainly handled by NER, which is also extensively

involved in the repair of a great number of different DNA insults caused by various environmental pollutants.

2.3. The principles of toxicogenomics

Toxicogenomics seeks to understand how the genome is involved in responses to environmental stressors and toxicants. Toxicogenomics combines studies of genetics, mRNA expression, cell and tissue-wide protein expression, and metabonomics to understand the role of gene-environment interactions in disease. Fundamental to toxicogenomics research is the use of technologies related to transcriptional profiling, e.g. microarrays. Due to the vast amount of biological information being generated in this field, the development and application of bioinformatic tools and databases are crucial in order to facilitate the mining, analysis, visualisation and sharing of data. This rapidly growing research area is apt to have a large impact on toxicological studies in terms of analysis and classification of chemicals, risk assessments, and will undoubtedly help unravel questions as to how components of biological systems work together and how different organisms respond to specific stresses, drugs, or toxicants.

2.4. The basics of microarray analysis

Microarray technology provides a powerful tool that is essential to the field of toxicogenomics. DNA or oligonucleotide microarrays are used to simultaneously assess the transcriptional states of tens of thousands of genes in response to treatment. For instance when an organism or cell is exposed to a foreign toxic chemical or other detrimental insults, gene expression patterns get altered, e.g. by elevated transcription of protective enzymes or cell-cycle regulators. The change in mRNA levels of affected genes is probably the first detectable response, and those changes can be discovered by microarrays.

Briefly described, thousands of short DNA segments, called probes, are fixed on a surface (glass or nylon) and constitute a microarray, each of these segments of DNA representing a single gene. Isolated mRNA from treated cells or tissues is used to make complementary DNA (cDNA) using reverse transcription (in cases with very little RNA, the cDNA may be amplified by a polymerase chain reaction (PCR)). The cDNA, called the target, is tagged with a fluorochrome and hybridised onto the probes on the microarray. Likewise a cDNA target is prepared from a control sample (usually untreated cells of the same type). If only one fluorochrome is used, the control and experimental samples are hybridised onto separate microarrays. If two fluorochromes are used, the samples are hybridised onto a so-

called two-colour microarray. Only the latter approach is used and will be described in this thesis. Following hybridisation the fluorochromes are excited by laser simultaneously as the array is scanned. Intensity ratios of emitted light from the fluorochromes are calculated by computer programmes and represent the amount of control and experimental target that has hybridised to each probe on the microarray. Through the use of specially designed software for microarray analysis, each target-to-probe hybridisation (spot) is examined, discarding spots of poor quality. Statistical tools are used to normalise the remaining data in order to exclude technical variance. Intensities from the spots are transformed into numeric values, which are used to classify genes of the treated sample as either up or down-regulated, compared to the control. Further processing usually includes sorting the genes into clusters, which is a reasonable approach; for one, genes displaying similar expression patterns tend to be co-regulated and second, co-regulated genes may be involved in the same metabolic pathways. Two methods of gene clustering extensively used in toxicogenomics are;

- Hierarchical clustering: Total gene expression profiles from different or parallel experiments are compared to each other and related in terms of similarity. The design of hierarchical clustering is much like a family tree; clusters divided by short branches share many of the same properties in response to treatment, while those further apart have less in common.

- K means clustering: Genes are sorted into clusters on account of similarity, but the number of clusters is already determined in advance. Each expressed gene is assigned to one cluster, and then reassigned to the next. The method “forces” each gene into the closest related cluster, minimising the differences within each cluster, while at the same time maximising differences between the cluster groups. After clustering, the data can be more thoroughly investigated and used to create individual or overall gene-expression profiles for the treatment in question.

The transcriptional profiles elicited by microarrays show great promise in identification and characterisation of hazardous chemicals. The profiles obtained for known toxicants can be used to prioritise further investigation of those compounds that show similar expression patterns. The concept is based on the assumption that chemicals that exert similar effects also invoke similar transcriptional responses.

2.4.1. Toxicogenomic databases

Two of the greatest challenges facing toxicogenomics are; how to share the enormous amount of data created by microarray experiments, and how different data sets can be related to one and other across laboratories (Hayes *et al.*, 2005). Several web accessible databases have been created in order to facilitate the processing and distribution of microarray results. There are however many different protocols and platforms available to the scientific community, hence a standardised technical report on how the data is obtained is crucial in order to interpret and compare related conclusions from other studies. To overcome this problem, the Microarray Gene Expression Data Society proposed the “Minimum Information About a Microarray Experiment” (MIAME) -guidelines, for reporting and publication of microarray experiments. Most journals today require that papers containing microarray experiments follow the MIAME guidelines. Standardisation has helped reducing variation across laboratories, but the work is difficult. The matter is further complicated by different nomenclatures used across laboratories to annotate targets, a result of differing informatics tools. But the situation is improving, and several open-access genomic databases with relevance to toxicology are now available providing toxicologists with resources for improved interpretation of profiling experiments. The largest databases in use today are Gene Expression Omnibus (GEO), at the National Centre for Biotechnology Information (<http://cebs.niehs.nih.gov/microarray/index.jsp>), and Array Express (<http://www.ebi.ac.uk/arrayexpress/>) both are adherent to MIAME guidelines. GEO houses over 18000 microarray experiments from a large number of biological fields, however only a small fraction of the database contains data from toxicological experiments. Array Express is based at the European Bioinformatics Institute (EBI) and contains data from over 5000 microarray hybridisations. Array Express has developed a subsystem called Tox-MIAME express, which works by incorporating descriptive data about toxicological experiments, e.g. dose used, route of administration, duration of treatment etc. There are also numerous databases under development with direct application to toxicogenomics such as the dbZach database and the Comparative Toxicology Database (CTD). dbZach for instance will house microarray data and provide analysis tools with particular emphasis on endocrine disruption and testicular toxicity, whereas CTD will focus on associations between toxic agents and biological systems. Eventually all such databases will be linked providing toxicologists with a rich online resource, facilitating the understanding of chemical actions, classification of hazardous compounds, and ultimately also (of course) risk assessment.

2.5. Cadmium

Cadmium is a relatively rare element (0.2 mg/kg in the earth's crust); it is not found in pure state in nature and is almost always recovered as a by-product from the processing of sulphide ores of zinc, lead and copper (WHO, 2000). Little cadmium has been produced prior to the early 1920s and over 65 % of the cumulative world production has taken place in the last few decades.

2.5.1. Use

Cadmium is used in a wide variety of consumer and industrial materials, the main applications falling into five categories: 1) protective plating on steel, 2) stabilisers for polyvinyl chloride (PVC), 3) pigments in plastics and glasses, 4) electrode material in nickel-cadmium batteries, and 5) as a compound in various alloys (IPCS, 1992; WHO, 2000; IARC, 1993).

2.5.2. Environmental exposure

Atmospheric emissions of cadmium from anthropogenic sources exceed those of natural origin (volcanoes and forest fires) by an order of magnitude (IARC, 1993). Sources of manmade emissions to the atmosphere include mining for zinc and cadmium, copper and lead, fossil fuel combustion, iron and steel production, sewage sludge and waste incineration, phosphate fertiliser and cement manufacture. At the global level the smelting of nonferrous metal ores has been estimated to be the largest human source of cadmium released into the aquatic environment through mine drainage water, wastewater and rainwater run-off from mine areas. Other human sources are spent solutions from plating operations and phosphate fertilisers, solid-waste deposits and wastewater of both municipal and industrial origin. With increasing acidification of soil due to acid rain and the use of fertilisers, increased uptake of cadmium from soil may occur. Plants may be contaminated by cadmium through two routes: soil-plant transfer, due to absorption of mobile forms of cadmium through the roots, or by air-plant transfer, due to deposition of soluble forms of cadmium particles on the epigleal parts of the plant. Figure 2.1 shows the percentage contribution of cadmium discharge to the environment from different sources in Norway 2002 (www.sft.no/2005).

Contribution of cadmium to the environment from different sources
Norway 2002 (Total of 2 tonnes)

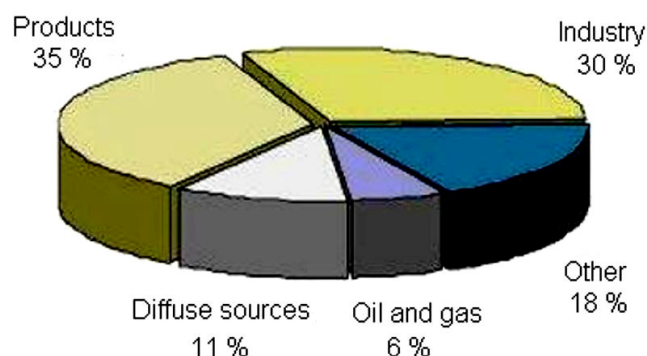


Figure 2.1 The Norwegian national emission of cadmium into the environment has been strongly reduced during 20 years. In 1985 a total of ~ 43 tonnes of cadmium was released into the environment compared to 2 tonnes in 2002. Atmospheric deposition of cadmium however is far greater than the national input. Data from Norwegian Pollution Control Authority (SFT) 2005

2.5.3. Human exposure

2.5.3.1. Exposure through air

Most of the cadmium found in air is associated with particular matter in the respirable range (diameter 0.1-1 μm) (WHO, 2000). Cadmium is emitted to the atmosphere predominantly as elemental cadmium and cadmium oxide, but also as cadmium sulphide from coal combustion and nonferrous metal production or as cadmium chloride from waste incineration. The relative deposition of inhaled cadmium in the lungs varies between 10-50 % depending on the size of the airborne particles, and the absorption of cadmium depends on the chemical nature of the particles deposited. Absorption of cadmium oxide is about 50 % but considerably less for insoluble salts like cadmium sulphide.

2.5.3.2. Occupational exposure

The major occupational exposures occur in smelting and refining of zinc, lead and copper ores, electroplating, manufacture of cadmium alloys and of pigments and plastic stabilisers, production of nickel-cadmium batteries and welding. Other exposed occupations include paint production and use, pesticide production and use, phosphorus production, textile printing, glasswork, laser cutting and more (IPCS, 1992; IARC, 1993).

2.5.3.3. Smoking

Tobacco plants naturally accumulate relatively high concentrations of cadmium in the leaves (Waalkes, 2003; IARC, 1993). The cadmium content of cigarette tobacco is generally 1-2 µg per cigarette, so a person who smokes 20 cigarettes per day has an estimated daily intake of 2-4 µg and accumulates 0.5 mg cadmium in one year. Smoking is thought to double the life time body burden of cadmium in non-occupationally exposed persons.

2.5.3.4. Food

For non-smokers and non-occupationally exposed people, food constitutes the principal environmental source of cadmium (WHO, 2000). The lowest concentrations are found in milk (around 1 µg/kg). The concentration of cadmium is in the range 1-50 µg/kg in meat, fish and fruit and 10-300 µg/kg in staple foods such as wheat, rice and potatoes. Highest cadmium levels (100-1000 µg/kg) are found in the internal organs (kidney and liver) of mammals and in certain species of mussels, scallops and oysters. Some crops, such as rice, can accumulate considerable amounts of cadmium (more than 1000 µg/kg). The average daily intake of cadmium via food in European countries and North America is 15-25 µg but there may be large variations depending on age and dietary habits. The gastrointestinal absorption of cadmium in humans amounts to about 5 % but may be increased by nutritional factors (up to 15 % in iron deficiency). The average amount of cadmium absorbed via food can thus be estimated at 1 µg/day. The World Health Organisation (WHO) has established a provisional tolerable weekly intake (PTWI) for cadmium of 7µg/kg body weight (FAO/WHO, 2003). This illustrates a low margin of safety and that even a minor increase in cadmium intake may lead to significant health problems.

2.5.4. Toxicokinetics

The main metabolic feature of cadmium is an exceptionally long biological half-life resulting in a virtually irreversible accumulation of the metal in the body throughout life (WHO, 2000). The two main storage sites for cadmium in the body are the liver and the kidney. For low-level exposures such as those occurring in the general environment, about 30-50 % of the cadmium body burden is stored in the kidneys alone, with concentrations in the renal cortex about 1.25 times higher than in the kidney as a whole. In non-occupationally exposed subjects the concentration of cadmium in the liver increases continuously with age. The concentration of cadmium also increases in the renal cortex but only until the age of 50-60 years, after which it levels off or even decreases. In Europe, mean concentrations of cadmium in the renal cortex in the age group 40-60 years are in the range 15-50 mg/kg.

Concentrations are usually 50-100 % higher in current or ex-smokers than in non-smokers. In industrial workers the concentrations can be considerably higher. In the tissues, cadmium is mainly bound to metallothioneine (MT), a low-molecular-weight protein (MW 6.6 kD) rich in cysteine residues. Metallothioneine is involved in the transport of cadmium from the liver to the kidney, the cadmium-metallothioneine complex released from the liver being rapidly filtered through the glomeruli and then reabsorbed by the tubules. Cadmium is eliminated mainly via urine, however the amount excreted is very small; it represents only about 0.005-0.01 % of the total body burden. Consequently the biological half-life for cadmium is 20-40 years.

2.5.5. Toxicological effects

Acute toxic effects of short term exposures to high levels of inhaled cadmium fume include chemical pneumonitis with pulmonary oedema, which may be lethal (IPCS, 1992;WHO, 2000). High levels of ingested soluble cadmium salts cause nausea, stomach-ache and acute gastroenteritis. Effects of long-term occupational exposure to cadmium include respiratory illnesses such as chronic obstructive lung disease, bronchitis and emphysema. The kidney is the critical organ in both long-term occupational and environmental exposure. The accumulation of cadmium in the renal cortex leads to renal tubular dysfunction with impaired re-absorption of e.g. amino acids, glucose and proteins. Exposure to cadmium has been linked to a number of adverse health effects in a variety of tissues and organs (reviewed in (Waisberg *et al.*, 2003); (Satarug *et al.*, 2003); (Waalkes, 2003). These include irreversible renal tubular injuries, eosinophilia, hypertension, osteoporosis, anaemia and cancer in several organs such as the lung, kidney, urinary bladder, pancreas, breast and prostate.

2.6. Carcinogenic effects of cadmium

In 1993, the International Agency for Research on Cancer (IARC) classified cadmium and cadmium compounds as group 1 human carcinogens. (IARC, 1993). The classification was based primarily on cadmium characteristics as a lung carcinogen. The carcinogenesis of cadmium is not yet fully understood but has been linked to several properties; these include inhibition of DNA repair mechanisms, elevated levels of reactive oxygen species, induction of oncogens, inhibition of tumour-suppressor genes, disruption of cell adhesion, and selection for apoptosis resistant cells (Waisberg *et al.*, 2003). Molecular effects of cadmium are summarised in Figure 2.2. In the present study we have primarily focused on aspects concerning DNA repair.

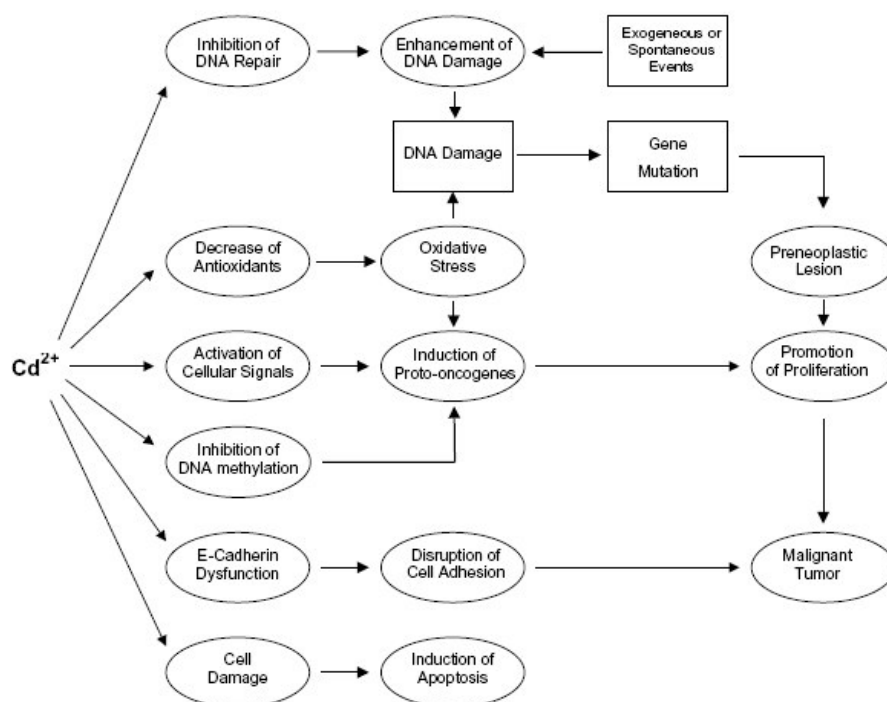


Figure 2.2 The figure displays the numerous ways in which cadmium is thought to elicit its genotoxicity (Waisberg et al., 2003).

2.6.1. DNA repair

The human genome is built up by 3 billion nucleic acid base-pairs encoding for some 20 000 – 25 000 genes (Human Genome Project). It consists of large double helix molecules which in non-dividing cells are supercoiled and tightly packed into chromosomes residing within the nucleus. The DNA is accessed by a wide range of different proteins which act in concert to relax the chromosome structure, unwind DNA strands, and initiate transcription of genes required for cell maintenance, tissue functions or cell proliferation. Due to its chemical properties and size the DNA is susceptible to a great many mechanical and chemical stresses both by endogenous processes as well as by attacks from a large number of exogenous DNA damaging agents. Endogenous (by)products include reactive oxygen species (ROS) like superoxide anions, hydroxyl radicals and hydrogen peroxide, derived from normal oxidative respiration and lipid peroxidation. The consequences of allowing DNA damage go unchecked is all evident in cancer development, which ultimately is caused by mutations in critical genes like oncogenes or tumour-suppressor genes resulting in faulty DNA replication and uncontrolled cell proliferation. To overcome the otherwise deleterious effects of DNA damage, evolution has provided us with several highly efficient DNA repair mechanisms.

There are four main mechanisms: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and recombination repair which constitutes homologous repair (HR) and end joining (EJ) (Hoeijmakers, 2001b; Hoeijmakers, 2001a; Fleck *et al.*, 2004). In this study we have focused predominately on NER, but also on BER since cadmium is involved in ROS generation.

2.6.2. Nucleotide excision repair (NER)

Of all the repair mechanisms NER is the most versatile when it comes to recognition of DNA lesions, and it removes a wide variety of DNA damage including photoproducts induced by UV and (other) bulky adducts caused by exogenous agents e.g. polycyclic aromatic hydrocarbons (PAHs) (Fleck *et al.*, 2004; Hoeijmakers, 2001b; Olsen *et al.*, 2005). At least three human syndromes are associated with aberrant NER: 1) Xeroderma pigmentosum (XP) caused by mutations in one of seven genes (*XP*-complementation group *A-G*), 2) Cockayne syndrome (CS) caused by mutations in the *CSA* or *CSB* genes, and 3) Trichothiodystrophy (TTD). All three syndromes are characterised by extreme sensitivity to sunlight. NER is divided into two sub-pathways (Figure 2.3): ●global genome repair (GGR), which removes damage in the genome overall, and ●transcription-coupled repair (TCR), which especially repairs the transcribed strand of active genes. The main difference between GGR and TCR is the recruitment of different factors during the initial recognition of DNA damage. A UV-DNA damage binding protein (UV-DDB consisting of DDB1 and DDB2), and XPC are involved in the recognition step of GGR, while TCR is thought to be recruited by RNA polymerase II stalled at a lesion. The stalled polymerase needs to be displaced to make the lesion accessible for repair, and this requires at least two TCR-specific factors, *CSA* and *CSB*. The proteins acting further downstream in GGR and TCR are likely to be identical. First a complex consisting of nine subunits called transcription factor IIIH (TFIIH) is recruited to the damage site, where after the initial recognition factors are probably released from the damaged DNA. Two helicases which are subunits of TFIIH; *XPB* and *XPD*, unwind ~30 base-pairs around the lesion through helicase activity. Next the factors *XPG*, *XPA* and *RPA* (replication protein A) bind to the damaged site. *XPA-RPA* verifies whether the NER complex is correctly assembled and ensures proper incision of the damaged DNA strand. *XPG* and *XPF-ERCC1*, which are endonucleases, perform a dual incision; they cut 3' and 5' to the damage, respectively. The damage is released in a 24-32 nucleotide long oligonucleotide. Subsequently the resulting gap is filled by DNA polymerase and ligated. 25 proteins or more are thought participate in NER.

Nucleotide excision repair (NER)

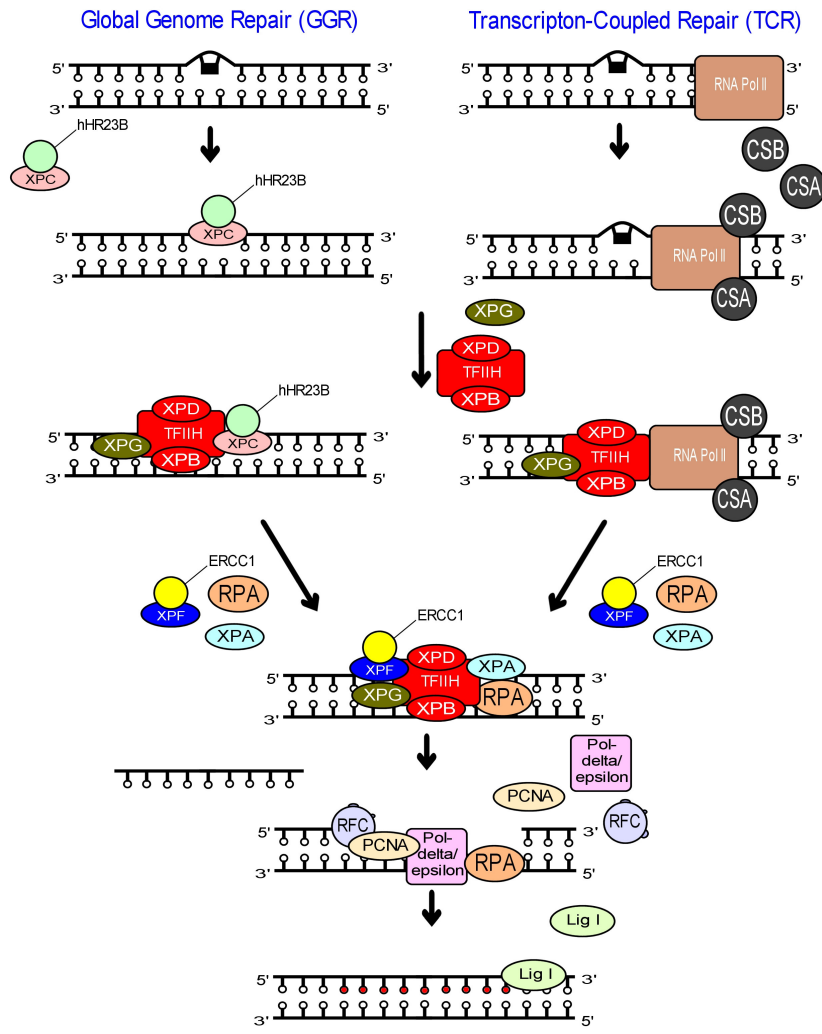


Figure 2.3 The Nucleotide Excision Repair (NER) pathway. See text for description of the pathway. Abbreviations: XPA-G, xeroderma pigmentosum complementing group A-G; RNA Pol II, RNA polymerase II; CSA and CSB, Cockayne syndrome factors A and B; TFIIH, general transcription factor IIIH; ERCC1, excision repair cross complementing group 1; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; Pol δ/ϵ , DNA polymerase delta/epsilon; Lig I, ligase 1 (Olsen *et al.*, 2005).

2.6.3. Base excision repair (BER)

BER mainly repairs non-bulky DNA lesions produced by alkylation, oxidation or deamination of bases, caused by either endogenous processes or exogenous DNA damaging agents (Hoeijmakers, 2001b; Fleck *et al.*, 2004; Olsen *et al.*, 2005). BER is divided into two pathways (Figure 2.4); short-patch repair (SPR) which is the main pathway in mammals, and long-patch repair (LPR). Cells have several DNA glycosylases, each with high specificity to DNA substrates, which recognise and remove damaged or modified bases. The glycosylases remove the damaged base by cleaving the N-glycosylic bond to the DNA sugar-phosphate

backbone, leaving an (abasic) apurinic/aprimidinic site (AP site). The AP site can also occur spontaneously by hydrolysis and represent damage itself. There are two main types of DNA glycosylases; mono and bi-functional. Bifunctional glycosylases cleave the sugar-phosphate backbone 3' to the AP site through an associated AP-lyase activity, which leaves a single strand break (SSB). The resulting abasic sugar residue is removed by either an AP-endonuclease or by DNA polymerase β (Pol β). The one-nucleotide gap is then filled by polymerase β and ligated. The processing of AP sites created by monofunctional glycosylases on the other hand, first require a 5' incision by an AP-endonuclease, which is usually preformed by human AP-endonuclease 1 (HAP1). After this incision polymerase β inserts the missing nucleotide and removes the 5' moiety through its deoxyribophosphodiesterase (dRPase) activity. The remaining nick is sealed by a complex consisting of DNA ligase III, and X-ray cross complementing protein 1 (XRCC1). The second pathway, LPR is probably required for AP sites where the 5' moiety cannot be removed by dRPase activity. LPR involves the DNA polymerases β and δ or ϵ , which together with proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) create a flap-structure of 2-8 nucleotides that include the AP site. The flap structure is then removed by flap-structure specific endonuclease 1 (FEN1). DNA ligase 1 then seals the gap.

Base excision repair (BER)

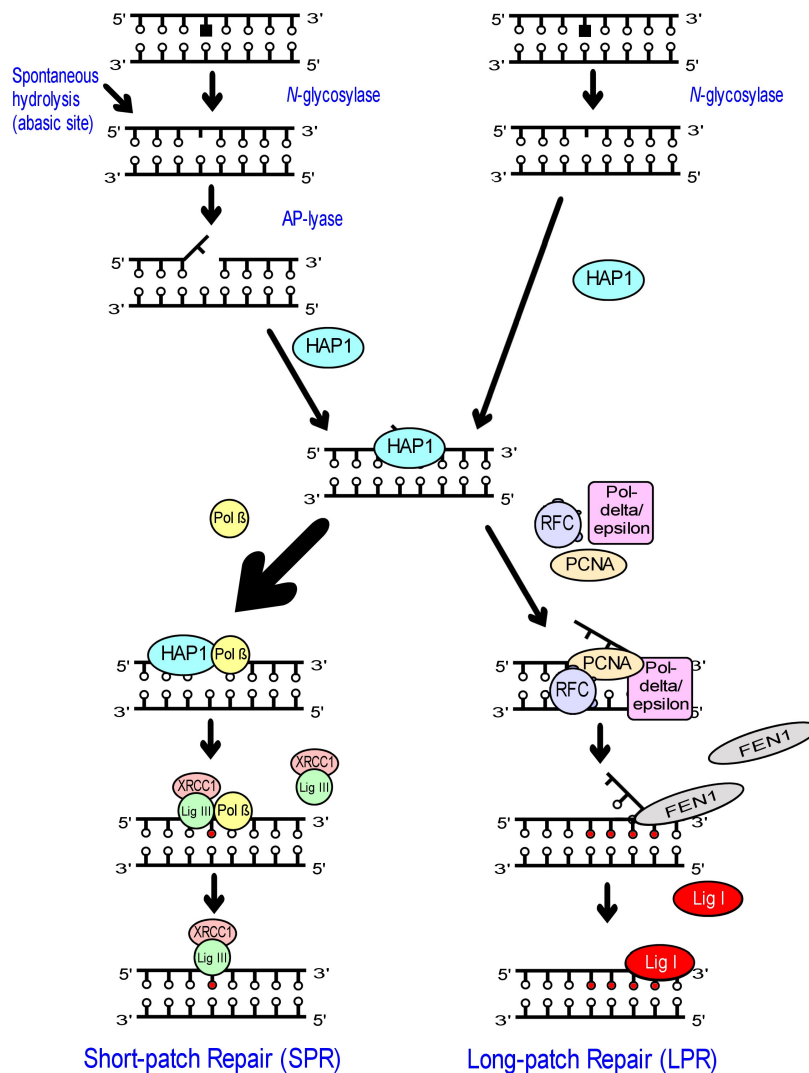


Figure 2.4 The Base Excision Repair (BER) pathway. See text for description of the pathway. Abbreviations: HAP1, human AP endonuclease I; Pol β , DNA polymerase β ; XRCC1, X-ray cross complementing protein 1; Lig III, DNA ligase III; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; Pol δ/ϵ , DNA polymerase delta/epsilon; FEN1, Flap endonuclease; Lig I, DNA ligase I (Olsen *et al.*, 2005).

2.6.4. Cadmium Inhibition of DNA repair

Cadmium is not able to catalyse the Fenton reaction and create oxygen radicals in its own right, but several studies have shown cadmium to cause oxidative DNA damage in a dose-dependent manner (Atesi *et al.*, 2004; Stohs *et al.*, 1995). Cadmium tends to reduce cellular content of glutathione (GSH), an important detoxifier of cadmium, and in general depresses levels of antioxidants and protective enzymes like superoxide dismutase (SOD), peroxidase (GPx) and catalase (CAT), resulting in an indirect increase of oxidative stress in cells (Uchida

et al., 2004; Casalino *et al.*, 2002). A study by Bialkowski and co-workers (Bialkowski *et al.*, 1999) showed that cadmium treatment resulted in a loss of up to 50 % in activity of the enzyme 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) in male rats given a single sub coetaneous injection of Cd(II) acetate (20 $\mu\text{mol/kg}$ body wt). 8-Oxo-dGTPase normally prevents DNA incorporation of premutagenic 8-oxo-2'-deoxyguanosine (8-oxo-dG) derived from a deoxynucleotide pool, damaged by endogenous oxidants. Cadmium is also thought to replace metal ions, e.g. copper and iron from metal binding proteins, (Fpg) and the mammalian XPA protein (Hartwig *et al.*, 2002a). Fpg is a glycosylase that initiates base excision repair in *Escherichia coli*, by recognising and removing some oxidative DNA base modifications including 8-oxoG. DNA binding by Fpg is mediated by a single zinc finger domain, where zinc is complexed by four cysteins. Asmuss and co-workers (Asmuss *et al.*, 2000a; Asmuss *et al.*, 2000b), showed that cadmium inhibited Fpg in a dose-dependent manner, and that simultaneous treatment with zinc partly counteracted the inhibition. Also XPA contains a four-cysteine single zinc-finger motif which is a part of a minimal DNA-binding domain (MBD), and as for Fpg, cadmium strongly reduces the binding of XPA to DNA, or to other NER proteins. As a consequence DNA repair by NER is weakened. Inhibition of XPA by cadmium is counteracted by simultaneous treatment with zinc.

Taken together, cadmium is a weak mutagenic in itself but may act synergistically when cells or organisms are co-exposed to other genotoxic agents. This is a central hypothesis studied in the present work

2.6.5. Induction of apoptosis

There are several reasons for a cell to commit suicide, via the process known as apoptosis. A signal that can lead to apoptosis is DNA damage provoked by toxicants, when such lesions are too extensive for recovery by DNA repair mechanisms. In this study we have examined the induction levels of a few proteins involved in apoptosis, three of them; p53, p38 and ERK, and some of their functions are described in this section.

One of the best known pro-apoptotic proteins is p53, which is an important tumour suppressor (Soussi, 2005). p53 function is very often modified in cancers. Exposure of rat primary epithelial lung cells (Type 2 and Clara cells) to low levels (1-10 μM) of cadmium has been shown to promote apoptosis by inducing p53 and BAX, also a mediator of apoptosis (Lag *et al.*, 2002). Further more, the induction of Apoptosis was seen at very low ROS levels invariable to antioxidant treatment of the cells, indicating that other than oxidative pathways

were involved. Another study by Låg and co-workers (Lag *et al.*, 2005), demonstrated that cadmium increased the activity of several proteins such as mitogen activated protein kinases (MAPKs); extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2), c-jun N-terminal kinase (JNK), protein kinase C (PKC), and p38, in the same rat primary epithelial lung cells. It was found that increased levels of p38 induced apoptosis, and some isoforms of PKC were probably involved in the promotion of p38 activity. The activation of ERK1 and 2, and JNK on the other hand seemed not to be involved in the apoptotic pathway. These findings coincide with a study (Chao *et al.*, 2001) which demonstrated that cadmium persistence activated ERK and p38 MAPKs in human lung adenocarcinoma cells (CL3 cell line). This was observed particularly in the G2/M phase where cadmium induced the highest levels of intracellular peroxide and genotoxicity. The activation of ERK and p38 by cadmium was associated with increased levels of ROS rather than cadmium accumulation. The authors concluded that ERK potentially plays a role in guarding genome integrity, while p38 may trigger genome instability and apoptosis in cadmium-treated cells.

2.6.6. Induction of oncogens

Cadmium has been shown to interact with various oncogens. The signal transducers c-jun, c-myc and c-fos are important mediators of cell growth and proliferation, and are linked to tumour promotion. The genes encoding them are so called oncogens. Low levels of cadmium have been shown to induce *c-jun*, *c-myc* and *c-fos* (Jin *et al.*, 1990; Joseph *et al.*, 2001). Joseph and co-workers (Joseph *et al.*, 2001) showed that cadmium-induced transcriptional activation of the oncogens was linked to elevated intracellular Ca^{2+} and ROS levels. Furthermore, activation of *c-jun* and *c-fos* (but not *c-myc*) was dependent on PKC and MAPK pathways in both tumour cells (transformed BALB/c-3T3 cells) and untransformed cells (BALB/c-3T3). Similarly, a study conducted on immortalised but untransformed human prostate cells (RWPE-1 cells) showed an initial rapid increase in mRNA levels for *c-myc*, *c-jun* and *p53*, in response to treatment with 10 μM cadmium (Achanzar *et al.*, 2000). While *c-myc* and *p53* mRNA levels receded after few hours, *c-jun* levels increased up to 20-fold during 24 hours. After 48 hours a substantial increase in apoptosis was seen, but 35 % of the cells were viable and appeared normal. The surviving cells had a 2.5-fold increase in cellular metallothionein content. From their results the authors proposed that cadmium in this way could promote the survival of tumour-prone cells.

2.6.7. Disruption of cell adhesion

Cadmium can disrupt the tight junctions between epithelial cells by interfering with the normal function of E-cadherin, a Ca^{2+} -dependent cell adhesion molecule that plays a key role in epithelial cell-cell adhesion (Prozialeck *et al.*, 1991; Prozialeck *et al.*, 1997). This is thought to occur via displacement of Ca^{2+} ions in the extra-cellular domains of E-cadherin, which leads to a distortion of the protein and loss of adhesive binding to E-cadherin of a neighbouring cell. In a review written by Pearson and Prozialeck (Pearson *et al.*, 2001), it is suggested that the disruption of E-cadherin plays an essential role in both the initiation of cancer by cadmium as well as in tumour promoting effects of cadmium. The assumption is that the disruption of E-cadherin could lead to increased transcription of oncogens mediated by β -catenin, and that existing cancer cells might more easily detach and invade neighbouring tissues (Figure 2.5).

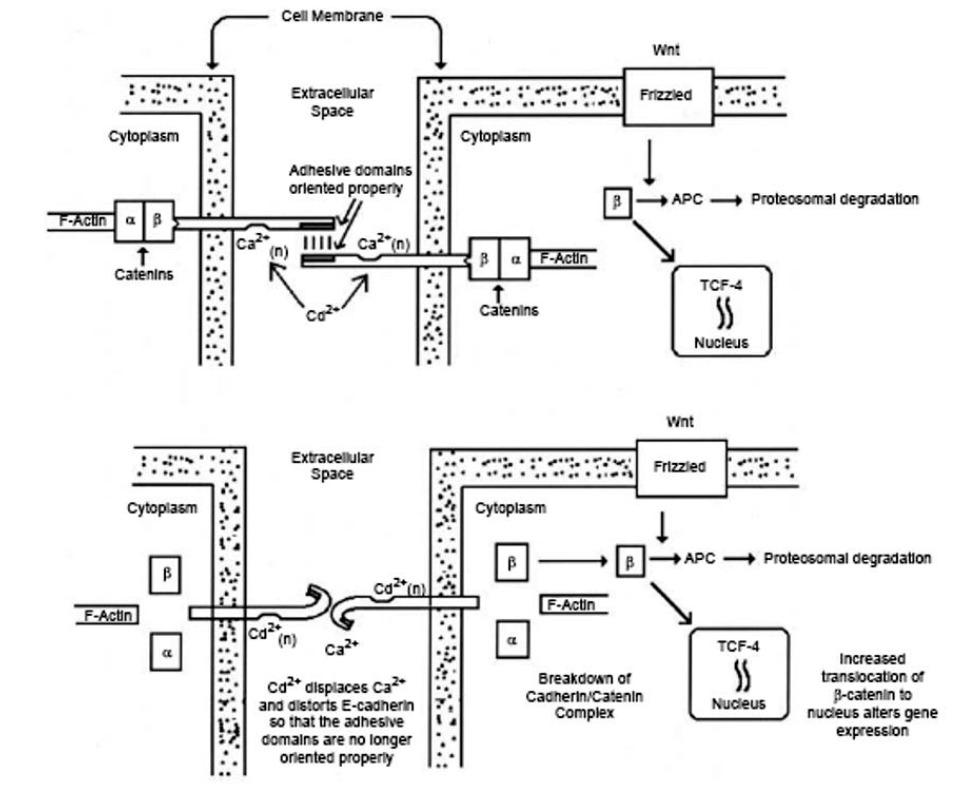


Figure 2.5 The figure shows how Cd^{2+} is thought to displace Ca^{2+} in the extra cellular domain of E-cadherin, severing the connection between cells. The disruption of E-cadherin is also thought to alter gene expression due to translocation of β -catenin to the nucleus, indicating a possible mechanism of cadmium activation of oncogens (Pearson *et al.*, 2001).

2.6.8. Effects of cadmium on DNA methylation

Cadmium also interferes with methylation. Methylation of DNA is a vital cellular function related to genomic imprinting and regulation of gene transcription, and constitutes an important field in cancer research (reviewed by Worm *et al.*, 2002). Generally DNA methylation causes gene silencing. Sustained methylation (hypermethylation) of particular DNA sequences, e.g. promoter regions of tumour-suppressor genes, can potentially enhance the risk of tumour development due to loss of protein production. Similarly, continuous repression of DNA methylation (hypomethylation) can result in elevated transcription of oncogens, thereby enhancing cancer risk. Both types of defects in DNA methylation have been linked to carcinogenesis. In a study by Takiguchi and co-workers (Takiguchi *et al.*, 2003) performed on rat liver cells, it was shown that cadmium inhibited the enzyme DNA (5-cytosine) methyltransferase (DNA MeTase), which methylates cytosine residues. In mammals the methylation of cytosine residues is the predominant post-replication base modification. It was also found that even if cadmium initially inhibited DNA MeTase, prolonged exposure (10 weeks) to cadmium led to an increased activity of the enzyme, resulting in hypermethylation of DNA. The rat liver cells also showed signs of transformation in response to continuous cadmium treatment.

3. Materials and methods

3.1. Cell culture and treatments

3.1.1. Cell culture

The Human Testicular Germ Cell Tumour (TGCT) cell line 833K, is derived from the embryonic carcinoma component of a mixed histology testicular germ cell tumour metastasis (metastasised, nonseminomatous germ cell tumour) (Bronson *et al.*, 1980). We used 833K as a model cell line in our study. The cells are a generous gift from John Master.

The 833K cells were routinely maintained in RPMI 1640 medium (with L-glutamine and phenol red), supplemented with 10 % heat-inactivated Australian calf serum (AS) and 1 % Penicillin & Streptomycin (P/S). The cells were grown to confluence (70-80 %) in 162 cm² Costar cell culture flasks (Corning Inc.), at 37 °C with 5 % CO₂ in air under saturated humidity. Cells were passaged using trypsin-EDTA every 3 or 4 days. Passages over 20 were discarded and replaced by cells from frozen stock (passage 7) stored in liquid nitrogen.

3.2. Cytotoxicity assays

3.2.1. Determination of cadmium concentration

We wanted determine the concentration of Cd^{2+} ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) that would trigger cellular responses but had little effect on cell viability (Table 3.1). Viability was determined by staining the cells with the fluorophores Propidium Iodide (PI) and Hoechst 33342, and then examining the cells using a fluorescence microscope. PI intercalates between the bases of nucleic acids (RNA as well as DNA) and may be excited either by UV or blue light giving red fluorescence. Hoechst 33342 binds preferentially to AT-rich regions in the small groove of DNA and fluoresces blue when excited by UV light. Unlike Hoechst 33342, PI is not able to pass through intact plasma membranes. So whilst Hoechst 33342 colours all nuclei blue, PI will only enter cells which have a disrupted plasma membrane (necrotic and late apoptotic cells) and will stain the nuclei red. Early apoptotic cells have intact plasma membranes and thus do not allow PI to enter the cells. However apoptotic cells are recognised by distinct morphologies (e.g. condensation of cellular compartments, small and spherical apoptotic bodies). The cells were divided into three different categories: 1. Viable, 2. Necrotic, 3. Apoptotic.

Table 3.1 Cell culture treatments.

Treatment	$[\text{Cd}^{2+}] \mu\text{M}$	Incubation (h)
1(Control)	0	20
2	2.5	20
3	5	20
4	10	20
5	30	20
6	50	20
7	200	20

Procedure

1. Medium with cadmium was prepared by adding freshly made cadmium stock solution to medium that contained calf serum.

2. 833K cells were plated onto Costar cell culture dishes (28 cm²), 5×10⁵ cells per dish in 2 ml medium (sub-confluent cell density), and incubated for 4 h to allow cells to attach.
3. After incubation the medium was removed and new medium that contained different concentrations of cadmium was applied to the samples.
4. The cells were incubated for 20 h under standard conditions.
5. Medium from each sample was transferred to 15 ml Falcon tubes, and the cells were washed twice with 2 ml Ca²⁺ and Mg²⁺ -free PBS, and detached with 200 µl trypsin-EDTA. The cells were then added to the medium.
6. Cell suspensions were briefly centrifuged at 280 x g for 5 min, and the supernate was discarded.
7. Cells were re-suspended in 1 ml fresh medium.
8. Half (500 µl) of each sample was fixed in an equal volume of 0.2 % para-formaldehyde for flow cytometric analysis.
9. The remainder of the samples were given 5 µl of PI (0.5 mg/ml), 5 µl of Hoechst 33342 (1 mg/ml) and 5 µl of Bovine Serum albumin (BSA) (BSA reduces clumping of the cells).
10. The samples were re-suspended and incubated for 10 min before they were examined using a fluorescence microscope.

3.2.2. Effects of UVC, alone or in combination with cadmium, on cell viability

We wanted to find a UVC dose that would induce cellular responses (such as DNA single-strand breaks) in 833K cells pre-incubated with or without 5 µM Cd²⁺, and at the same time had little effect on cell viability. Viability was measured using PI/Hoechst.

Table 3.2 Cell culture treatments.

Treatment	[Cd ²⁺] µM	UVC (J/m ²)	Incubation (h)
1 (Control)	0	0	24
2	5	0,5	24
3	5	1	24

4	5	3	24
5	5	5	24
6	5	10	24
7	0	0,5	24
8	0	1	24
9	0	3	24
10	0	5	24
11	0	10	24

Procedure

1. 833K cells were plated onto Costar cell culture dishes (28 cm²), 5x10⁵ cells per dish in 2 ml medium, and incubated for 4 h for cell attachment.
2. After incubation the medium was removed and 2 ml fresh medium with or without 5 µM cadmium was applied to the samples.
3. Samples were incubated for 20 h before they were cooled down to ~ 4 °C on aluminium plates.
4. Samples were exposed to different doses of UVC and further incubated for 4 h.
5. Each sample was processed as described in section 3.2.1
6. Half of each sample was fixed for flow cytometric analysis, and the remaining half were stained with PI and Hoechst for viability count in the fluorescence microscope.

3.2.3. Flow cytometric analysis of cell cycle stages

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells (or other particles) e.g. cell size, DNA content, shape and internal complexity, as they travel in suspension one by one past a sensing point. The flow cytometer consists of a light source, collection optics, electronics and a computer to translate signals to data.

We wished to investigate if cadmium alone and/or UVC treatment had an effect on the cell cycle. The cell samples that were fixed with 0.2 % paraformaldehyde, were stained with Hoechst 33258, and analysed in an Argus 100 flow cytometer (Skatron, Lier, Norway). The percentages of cells in the different phases of the cell cycle were estimated from DNA histograms using the Multicycle Program (Phoenix Flow System, San Diego, CA, USA). It is

possible to distinguish different cell phases, as well as apoptotic cells/bodies and secondary necrotic cells, on the basis of their DNA content (Hoechst 33258 fluorescence) and cell size (forward light scatter) (Wiger *et al.*, 1998; Gorczyca *et al.*, 1993). All samples were analysed by Richard Wiger at (our) Department of Chemical Toxicology (MIKT), at the Norwegian Institute of Public Health.

3.2.4. Analytic quantification of cadmium in 833K cells

In order to investigate to what degree cadmium was taken up by the cells, we exposed 833K cells to two different concentrations of cadmium (5 μM and 10 μM) in medium containing calf serum (Table 3.3), where after the content of cadmium in the cells and in the medium was analysed by means of atomic absorption spectrometry.

Table 3.3 Cell culture treatments

Treatment	[Cd ²⁺] μM	Incubation (h)
1 (Control)	0	20
2	5	20
3	10	20

Procedure

1. 833K cells were plated onto six Costar cell culture dishes (28 cm²), 5x10⁵ cells per dish in 2 ml medium, and incubated for 4 h to allow the cells to attach before the medium was changed.
2. Samples were incubated for 20 h, and transferred to Falcon tubes together with the medium.
3. Samples were centrifuged at 500 x g for 5 min, and the supernates were transferred to new Falcon tubes. These tubes were named “wash 1”.
4. Cell pellets were re-suspended in 2 ml PBS and centrifuged at 500 x g for 5 min. Supernates were transferred to new Falcon tubes called “wash 2”.
5. Cell pellets were re-suspended in 2 ml PBS and centrifuged at 500 x g for 5 min. Supernates were transferred to new Falcon tubes, called “wash 3”.

6. 2 ml of HNO₃ : H₂O (1 : 1), was added to each of the “wash” tubes as well as the pellets.
7. All samples were incubated at 90 °C for 1 h.
8. All volumes were adjusted to 5 ml with deionised water.
9. Cadmium content was measured by Ewa Andruchow at the Department of Analytical Chemistry (MIAN), at the Norwegian Institute of Public Health.

3.3. DNA repair assays

3.3.1. DNA alkaline filter elution

The method is used to quantify DNA damage levels in the form of single strand breaks (SSBs). Cells were lysed, the DNA unwound, purified, eluted and collected as two-hour fractions in a semi automated setup. DNA contents in the fractions of eluted DNA are measured fluorometrically using Hoechst 33258 (excitation max: 360 nm, emission max: 450 nm) in an automated sampler connected to a recorder and a computer. Hoechst 33258 binds to DNA with a strong preference for A-T rich sequences. The resulting peaks from the recorder are manually read and then fed to a computer and calculated by software. The data output includes total amount of DNA in each sample, summarised from the fractions, and elution profiles which are used to calculate DNA damage levels. DNA damage levels are determined by the Normalised Area Above (the elution) Curve (NAAC) (Brunborg *et al.*, 1996). Calibration of NAAC values is achieved by exposing cells to a known dose of X- rays and analysing them by alkaline elution.

To study the effects of cadmium on DNA repair in 833K cells we combined alkaline elution with a crude extract of the enzyme bacteriophage T4 pyrimidine dimer glycosylase (T4-pdg), formerly known as T4 endonuclease V (Lloyd, 2005). T4-pdg has a high affinity for cyclobutane pyrimidine dimers (CPDs) which is the main type of DNA lesions induced by UVC. The enzyme binds to and incises the lesions leaving gaps in the DNA. Consequently if cadmium inhibits mechanisms involved in DNA repair of UVC induced lesions, we would expect cells co-exposed to cadmium and UVC to show higher levels of DNA damage (NAAC values) than cells exposed to UVC only. In an alternative approach we made use of the DNA repair inhibitors (RI) Cytosine Arabioside (AraC) and Hydroxyurea (HU). AraC and HU are so called anti-metabolites used in cancer treatment. AraC is metabolised intracellularly into its active form; cytosine arabioside triphosphate (AraCTP). AraCTP inhibits both the initiation and elongation steps of DNA synthesis. HU inhibits the enzyme deoxyribonucleotide

reductase which synthesises deoxyribonucleotides. In effect the two drugs knock out the polymerisation and ligation steps of DNA repair. After cadmium and UVC exposure the cells were incubated with the inhibitors before SSBs were measured. Cells co-exposed to cadmium and UVC should show a lower NAAC value than cells treated with UVC only, if cadmium was to inhibit enzymes involved in the incision step of DNA repair. The alkaline elution procedures are based on Brunborg et al. (1988) with some modifications. All cell cultures were protected from light during the entire treatment period to avoid additional light-induced DNA damage.

Procedure

1. 833K cells were plated onto 36 Costar cell culture dishes (28 cm²), 5x10⁵ cells per dish in 2 ml medium, and incubated for 4 h for cell attachment.
2. After incubation the medium was removed and replaced by fresh medium with or without 5 µM cadmium, and further incubated for 20 h under standard conditions.
3. Medium with repair inhibitors (RI) was prepared during the last 15 min of incubation.
Step 3 does not apply to treatment with T4-pdg.
4. The cells were cooled to ~ 4 °C on aluminium plates before exposure to various doses of UVC.
5. When DNA alkaline elution was to be combined with T4-pdg treatment, the cells were incubated for 4 h at 37 °C for repair after the UVC treatment. Alternatively the samples were given 222 µl of RI solution (final concentration: 0.1 mM AraC, 2mM HU per sample), and incubated for 1 h.
6. Following incubation the samples were kept cold (~ 4 °C), until they were loaded onto designated filters in an automated DNA alkaline elution system (Brunborg *et al.*, 1988). The filters were submerged at all times in a temperature regulated water bath.
7. Samples were washed with Merchant buffer for 30 min at a flow rate of 0.6 ml/min for each filter. Temperature ~ 4 °C.
8. After the wash with Merchant's buffer the cells were lysed for 1 h with lysis buffer. For the initial 5 min the flow rate was 0.6 ml/min, during which the water temperature was quickly increased up to ~ 22 °C. For the remaining 55 min the flow rate was 0.09 ml/min.

9. Lysis buffer was completely washed out with 20 mM Na₂EDTA, pH 9.6 for 35 min at a flow-rate of 0.25 ml/min.
10. The samples were washed with BE1 buffer for 1 h at a flow-rate of 0.25 ml/min. *Steps 10 and 11 apply only to the treatment with T4-pdg.*
11. A crude extract of T4-pdg dissolved in BE1 buffer was added. The mix was first flushed through each filter (0.25ml/min) for 8 min at 22 °C, and then 30 min (0.034 ml/min) at 37°C.
12. DNA remaining on the filters was washed with 20 mM Na₂EDTA, pH 9.6, for 37 min at 22°C (0.25 ml/min).
13. DNA was eluted with Elution buffer at a flow rate of 0.03 ml/min, for 16 h, producing 8 two-hour fractions of eluted DNA for each sample.
14. Elution buffer (4 ml) was flushed through the system for each sample to produce fraction number 9.
15. Each chamber containing the filters was opened and the filters were incubated in 10 ml elution buffer and stirred for 45 min in a water bath at 42 °C. These were designated fraction 12.
16. The chambers were filled with elution buffer and flushed twice after 30 min to produce fractions 10 and 11.
17. DNA content in all of the fractions was quantified fluorometrically with Hoechst 33258.
18. DNA-damage levels were calculated from the elution profiles.

3.4. Gene expression analysis

3.4.1. Cell culture treatment

The cells were protected from light sources during the entire experiment to avoid additional photo-induced DNA damage. The cultures were treated as shown in table 3.4.

Table 3.4 Cell culture treatments

Treatment	[Cd ²⁺] μM	UVC J/m ²	Incubation (h)
1 (Control)	0	0	24
2	5	0	24

3	0	3	24
4	5	3	24

Procedure

1. 833K cells were plated onto Costar cell culture dishes (78 cm²), 5-6 x10⁶ cells per dish in 5 ml medium, and incubated for 4 h to allow the cells to attach.
2. The medium was removed and replaced by fresh medium with or without 5 μM cadmium, and the cells were further incubated for 20 h.
3. After incubation the cells were cooled down to ~4 °C on aluminium plates, exposed to 3 J/m² UVC, and incubated for additional 4 h.
4. Total-RNA or protein isolation.

3.4.2. Total RNA isolation

Isolation was performed according to manufacturer protocol (GenElute™ Mammalian Total RNA Kit). Samples were lysed and homogenised in guanidine thiocyanate and β-mercaptoethanol (β-ME) to release RNA and inactivate RNases. Lysates were spun through a filtration column to remove cellular debris and shear DNA. Next the filtrates were applied to high capacity silica columns to bind total RNA, followed by wash and elution.

Procedure

1. The β-mercaptoethanol was added to the lysis solution (10 μl β-ME/1 ml lysis solution).
2. Medium was removed from the cell-cultures and the cells were washed twice with PBS.
3. Cells were lysed by adding 500 μl of lysis solution/β-ME mixture directly to each sample, covering the whole surface of the dish.
4. The lysates were transferred to filtration columns provided in the kit, and spun for 2 min at maximum speed (14000 x g). This step removes cellular debris and shears DNA.
5. An equal volume of 70 % ethanol was added to the filtrates and thoroughly mixed.

6. The lysate/ethanol mixtures were transferred to binding columns, and spun for 30 s at maximum speed. Flow-through was discarded.

Wash to remove contaminants

7. First 500 μ l wash solution 1 was added to each column, where after the columns were spun for 30 s at maximum speed. Flow-through was discarded.
8. Likewise 500 μ l wash solution 2 was added to each column, and the columns were spun for 30 s at maximum speed. Flow-through was discarded.
9. A second 500 μ l wash solution 2 was added to each column, and the columns were spun for 30 s at maximum speed. Flow-through was discarded.
10. Columns were spun for 2 min to remove remaining ethanol.

Elution of purified RNA

11. The columns were transferred to new collecting tubes.
12. A 50 μ l of elution solution was added to the columns and the columns were spun for 1 min at maximum speed.
13. A second 50 μ l of elution solution was added and the columns were spun for 1 min. (The second 50 μ l were added because more than 50 μ g RNA was expected per column).
14. The flow-through containing the RNA was collected and spun through the column a second time, to increase the yield of total RNA.
15. The purified RNA was stored at -70 °C.

3.4.3. Determining the concentration and purity of isolated total RNA

We used the “NanoDrop ND-1000 spectrophotometer” which is a full-spectrum (220-750 nm) spectrophotometer that measures 1 μ l samples with high accuracy and reproducibility.

When measuring nucleic acids there are two ratios of importance: 260/280 nm and 260/230 nm (Figure 3.1). The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA, whereas a ratio of ~2.0 is generally accepted as “pure” for RNA. Ratios appreciably lower in either case may indicate the presence of proteins, phenols or other contaminants that absorb strongly at or near 280 nm. The ratio of sample absorbance at 260 and 230 nm often have higher values than

the respective 260/280. They are commonly in the range of 1.8-2.2. Appreciably lower values may indicate the presence of co-purified contaminants. All samples of isolated RNA were analysed using NanoDrop. If the concentration of RNA was too low, the sample(s) were concentrated using a vacuum-centrifuge. The samples were stored at -70 °C.

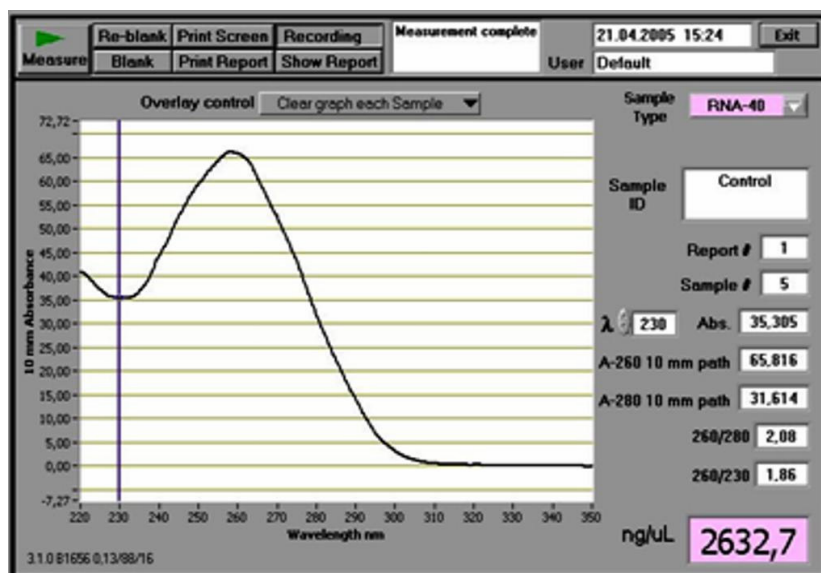


Figure 3.1 Concentration (ng/μl), 260/280 nm and 260/230 nm ratio-values for isolated RNA (control sample).

3.4.4. Total-RNA integrity and quality control

Although NanoDrop provides a good method of assessing the concentration and purity of nucleic acids, it says nothing about the integrity of the molecules. In order to ensure that the quality of our RNA samples was acceptable we used the “Agilent 2001 Bioanalyzer” and the “RNA 6000 Nano LabChip Assay” kit. The method provides a fast and accurate measurement of small amounts of RNA. The total-RNA samples are loaded into a 16-well chip that holds a separating gel which contains a luminescent dye. 12 samples can be measured per chip. RNA in the samples are separated in the gel and analysed by the Bioanalyzer software. The resulting data output includes graphs, RNA concentrations, the ratios of 28S rRNA and 18S rRNA, and a RNA integrity number (RIN). RIN has a value between 1 and 10 and is calculated from the whole area of the graph. RIN = 10 designates RNA of high integrity whereas RIN = 1 designates poor integrity. (Figure 3.2 A & B).

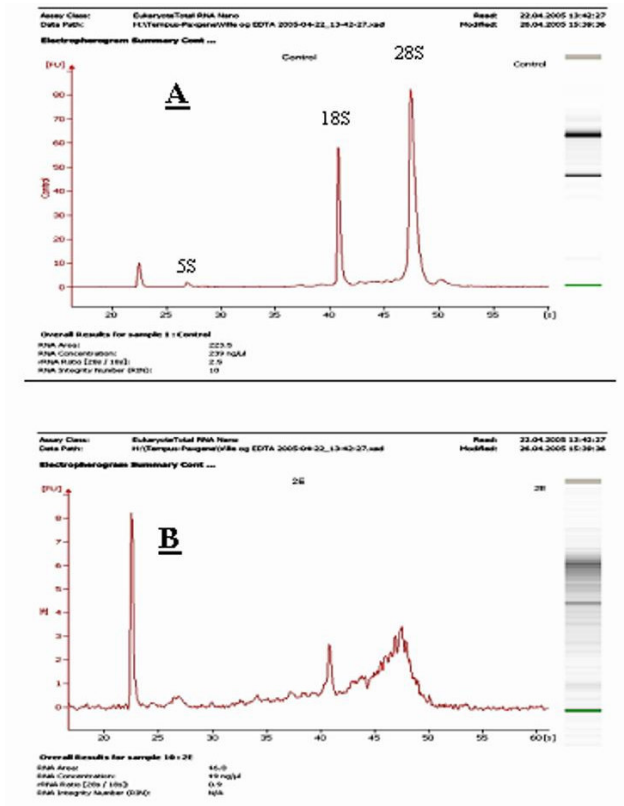


Figure 3.2: A) RNA of high integrity, RIN = 10 (control sample). B) Example of degraded RNA.

Procedure

(Measurements were performed according to manufacturer protocol.)

1. RNA ladder and RNA samples were denatured for 2 min at 70 °C, and equilibrated to room temperature for 30 min together with the rest of the reagents.
2. A 550 μl of gel matrix was filtered through a spin filter for 10 min at ~ 1500 x g.
3. The dye concentrate was vortexed for 10 s and briefly spun down to collect contents.
4. A 1 μl of dye concentrate was added to 65 μl of the filtered gel, and the two were mixed thoroughly by vortexing.
5. The gel-dye mix was spun for 10 min at 13000 x g.
6. The gel was loaded onto the chip according to manufacturer protocol.
7. A 5 μl of RNA marker was loaded into all wells.
8. A 1 μl of the RNA ladder was loaded into its designated well and 1 μl of sample was loaded into each of the 12 sample wells. The chip was vortexed for 1 min, and run in the Bioanalyzer.

3.4.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a technique used to make complementary DNA (cDNA) from mRNA, and then amplifying the cDNA (if needed). Transcribing mRNA into cDNA is termed reverse transcription, and is accomplished through the use of a primer that binds to the polyA-tail of mRNA and an mRNA-dependent DNA polymerase (Reverse Transcriptase). The resulting cDNA strands function as templates for a deoxyoligonucleotide primer and a DNA-dependent DNA polymerase. The cDNA is then exponentially amplified via PCR. The original mRNA templates are degraded by RNase. In our study we have not amplified our cDNA, but stopped cDNA generation after the first cycle.

Before cDNA generation was started, all sample parallels of isolated total-RNA were pooled together: “control 1” with “control 2 + 3”, “cadmium 1” “with cadmium 2 + 3”, and so on. The pooled samples from three parallel experiments were then measured in the NanoDrop to determine total RNA content and concentration, and subsequently used to make cDNA.

Procedure

(cDNA generation was performed accordingly to the “Fairplay Microarray Labeling Kit” protocol from Stratagene.)

1. A 20 µg of total RNA from each of the pooled samples was suspended in 12 µl RNase free water.
2. A 1 µl of 500 ng/µl oligonucleotide-dT primer (d(T)12-18) was added to each of the samples where after they were incubated for 10 min at 70 °C and cooled down on ice. Samples were spun briefly to collect content.
3. The following master mix was prepared (volumes are for one sample)

2 µl of 10x StrataScript reaction buffer

1 µl of 20x dNTP mix

1.5 µl of 0.1 M DTT

0.5 µl of RNase block (40 U/µl)

4. The master mix was added to the samples

5. A 1.5 µl of 50 U/µl StrataScript reverse transcriptase (RT) was added, and the samples were incubated for 30 min at 48°C.
6. Another 1.5 µl of 50 U/µl StrataScript reverse transcriptase was added, and the samples were incubated for additional 40 min.
7. A 10 µl of 1 M NaOH was added and the samples were incubated for 10 min at 70 °C. The heating hydrolyses RNA.
8. Samples were slowly cooled down to room temperature.
9. A 10 µl 1M HCl was added to neutralise the solutions.
10. A 4.6 µl of 2.6 M sodium acetate (pH 4.5) was added to each of the samples, as well as 1 µl of 20 mg/ml glycogen.
11. Finally 100 µl of ice cold 95 % ethanol was added, and the samples were stored at -70 °C.

3.4.6. Microarray analysis

As was described in the introduction, DNA and oligonucleotide microarrays are used to simultaneously assess the transcriptional states for thousands of genes. Fluorochrome coupled cDNA prepared from treated samples and control are hybridised to imprinted gene segments on the array. In our study we have used the fluorochromes Cyanine 3 (Cy3) and Cyanine 5 (Cy5). When scanning the microarrays, Cy3 spots are measured at ~ 532 nm, where as Cy5 spots are measured at ~ 635 nm. The result of the scanning is actually in greyscale, but the scanning software applies pseudo-colours, so that Cy3 is shown in green and Cy5 in red. We used the 21k human oligonucleotide microarray v2.3, printed at the Norwegian Microarray Consortium (NMC). The microarrays consist of 23.184 spots, including 21.329 oligos (70 mer) representing 21.329 genes from the UniGene database. The oligos are printed as 48 sub-grids on the array, each sub-grid printed by a separate print-tip (Figure 3.3). For more details on the arrays, we refer to: <http://www.mikromatrise.no/facility/Oslo>.

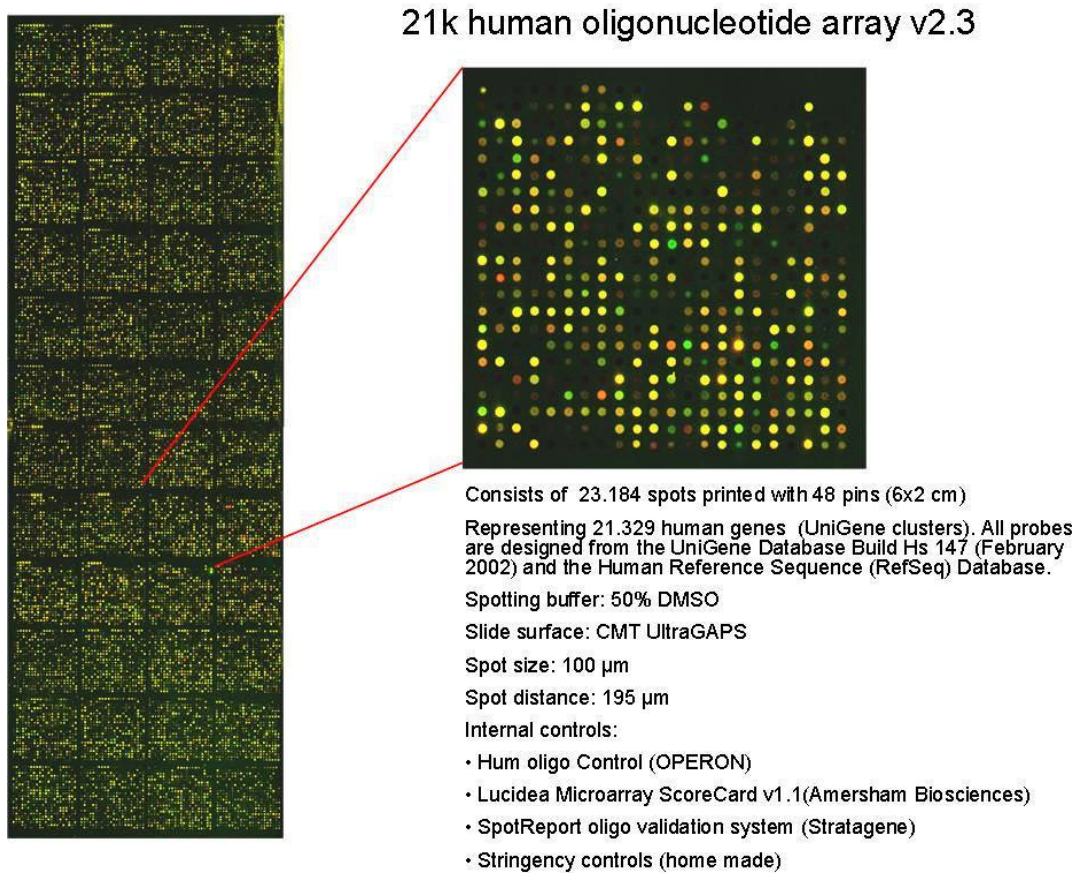


Figure 3.3 The 21k oligonucleotide microarray v2.3 from NMC

Dye coupling reaction

Procedure

(Dye coupling reaction was performed accordingly to the “Fairplay Microarray Labeling Kit” protocol from Stratagene.)

1. The cDNA samples stored in ethanol were spun down at 14,000 x g for 1 h at 4 °C, and the ethanol was gently poured out leaving the cDNA pellet attached to the tube wall.
2. The cDNA pellets were washed with 0.5 ml ice cold 70 % ethanol and spun at 14,000 x g for 30 min at 4 °C. The ethanol was again removed and the samples were briefly spun down to collect the last droplets of ethanol, which were removed with a pipette.
3. The cDNA pellets were air dried to remove residues of ethanol that might interfere with the dye coupling reaction.

4. The cDNA pellets were re-suspended in 5 μ l of 2x coupling buffer, and incubated for 15 min at 37 $^{\circ}$ C.
5. The Cy3(s) and Cy5(s) dyes were re-suspended in 45 μ l high purity DMSO, at room temperature.
6. The control sample cDNA was divided into two tubes, 5 μ l of Cy3 was added to one tube and 5 μ l of Cy5 to the other, as well as to the rest of the samples (Cadmium, UVC, Cadmium + UVC).
7. The cDNA-dye mixtures were gently vortexed for a few seconds, and spun down to collect contents.
8. The samples were incubated for 1 h in the dark at room temperature, and gently mixed for a few seconds every 15 min.

Dye-coupled cDNA purification

9. A 90 μ l of 1x TE buffer was added to each of the labelled cDNA samples.
10. DNA binding solution and 70 % ethanol was mixed together 1 : 1
11. A 200 μ l of the DNA-binding solution and ethanol mixture was added to each of the labelled cDNA samples, and mixed by vortexing.
12. The samples were transferred to microspin cups seated in 2 ml receptacle tubes, and spun for 30 s at maximum speed. The flow through containing uncoupled dye was discarded. The microspin cups were placed back into the receptacle tubes.
13. Another 200 μ l of the DNA-binding solution and ethanol mixture was added to the microspin cups and the samples were spun again for 30 s at maximum speed. Flow through was discarded, and the microspin cups were placed back into the receptacle tubes.
14. A 750 μ l 75 % ethanol was added to the microspin cups and the samples were spun for 30 s at maximum speed. Flow through was discarded, and the microspin cups were placed back into the receptacle tubes
15. Second wash with 750 μ l 75 % ethanol (previous step repeated).
16. The samples were spun for 2 min at maximum speed in order to remove all wash buffers. The receptacle tubes were discarded and the microspin cups were placed into fresh 1.5 ml microcentrifuge tubes.

17. A 50 μ l of 10 mM Tris base, pH 8.5 was added directly on top of the fibre matrix at the bottom of the microspin cups, and the samples were incubated for 5 min at room temperature.
18. The tubes were spun for 30 s at maximum speed.
19. Additional labelled cDNA was eluted by loading the flow through back onto the fibre matrix, incubating the samples for another 5 min, and spinning them for 30 s at maximum speed
20. A final elution was performed by repeating the last elution step.

Analysis of fluorescence-labelled cDNA with NanoDrop ND-1000

The NanoDrop ND-1000 includes a module for microarrays which is used to measure dye incorporation, concentration and purity of nucleic acid. Nucleic acids are measured at 260 nm, Cy3 at 550 nm, and Cy5 at 650 nm. The NanoDrop calculates the amount of cDNA produced (ng/ μ l) and pmol of dye incorporated (pmol/ μ l of Cy3 and Cy5). The control sample was measured in the NanoDrop to determine Cy3 incorporation, cDNA and Cy3 concentration, while the rest of the samples were measured to determine Cy5 incorporation, cDNA and Cy5 concentration. Total amount of dye incorporated was calculated by multiplying the concentration (pmol/ μ l) Cy3 or Cy5 with the total volume of the eluted cDNA for each sample. The control sample was added to each of the other samples in appropriate volumes so that the ratio of Cy3 and Cy5 labelled cDNA was acceptable. The combined samples were measured again in the NanoDrop, before hybridisation to the microarrays (Table 3.5).

Table 3.5 *Dye incorporation and hybridisations*

Cell treatment	Dye incorporated	Hybridisations
Control	Cy3	
Control*	Cy5	Control vs. Control*
Cadmium	Cy5	Control vs. cadmium
UVC	Cy5	Control vs. UVC
Cadmium + UVC	Cy5	Control vs. Cadmium + UVC

Oligo array pre-hybridisation

Pre-hybridisation solution contains a large excess of Bovine Serum Albumin (BSA), which physically binds to reactive groups on the surface of the microarray slides, rendering them inactive (physical blocking). Physical blocking prevents unbound DNA in printed elements associating with the reactive groups on the slide surface, which would otherwise lead to elevated background and “streaking” or “comet-tailing” on the slide.

21. Four 50 ml Falcon tubes, one for each array, filled with pre-hybridisation solution were heated to 42 °C in a water bath for 30 min, to ensure that all solids were dissolved.
22. The arrays were incubated in the tubes for 1 h at 42 °C to block the slide surfaces.
23. After blocking, the arrays were washed twice by quickly placing them in Falcon tubes filled with sterile water, and rinsed by agitating the tubes for 30 s. The wash was then repeated in new Falcon tubes with sterile water.
24. The arrays were quickly immersed in Falcon tubes with iso-propanol and agitated for 30 s.
25. The arrays were dried in Falcon tubes by centrifugation (480 x g for 5 min), after which they were ready for hybridisation.

Manual hybridisation

26. The labelled cDNA samples were mixed with hybridisation solution and heated for 3-5 min at 100 °C, and then cooled down to 48 °C.
27. The samples were quickly centrifuged to spin down contents, and incubated further at 48 °C until hybridisation.
28. The samples were applied onto the gasket slides of the microarrays, already mounted in the hybridisation chamber bases.
29. The microarray slides were gently lowered onto the gasket slides, and the chamber covers were placed on top. The chambers were closed and tightened by using the chamber clamp.
30. The flow of the hybridisation solution was inspected by slowly rotating the chambers. It is important to ensure a continuous flow of the solution and to avoid stationary bubbles.

31. The chambers were placed in an incubator at 50 °C where they slowly rotated for 16-17 h.

Post hybridisation wash

32. For each array 3 x 50 ml Falcon tubes were filled with wash solution 1 and heated to 48 °C in a water bath for minimum 30 min.
33. After dismantling the hybridisation chambers the arrays were quickly transferred to the Falcon tubes containing wash solution 1, and gently agitated until the gasket slides let go of the arrays.
34. The arrays were placed in the second Falcon tubes with wash solution 1 and incubated in the water bath for 5 min at 48 °C.
35. The arrays were quickly transferred to the third Falcon tubes and the previous wash step was repeated.
36. The arrays were transferred to Falcon tubes (wrapped in tin foil to avoid photo bleaching of the arrays), with wash solution 2 and gently agitated for 1 min in a rotator at room temperature.
37. Second wash with wash solution 2, (previous step repeated).
38. In the same manner the arrays were washed in wash solution 3 for 1 min in the rotator.
39. Incubation in new Falcon tubes with wash solution 3 for 1 min, no agitation.
40. The arrays were briefly dipped in a Falcon tube containing sterile water before they were dried by centrifugation at 480 x g for 5 min.

3.4.7. Scanning and data processing

The slides were scanned by an Agilent microarray scanner (Agilent, Inc.) at the Norwegian Radium Hospital. The GenePix 4.1 image analysis software (Axon Instruments, Inc., Union City, CA) was used for spot segmentation, intensity calculation and flagging spots with inadequate measurements. Raw-data results from the GenePix 4.1 image analysis software were normalised using print-tip LOWESS, and dye-normalisation by DN MAD ([http:// damad.bioinfo.cnio.es/](http://damad.bioinfo.cnio.es/)). The normalised data was then further processed by GEPAS (Gene Expression Pattern Analysis Suite v1.1), filtering out flagged spots, removing inconsistent replicates, merging replicates, calculating missing values and removing missing values (this option is intended for removing the patterns with many missing values. For

example, if you have a dataset with 10 conditions and you set up the minimum percentage of existing values to 70 %, all the patterns with less than 7 existing values will be removed, i.e. all the patterns with more than 3 missing values will be removed). The methods of normalisation will be further explained together with the results.

3.5. Protein related methods

3.5.1. Cell treatment

The cells were plated and treated simultaneously with the cells used for total RNA isolation in section 3.4.

3.5.2. Protein extraction

Procedure

All samples were kept cold during the procedure to avoid protein degradation

1. The medium was removed and the cadmium and/or UVC treated cell cultures were washed twice with 5 ml PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$).
2. A 500 μl of lysis-buffer was added to each of the 78.5 cm^2 cell culture dishes.
3. The cell culture dishes were scraped and the cell lysates transferred to 15 ml Falcon-tubes, and put on ice for 10 min.
4. The cell lysates were sonicated on ice at amplitude 40, using approximately one pulse per second, 3 x 10 pulses with 5 s intervals.
5. A 20 μl of each cell lysate was put aside for measuring protein concentration.
6. All cell lysates were “snap-frozen” in liquid nitrogen and stored at $-70\text{ }^\circ\text{C}$.

3.5.3. Measurement of protein concentration with the Bio-Rad DC Protein Assay

The reaction is similar to the Lowry assay, and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay there are two steps which lead to colour development: The reaction between protein and copper in an alkaline medium (reagent A), and the subsequent reduction of Folin reagent (reagent B), by the copper-treated protein. Colour development is primary due to the amino acids Tyrosine and Tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Foline reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with

maximum absorbance at 750 nm and a minimum absorbance at 405 nm (Description from manufacturer manual. www.bio-rad.com). Bovine serum albumin (BSA) dissolved in lysis solution was used to make protein standards.

Procedure

1. Five different standards were made from a Bovine Serum Albumin (BSA) solution (1 mg/ml): 0, 0.25, 0.5, 1.0, and 2.0 $\mu\text{g}/\mu\text{l}$.
2. Each of the previously prepared cell lysates were diluted into two different concentrations 1/5 and 1/15 of stock.
3. A 5 μl of each of the diluted protein lysates and standards were loaded into wells on a 96 well micro-titre plate, three parallels were made for each lysate and standard.
4. Each of the wells were given 25 μl reagent A' (1ml reagent A + 20 μl reagent S (SDS)), and 200 μl of reagent B.
5. The plate was incubated for minimum 20 min at 37 °C to allow full colour development.
6. Absorption was measured in a plate-reader (Magellan V.1.11, Sunrise) at 750nm.
7. Protein concentration in the lysates was calculated from the standards, and adjusted with lysis buffer to 2.5 $\mu\text{g}/\mu\text{l}$.

3.5.4. Protein analysis (Western blot analysis)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting and immunodetection, was used to separate and measure concentrations of specific proteins in our prepared cell lysates. The method can be used to study protein modifications, how protein levels differ in different cells and tissues, and how these levels are affected by certain exposures. Effects on protein levels might also suggest modes of action caused by the exposure in question. Protein samples of equal concentration are loaded onto the SDS-polyacrylamide gel, which will separate the proteins according to size during electrophoresis. The separated proteins are then transferred onto a nitro-cellulose membrane by electroblotting. Next the protein of interest is tagged with a primary antibody which binds to protein specific epitopes. A secondary antibody conjugated to horseradish peroxidase (HRP) is used to connect to the primary antibody. Finally the protein is visualised through chemiluminescence by treating the nitro-cellulose membrane with a solution that contains a chemiluminescent substrate which, when activated by HRP, emits light. The emitted light can

be detected on film (Figure 3.4), or in a chemiluminescent imager system. Before the electrophoresis was started, we added Bromo Phenol Blue (BPB) to the protein lysates (25 μ l for each millilitre of lysate). BPB makes it possible to follow the buffer front through the gel during electrophoresis. We also added β -mercaptoethanol (β -ME) to each of the lysates (final concentration 5 %) before they were boiled at 95-98 $^{\circ}$ C for 4 min and cooled down to room temperature. β -ME together with the heating disrupts disulfide bonds, denatures and elongates the protein chains. This allows the SDS (Sodium Dodecyl Sulphate) to bind evenly to the proteins giving them a uniform negative charge. Consequently the proteins are separated in the gel according to size.

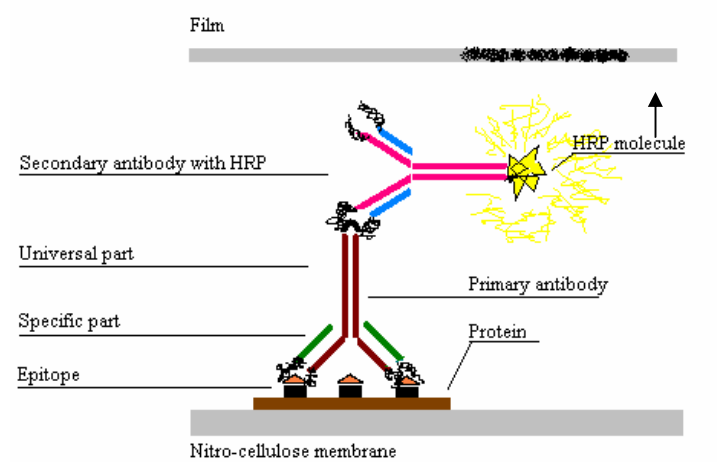


Figure 3.4 Photo development by chemiluminescence.

Procedure

Preparation of the SDS-PAGE gel

1. Two glass plates were mounted together with spacers between them on a gel-holder.
2. A well-comb was put between the two glass plates, and a line approximately 1 cm under the comb was drawn to mark the starting point of the separating gel.
3. Freshly made liquid SDS-acrylamide separating gel (6, 10 or 12 %) was poured between the two glass plates up to the drawn line.
4. Water saturated n-butanol was poured on top of the gel to prevent oxygenation and even-out the gel surface, and the gel was left to harden for approximately 1/2 h.
5. The n-butanol was thoroughly washed off with water.

6. Freshly made liquid SDS-acrylamide stacking gel (4%) was poured on the top of the separating gel and the well-comb was put in place to make 15 loading wells. The gel was hardened for approximately 1/2 h.
7. The gel was placed in a chamber filled with electrophoreses buffer and was now ready to separate the protein samples.

Loading the samples

8. A 10 µl of molecular weight marker (Precision Plus Protein™ standards, Kaleidoscope™. BIO-RAD) was loaded into the first well of the gel while the rest were loaded with 10 µl (25 µg protein) from each sample.

Electrophoreses

9. Electrophoreses was first run at 30 mAmp (per gel) until the samples had moved into the separating gel, after which the current was raised to 40 mAmp. Electrophoreses was stopped when the BPB was run out of the gel, or when separation of the molecular weight marker was satisfactory.

Electroblotting

10. Following electrophoreses the glass plates were removed and the separating gel was placed in 1x transfer buffer for 15-20 min to avoid the gel from shrinking during transfer to the nitro-cellulose membrane.
11. The gel was placed in an electroblotting-cassette submerged in transfer buffer, and run for 1 h at 100 Volt. The cassette was kept cold until protein transfer was complete.
12. After the run, the cassette was disassembled and the nitro-cellulose membrane was washed in 1x TBS for 15 min on a platform shaker.

Immunological detection of proteins

13. The nitro-cellulose membrane was incubated in blocking solution (5 % non-fat dry milk in 1x TBS) for 1 h on a platform shaker to avoid non-specific binding of antibodies to “sticky sites”.
14. Excess blocking solution was washed of with 1x 10 min in TBST with 1 % non-fat dry milk and 0.01 % Thimerosal.
15. The membrane was incubated over night with primary antibody (Table 3.6) in 10 ml 1x TBST with 1 % dry milk and 0.01% thimerosal, on a platform shaker at 4°C.

16. The membrane was washed 4 x 10 min in 1x TBST, and incubated with secondary antibody (Table 3.7) in 20 ml TBST with 1 % dry milk and 0.01 % thimerosal, for 1 h on a platform shaker at room temperature.
17. Excess antibodies were removed by 3 x 10 min wash with 1x TBST and 1x 10 min in 1x TBS, where the membrane remained, until used for visualisation by photo development.

Table 3.6 List of primary antibodies

Antigen	Host	Dilution	Manufacturer
DDB2	Goat	1 : 2000	Santa Cruz
XPA	mouse	1 : 2000	Santa Cruz
XPC	mouse	1 : 3000	Abcam
ERCC1	mouse	1 : 20000	Neomarkers
PCNA	mouse	1 : 40000	Abcam
Cdk2	rabbit	1 : 5000	Santa Cruz
phospho-Erk (Thr 202, Tyr 204)	mouse	1 : 5000	Cell Signalling tech.
p38	rabbit	1 : 1000	Cell Signalling tech.
phospho-p38 (Thr 180, Tyr 204)	rabbit	1 : 1000	Cell Signalling tech.
p53	rabbit	1 : 5000	Cell Signalling tech.
phospho-p53 (Ser 15)	rabbit	1 : 3000	Cell Signalling tech.
MGMT	mouse	1 : 5000	Neomarkers
PARP	mouse	1 : 150000	Clontech
GAPDH	mouse	1 : 200000	Biogenesis

Table 3.7 List of secondary antibodies

Antigen	Specificity	Conjugate	Host	Dilution	Manufacturer
IgG	mouse	HRP	donkey	1 : 40000	Jackson
IgG	rabbit	HRP	donkey	1 : 100000	Jackson
IgG	goat	HRP	rabbit	1 : 80000	Zymed

Chemiluminescence visualisation

The following steps are done in a darkroom for photo development.

18. Equal volumes (2ml) of two chemiluminescent solutions (Pierce, supersignal West Dura Extended Duration substrate, reagents) were mixed together.
19. The membrane was bathed in the combined solution for 5 min and placed between two transparent plastic sheets in a photo-developing cassette, the protein side of the membrane facing up.
20. An X-ray film (Fuji Medical X-ray Film, Super RX), was placed on top of the plastic sheets with the membrane, and the cassette was closed. The exposure of the film varied from 30 s up to 15 min, depending on the intensity of emitted fluorescence from the protein-bands.
21. The film was developed in developing solution for 5 min, rinsed in water for 1 min, and fixed in fixer solution for 5 min, washed again with water and finally dried.
22. The film was scanned and visually analysed.

3.5.5. Protein sample quality and uniformity control

To ensure that the extracted protein samples were not degraded and had uniform concentrations, each of the protein samples were separated by SDS-PAGE and visualised by staining the protein bands with Coomassie Brilliant Blue.

Procedure

1. After step 9 in the procedure above, the gels to be inspected were placed in Coomassie Brilliant Blue solution over-night.
2. The following day the gel was first placed in destain solution I, which removes the bulk of excess colour, and slowly agitated for 30 min.
3. The destain solution I was removed and replaced with destain solution II. Destain solution II was periodically changed until the gel background became clear.
4. The gel was dried on a filter in a combined vacuum and heater element.
5. Conformation of protein bands and uniformity between samples was visually determined.

4. Results

4.1. Cytotoxicity assays

We wanted to determine a cadmium concentration and a UVC dose which only slightly affected cell viability, to be used in the DNA-repair and gene-expression studies.

4.1.1. Determination of cadmium concentration

A dose – response experiment was performed in which 833K cells were treated with various concentrations of cadmium for 20 hours, whereupon the viability of the cells was determined using PI/Hoechst.

More than 90 % of the cells treated with 2.5, 5 or 10 $\mu\text{M Cd}^{2+}$, were viable. Cells treated with 30 or 50 $\mu\text{M Cd}^{2+}$ showed a ~ 15 % reduction in number of viable cells, where as all the cells treated with 200 μM died (Figure 4.1).

Viability of 833K cells in response to various concentrations of cadmium

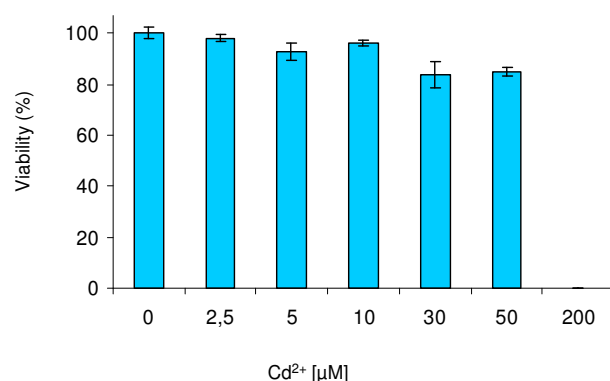


Figure 4.1 Viability of 833K cells exposed to various concentrations of cadmium.

4.1.2. Effects of UVC, alone or in combination with cadmium, on cell viability

The 833K cells were co-exposed to 5 $\mu\text{M Cd}^{2+}$ (24 h) and various doses of UVC or UVC alone, whereupon viability of the cells was measured using PI/Hoechst. No apparent differences in viability were observed between UVC-treated and co-exposed cells. Both groups showed a marked decline in viable cells when exposed to 3 J/m^2 UVC or higher (Figure 4.2).

Viability of 833K cells pre-incubated with or without 5 μM Cd^{2+} exposed to various doses of UVC

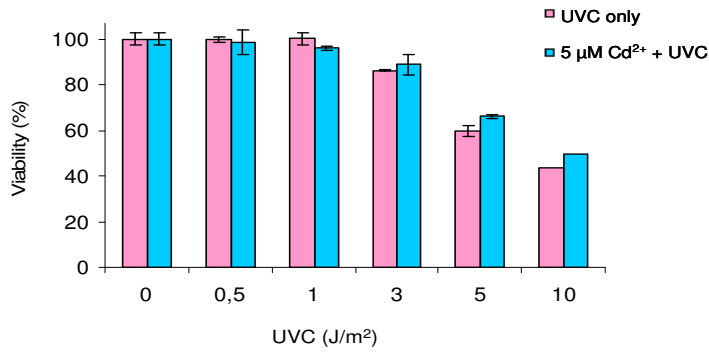


Figure 4.2 Viability of 833K cells after co-exposure to cadmium and UVC.

4.1.3. Flow cytometric analysis of cell cycle stages

Cell samples from all treatments were analysed using flow cytometry, to investigate if the percentage distribution of cells in different cell-cycle stages had been altered by the various treatments. No consistent differences were observed between the treated samples and control when comparing G1, S or G2 phases (Figure 4.3).

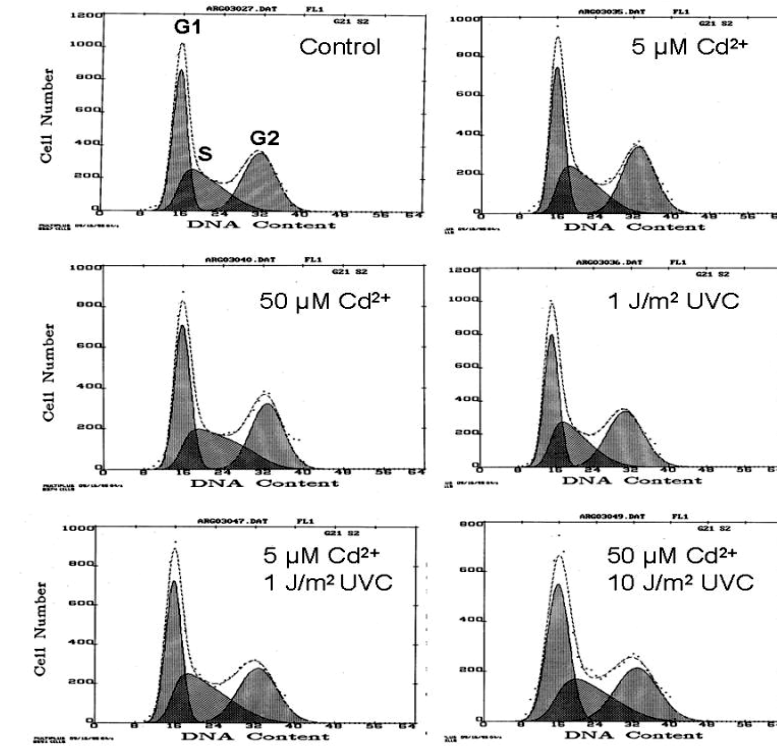


Figure 4.3 Flow data from six differently treated cell populations. No apparent treatment related effects were observed.

4.1.4. Analytical quantification of cadmium in 833K cells

Approximately one half million cells were incubated in medium containing 5 or 10 $\mu\text{M Cd}^{2+}$ for 20 hours. For analysis by atomic absorption the cells were centrifuged and washed three times. In both treatments 96 % of the Cd^{2+} remained in the medium and wash solutions, while 4 % was taken up by the cells. Mean amount of cadmium measured in the pellet of cells treated with 10 $\mu\text{M Cd}^{2+}$, was roughly twofold compared to cells treated with 5 $\mu\text{M Cd}^{2+}$. In both cases the levels of cadmium were considerably higher (47-78 fold) than in control cells. Thus, cellular uptake of cadmium was directly related to the concentration in the medium (Table 4.1).

Table 4.1 Cadmium uptake in 833K cells.

Sample	Fraction	Mean amount of Cd^{2+} (μg)
Control	Cell Pellet	1.435 x 10⁻³
	Total	2.945 x 10 ⁻³
5 μM	Cell pellet	0.0715
	Total	1.773
10 μM	Cell pellet	0.110
	Total	2.977

4.2. DNA repair assays

4.2.1. DNA alkaline elution combined with AraC and HU

To investigate if cadmium could inhibit the initial steps of DNA repair we blocked DNA-synthesis with the inhibitors AraC and HU. The results show no statistically significant differences between cells co-exposed to cadmium and UVC and cells exposed to UVC only (Figure 4.4).

833K cells pre-incubated with or without 5 μM Cd^{2+} and treated with repair inhibitors (RI) after exposure to various doses of UVC

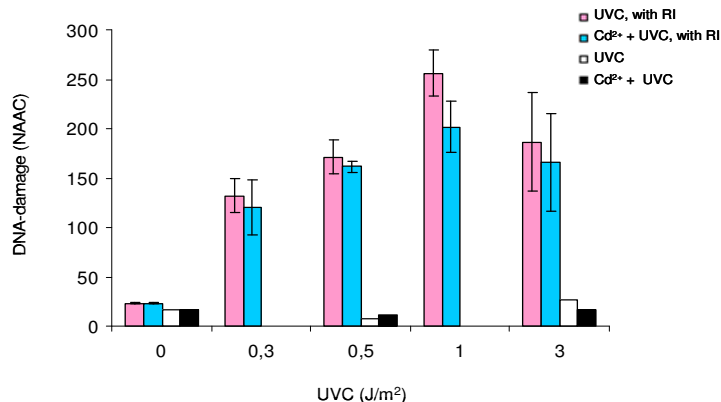


Figure 4.4 Results from one representative experiment. 833K cells were grown with or without 5 μM cadmium before exposure to different doses of UVC. All samples were then incubated for 1 hour in the presence of RI before DNA damage levels were measured by alkaline elution.

4.2.2. DNA alkaline elution combined with T4-pdg treatment

To study the effect of cadmium on DNA repair efficiency we pre-incubated 833K cells with or without 5 μM Cd^{2+} before DNA lesions were induced by UVC (1 J/m^2) irradiation. The cells were further incubated for repair where after DNA damage levels were measured by alkaline elution combined with T4-pdg. The results show a significantly (two-tailed t-test, $p \leq 0.01$) lower repair of UVC induced DNA lesions in the samples grown in the presence of cadmium, after three hours. After six hours the difference is still visible but not statistically significant (two-tailed t-test $p \leq 0.08$), (Figure 4.5).

833K cells pre-incubated with or without 5 μM Cd^{2+} and exposed to UVC (1 J/m^2)

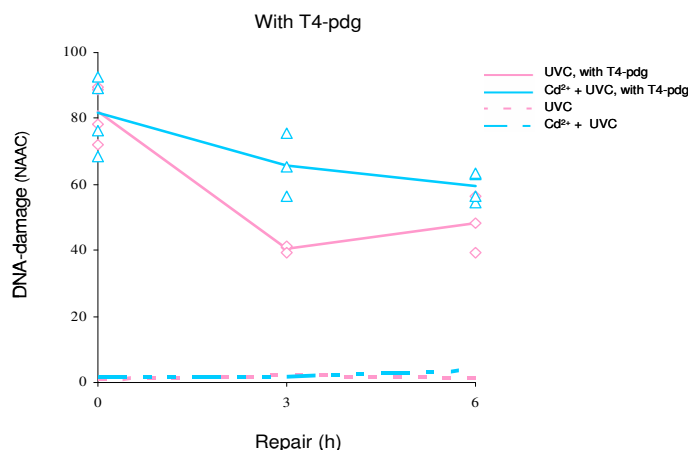


Figure 4.5 Results from one representative experiment. The cells were grown in medium either with or without 5 μM Cd^{2+} followed by exposure to UVC (1 J/m^2) and incubated 3 or 6 hours for repair. DNA-damage levels were then measured by DNA alkaline elution incorporating T4-pdg.

4.3. Gene expression analysis

4.3.1. Total-RNA purity and quality control

In order to get successful microarray hybridisations it is crucial that the RNA used is of high quality. Purity and integrity of our total-RNA samples was assessed using the NanoDrop ND-1000 spectrophotometer and the Agilent 2001 Bioanalyzer, respectively. The results of the analysis are listed in Table 4.2 and Figure 4.6, which show sample absorbance ratios (260/280 nm & 260/230 nm), rRNA ratio (28s/18s) and RIN values. The ratio of sample absorbance at 260 and 230 nm should ideally lie in the range 1.8-2.2, which indicates the presence of some co-purified contaminants in our samples. However, both the ratio of sample absorbance at 260 and 280, and the high RIN values show that the samples are of good quality.

Table 4.2 Purity and integrity of total-RNA samples

Sample	Mean 260/280	St.dev +/-	Mean 260/230	St.dev +/-	(28s/18s)	RIN
Control	2.08	0.007	1.75	0.163	2.5	10
Cadmium	2.06	0.028	1.70	0.092	2.6	10
UVC	2.07	0.014	1.45	0.318	2.6	10
Cadmium + UVC	2.09	0.014	1.59	0.318	2.3	10

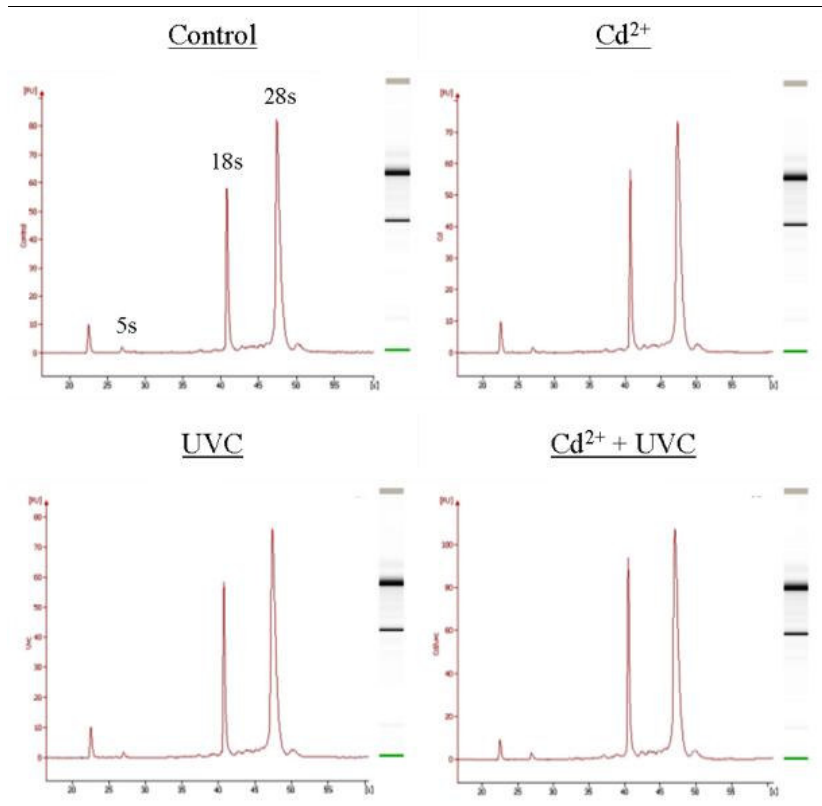


Figure 4.6 The graphs are well defined with distinct 28s and 18s peaks, signifying RNA of high quality.

4.4. Microarray analysis

Out of the twelve 21k oligonucleotide microarrays used we achieved eight hybridisations of acceptable quality that were further processed.

4.4.1. Results of normalisation

Essentially, the objective of the normalisation is to adjust for effects that are due to variations in the technology rather than the biology. In particular for differences in the red and green intensities caused, for example, by differences in the binding of the dyes (i.e., dye biases). Since these differences can be related to which print-tip printed each spot (when producing the array), the adjustment is carried out, generally, for each print-tip separately. Thus, the basic normalisation is based on a print-tip Lowess, which fits a robust local regression to the relation between **M** (difference in log ratios) and **A** (the "average" staining). The normalised **M** value is the original one minus the Lowess fitted one, and thus should correct for spatial effects (as reflected by print-tips) and for effects related to intensity.

MA plots

The MA plots show the relationship between A (the "average signal" [$0.5 * (\log R + \log G)$], where R is the background subtracted red [mean of F635 - median of B635] and G the background subtracted green [mean of F532 - median of B532]) and M (the log [base 2] differential ratio: $\log(R/G)$). These plots are shown both before and after normalisation, and with different colour lines for the Lowess lines of each print-tip (Figure 4.7).

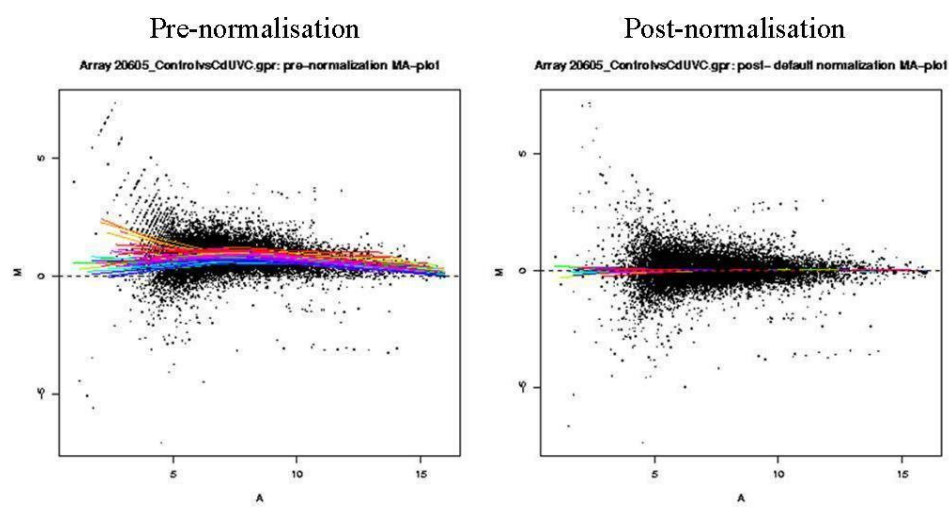


Figure 4.7 The figure shows results of MA plots for control vs. cadmium/UVC.

Diagnostic plots

First plots shown are \log_2 histograms of the raw intensities of each of the foreground channels. Similar information is also displayed in the density plots of the arrays. The densities shown are for the raw green and red channels (shown as red and blue, respectively). On the left, and for comparison, is the distribution of these channels for all the arrays. The image plots, displays the quality of the arrays and possible spatial problems. The image plots of the \log_2 intensities of the foreground and background are shown for both channels, as well as the un-normalised and normalised M values (the log [base 2] differential ratio: $\log(R/G)$) (Figure 4.8).

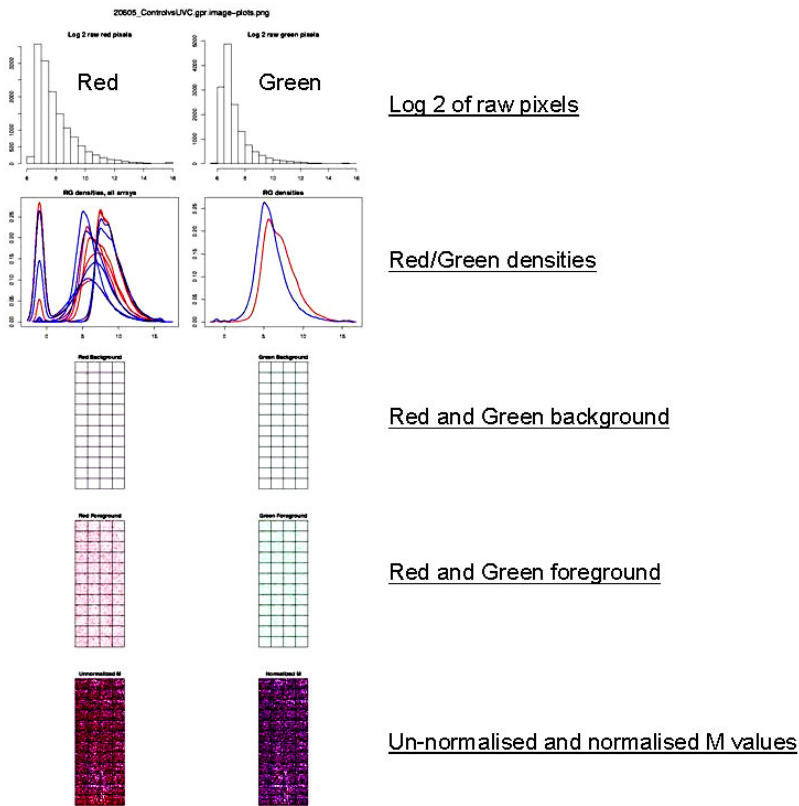


Figure 4.8 Diagnostic plots for control vs. cadmium/UVC.

Box plots

The box plots show the log intensities before and after normalisation for the 48 print-tips used to print each sub-grid on the array (Figure 4.9). This allows us to assess the quality of each sub-grid and remove, if necessary, those grids that deviate strongly before normalisation.

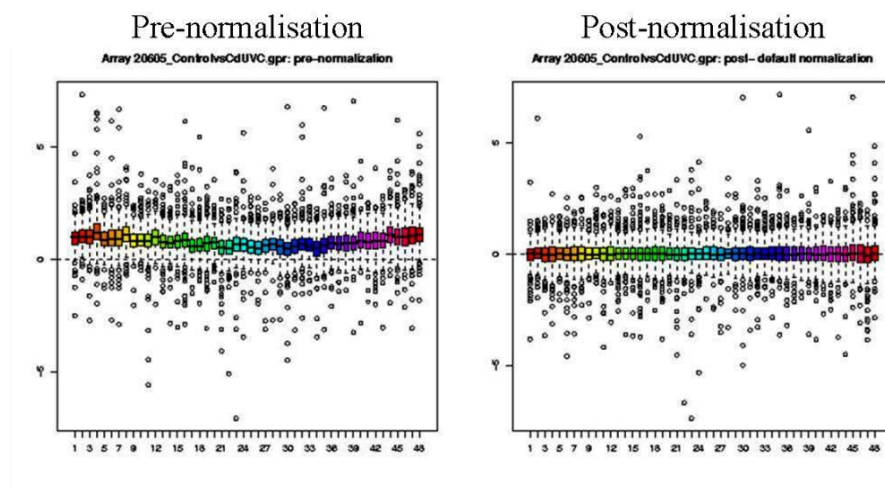


Figure 4.9 Each of the box-plots represents 1 of 48 tips used to print the oligos onto the array.

4.4.2. Finding differentially expressed gene profiles

We started out with ~21 000 genes per array, and after the above mentioned processing we ended up with ~11000 complete gene patterns. In order to find differentially expressed genes between the treatment groups, the genes were clustered by K-means and Hierarchical clustering, using J-express Pro 2.6 (www.molmine.com) (Figure 4.10, 4.11a-c and 4.12).

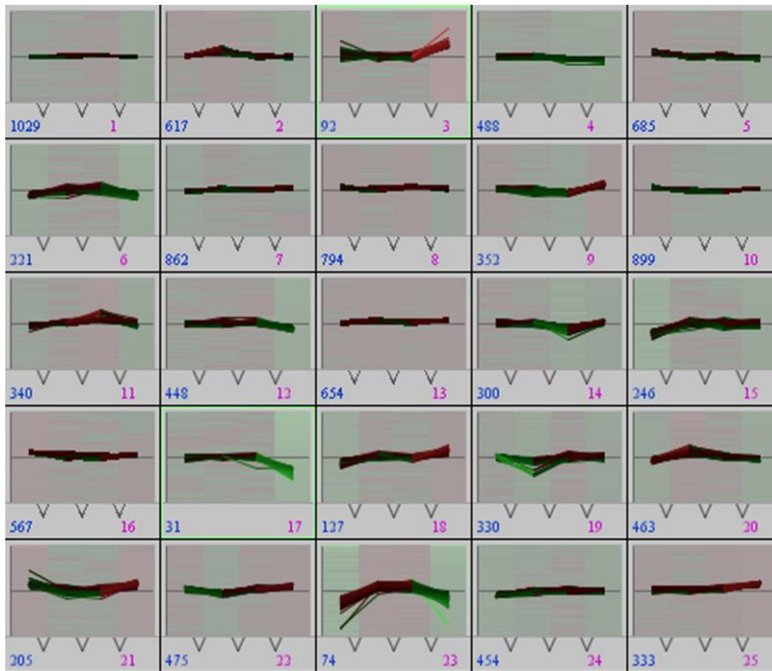


Figure 4.10 Each individual gene out of the 11 000 is sorted into a cluster that contains genes with resemblance to each other in terms of expression profile.

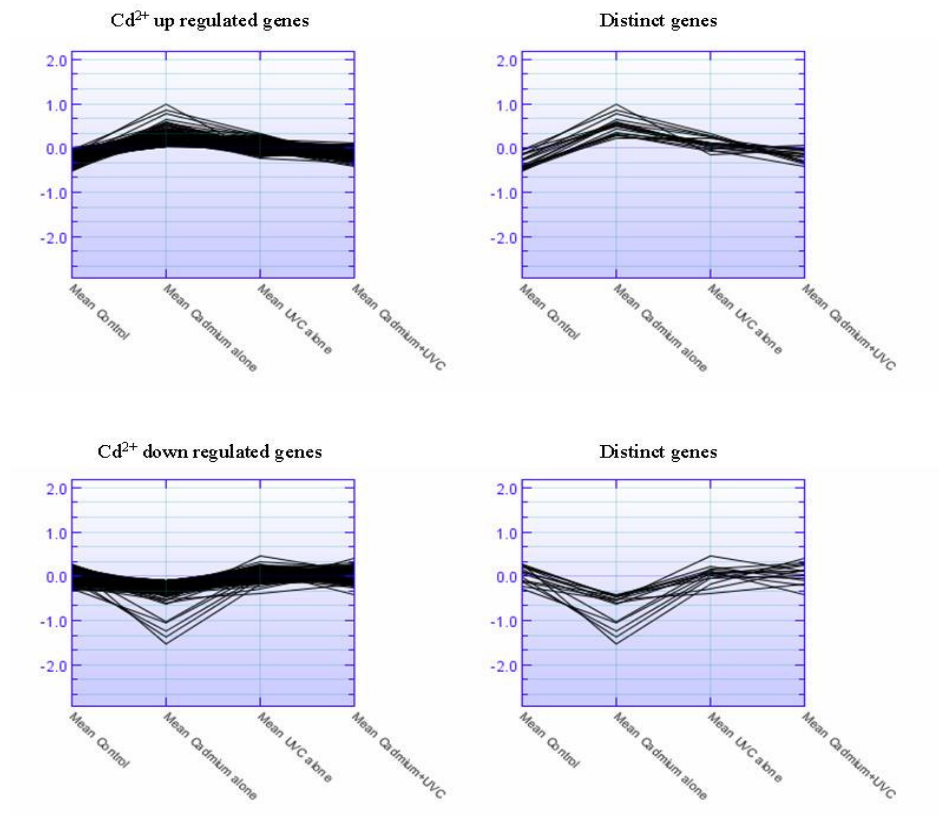


Figure 4.11a. Left upper diagram 463 genes, left lower diagram 330 genes. Total of 793 differentially expressed genes specific to treatment with cadmium.

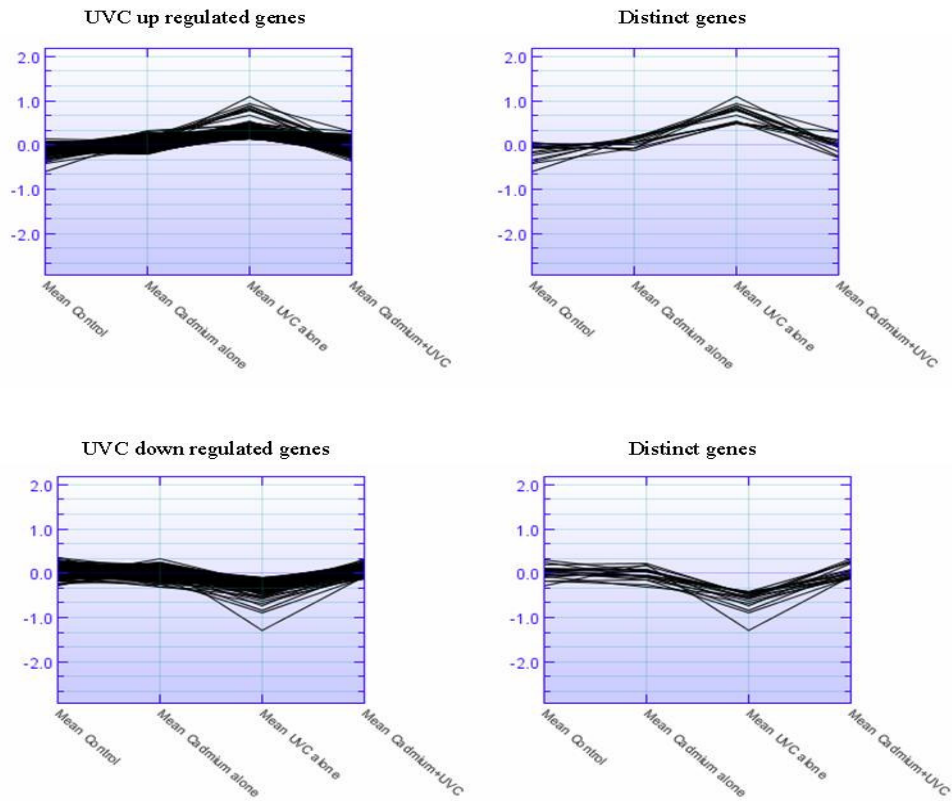


Figure 4.11b. Left upper diagram 340 genes, left lower diagram 300 genes. Total of 640 differentially expressed genes specific to treatment with UVC.

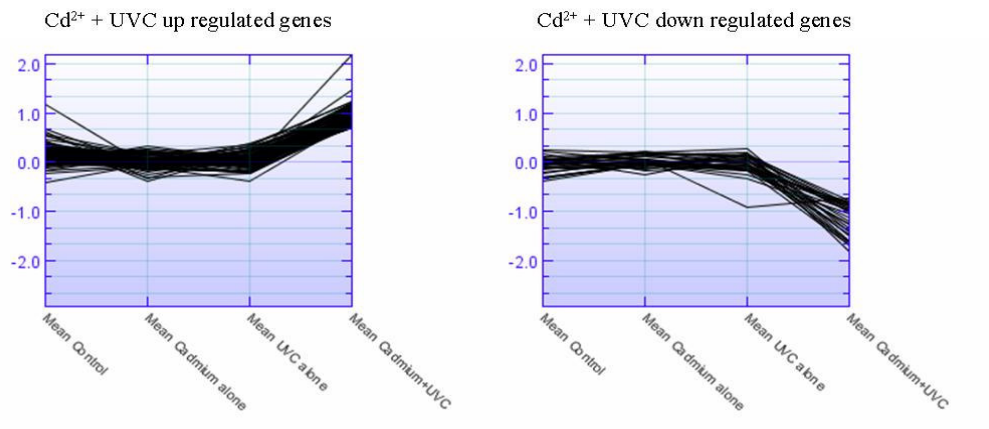


Figure 4.11c. Left diagram 92 genes, right diagram 31 genes. Total of 123 differentially expressed genes specific to treatment with both cadmium and UVC.

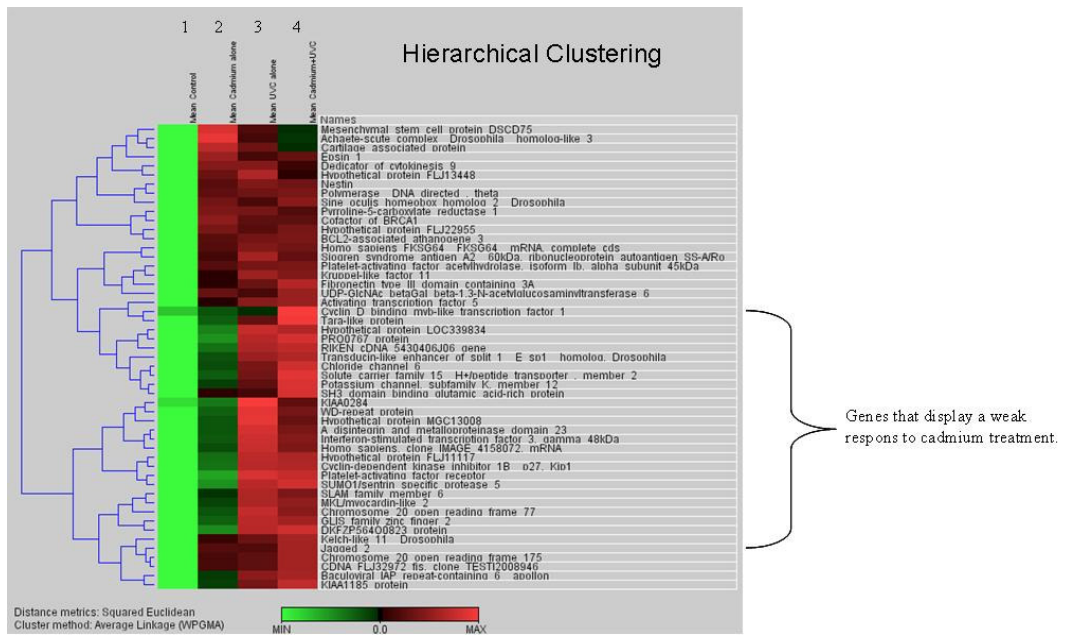


Figure 4.12 Column 1: Control, 2: Cadmium, 3: UVC, 4: Cadmium + UVC. The figure shows genes that are up-regulated in treated groups, but also genes less affected to cadmium alone (marked segment).

4.4.2.1. Gene annotation

Those genes that displayed differentiated patterns of expression were annotated and functionally categorised by GENETOOLS (<http://www.genetools.microarray.ntnu.no/adb/>). GENETOOLS is a collection of web-based tools on top of a database that brings together information from a broad range of resources, and provides this in a manner particularly useful for genome-wide analyses. Today, the two main tools connected to this database are the; NMC Annotation Database V2.0 and eGOn V2.0 (explore Gene Ontology). eGOn is a tool that facilitates use of biological background knowledge in analysis of genes selected from high throughput analysis like e.g microarray analysis. Lists of identifiers containing i.e. differentially expressed genes, are submitted to the annotation database, and eGOn automatically extends the list with Gene Ontology (GO) terms annotated to these genes.

4.4.2.2. Enriched genes

Having identified the genes, we selected those exhibiting the most noticeable changes in expression within each treatment group and exported these to a excel spreadsheet. For cadmium treated cells we found a total of 380 genes that were enriched (up- or down regulated) compared to control, likewise 188 genes for UVC treated cells, and 76 genes for cadmium and UVC co-treated cells (Table 4.3). P-values were calculated for the number of genes that were enriched due to UVC, or cadmium and UVC co-treatment, compared to cells treated with cadmium alone. For instance, our results show that 11 genes involved in cell

cycle were significantly ($p = 0.034$) enriched in cells co-treated with cadmium and UVC, while the 20 genes represented by UVC were not ($p = 0.093$). Similarly our results show that 14 genes involved in cellular catabolism were significantly ($p < 0.001$) enriched in cells treated with UVC alone, where as cadmium and UVC co-treated cells are represented by only one gene that is not significantly enriched ($p = 0.437$). The results show that 833K cells pre-treated with cadmium before UVC exposure, differ in expression of several genes compared to cells exposed to UVC alone.

Table 4.3 Genes are sorted into their respective categories of biological function, and the number of genes in each category is displayed for each treatment group. P-values assess the statistical significance of enriched genes for the groups.

GO	Category of Function	Cd alone	Cd+UVC	P-value	UVC alone	P-value
GO:0008150	biological_process	380	76		188	
GO:0043283	biopolymer metabolism	75	21	0.075	53	0.000
GO:0043412	biopolymer modification	53	17	0.025	38	0.001
GO:0009058	biosynthesis	49	4	0.033	21	0.360
GO:0009056	catabolism	13	1	0.479	14	0.000
GO:0007049	cell cycle	31	11	0.034	20	0.093
GO:0030154	cell differentiation	11	1	0.701	1	0.011
GO:0044249	cellular biosynthesis	45	4	0.048	19	0.342
GO:0044248	cellular catabolism	11	1	0.701	13	0.003
GO:0044237	cellular metabolism	221	52	0.051	124	0.003
GO:0050875	cellular physiological process	302	65	0.156	159	0.016
GO:0045184	establishment of protein localization	25	1	0.038	9	0.214
GO:0042592	homeostasis	2	2	0.040	1	1.000
GO:0051325	interphase	4	4	0.002	5	0.117
GO:0009057	macromolecule catabolism	7	1	1.000	9	0.033
GO:0008152	metabolism	234	53	0.114	128	0.011
GO:0000278	mitotic cell cycle	12	6	0.018	9	0.084
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	97	27	0.028	59	0.010
GO:0050874	organismal physiological process	53	6	0.098	15	0.001
GO:0007582	physiological process	330	67	0.850	170	0.048
GO:0031325	positive regulation of cellular metabolism	4	4	0.002	4	0.059
GO:0051242	positive regulation of cellular physiological process	13	6	0.028	7	0.785
GO:0009893	positive regulation of metabolism	4	4	0.002	4	0.059
GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid...	3	4	0.006	4	0.209
GO:0043119	positive regulation of physiological process	13	6	0.028	7	0.785
GO:0045941	positive regulation of transcription	3	4	0.006	4	0.209
GO:0050789	regulation of biological process	117	37	0.000	66	0.076
GO:0031323	regulation of cellular metabolism	69	23	0.004	42	0.046
GO:0051244	regulation of cellular physiological process	97	33	0.000	61	0.002
GO:0050794	regulation of cellular process	110	34	0.001	62	0.091
GO:0019222	regulation of metabolism	72	23	0.008	42	0.116
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	65	22	0.004	38	0.134
GO:0050791	regulation of physiological process	98	35	0.000	62	0.002
GO:0009966	regulation of signal transduction	15	1	0.322	2	0.006
GO:0045449	regulation of transcription	64	22	0.003	38	0.100
GO:0006355	regulation of transcription, DNA-dependent	61	22	0.001	37	0.069
GO:0050896	response to stimulus	70	11	0.408	22	0.001
GO:0016070	RNA metabolism	8	2	0.663	12	0.040
GO:0046903	secretion	11	2	1.000	9	0.034
GO:0006350	transcription	69	23	0.004	39	0.231
GO:0006366	transcription from RNA polymerase II promoter	19	9	0.005	10	0.818
GO:0006351	transcription, DNA-dependent	64	23	0.001	38	0.100
GO:0006810	transport	71	8	0.048	33	0.601

4.5. 3.6 Western blot analysis

All results concerning Western analysis are derived from a preliminary screening for proteins associated with UVC and/or cadmium exposure, and must be recognised as such. Consequently there are a limited number of parallels to collaborate the results. Protein levels of the house-keeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a loading control, when possible (Data not shown).

Coomassie Blue staining confirmed that the protein concentration in the samples was relatively uniform, and that the proteins were intact (Figure 4.13). Degraded protein would have produced a continuous smear running down each well in stead of bands.

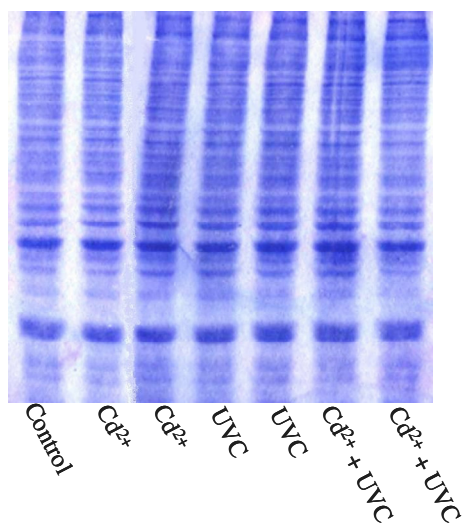


Figure 4.13 The protein bands are well defined suggesting that protein integrity is good. Concentrations appear to be of similar strength in the samples.

4.5.1. Proteins associated with DNA damage repair

Both XPC and UV-DDB are needed for DNA damage recognition in global genome repair of the NER pathway. Our results showed a lower expression of XPC in cells exposed to UVC, indifferent of cadmium pre-treatment. As for DDB2 (subunit of UV-DDB); there seemed to be a slightly higher induction in the sample from cadmium treated cells compared to the other (Figure 4.14 a-b). XPA and ERCC1 play crucial roles in confirming correct assembly of the NER complex, and incising the DNA damage. There was a slightly higher induction of XPA in cells exposed to UVC, ignoring cadmium pre-treatment, whereas the same treatments showed a reduction in ERCC1 (Figure 4.14 c-d). Proliferating Cell Nuclear

Antigen (PCNA), also called polymerase delta auxiliary protein (because of its function as co-factor in polymerase δ), is involved in replication and in both NER and BER. We found that PCNA was strongly and evenly expressed in all the samples (Figure 4.14 e). PARP: Poly (ADP-ribosyl)ation is a radical means of post-translational modification of proteins in Eukaryotic cells, and is preformed by enzymes called poly(ADP-ribose) polymerases (PARPs). PARPs are involved in a great many biological processes, including DNA repair (especially BER) and maintenance of genomic stability, transcriptional regulation, centromere function and mitotic spindle formation, apoptosis and necrosis. In the occurrence of DNA strand breaks, PARP activity can increase over a 100 fold in cells (Burkle, 2005). Furthermore, PARP is cleaved during apoptosis giving rise to a protein (subunit) of 85 kD. Our results showed a clear expression of PARP in all of the treated cell groups as well as control. Moreover, both UVC-treated cells and Cd^{2+} + UVC-treated cells displayed the additional lower band which is often produced during apoptosis (Figure 4.14 f). Finally we assessed the expression of O6-Methylguanine-DNA Methyltransferase (MGMT). MGMT removes highly mutagenic lesions caused by alkylation of guanine at the O6-position. MGMT is called a “suicide protein”, because it transfers the methyl or other alkyl groups bound in the O6-position of guanine to a cysteine residue within its active site. The alkyl group is irreversibly bound to MGMT, leaving the protein functionally inactive (Srivenugopal *et al.*, 1996). The expression of MGMT seemed to be particularly low in the cells co-exposed to Cd^{2+} and UVC, compared to the other samples (Figure 4.14 g).

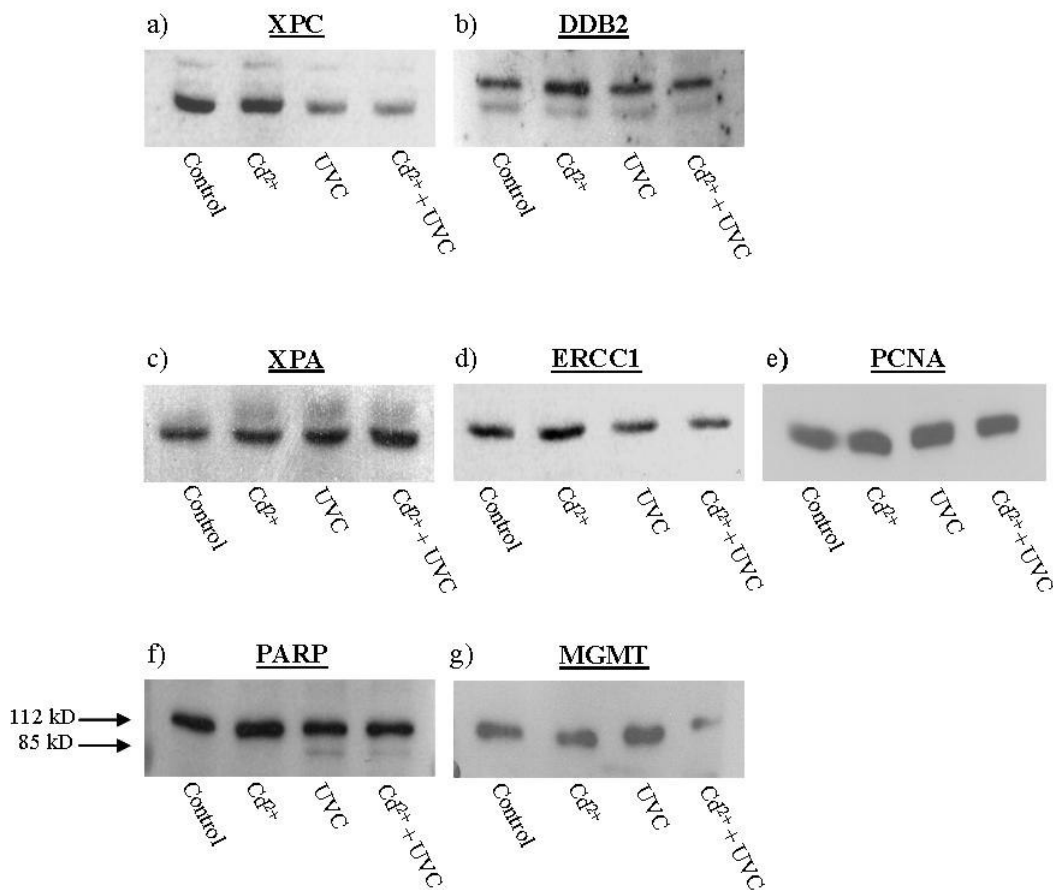


Figure 4.14 a-g. Proteins involved in DNA damage repair.

4.5.2. Proteins related to cell cycle control and apoptosis

The p53 protein functions as a master switch that coordinates and concentrates a plethora of stress signals and transforms them into a series of responses, such as arrest of cell growth, apoptosis or DNA repair (Soussi, 2005). Our results showed an induction of p53 protein levels in 833K cells exposed to UVC with or without cadmium pre-treatment. In contrast p53 phosphorylated at serine15 [phospho-p53(ser15)], was strongly induced and visible only in 833K cells pre-treated with cadmium, independent of UVC exposure (Figure 4.15 a-b). Cyclin Dependent Kinase 2 (Cdk2), when associated with cyclin E, is a mediator of cell transition from G1 to S phase through phosphorylation of a restricted number of proteins, including the tumour suppressor protein Retinoblastoma (Rb). Cdk2 also mediates cell transition from S to G2 phase, but then in association with cyclin A (Malumbres *et al.*, 2005). The active form of Cdk2 is phosphorylated, which makes the protein appear as a dual band in the gel (un-phosphorylated/phosphorylated). Our results showed reduced levels of Cdk2 in the cells treated with UVC, the lowest levels seen in co-exposed cells, but also a slight induction in the

cells treated to cadmium alone (Figure 4.15 c). Erk and p38 are both kinases that initiate Mitogen Activated Protein kinase (MAPK)-cascades vital to cell growth, differentiation, and apoptosis. Erk is generally activated by growth factors and initiates cascades required for cell survival and proliferation, whereas p38 is typically induced by genotoxic agents e.g. cadmium, and activates MAPK pathways involved in cell cycle arrest and apoptosis (Chao *et al.*, 2001;Lag *et al.*, 2005; Miguel *et al.*, 2005). Activation of signal mediators (i.e. Erk and p38) is often achieved by phosphorylation; we therefore measured the expression of phospho-Erk, p38 and phospho-p38. All three treatments increased the levels of phospho-Erk compared to control; the strongest induction seen in cells treated with cadmium (Figure 4.15 d). As for both p38 and phospho-p38 the induction levels displayed no differences between the samples (Figure 4.15 e-f).

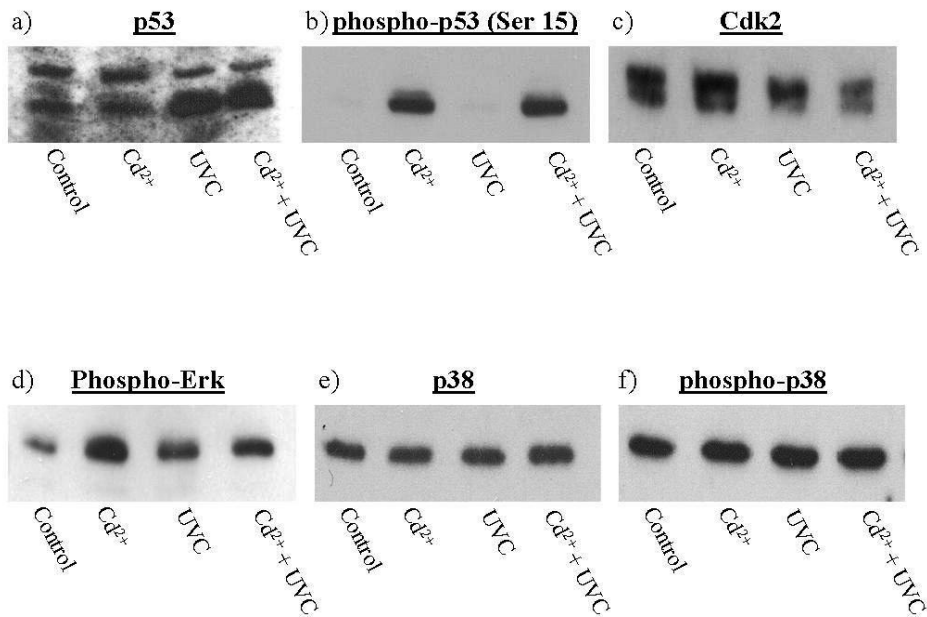


Figure 4.15 a-f. Regulators of cell cycle, growth and apoptosis.

5. Discussion

In this study we have combine traditional toxicological in vitro tests with modern microarray technology, in an attempt to assess the effects of co-exposures to harmful agents in biological systems. We chose cadmium and UVC as model agents because of their well documented genotoxic properties, and because they represent agents that may – based on their mechanism of action - exert synergistic toxic effects.

5.1. Cell culture

We have used the human TGCT cell line 833K derived from metastasised testicular tumour cells as a model cell line in our study. The 833K cells exhibit rapid growth and proliferation, and are known to have functional nucleotide excision repair. Considering that cancer cells generally have abnormal cellular functions, such as irregular transcription of genes leading to gain and/or loss of important protein functions, it could be argued that an untransformed cell line would have served as a better model. Normal cells would for instance display normal gene transcription, cell cycle progression and arrest, induction of apoptosis, etc. However, bearing in mind that the feasibility of the exploratory approach was more important than the results of exposure *per se*, we felt that the type of cell line used played a minor role.

5.2. Exposure of cells

Based on the viability of 833K cells using Propidium Iodide and Hoechst 33342, we determined the cadmium concentration and the UVC doses to be used in the DNA-repair assays and the gene-expression analysis. All treatments were performed in medium that contained both serum and phenol-red. It is possible that cadmium ions can bind to serum proteins in the medium and thereby reduce the accessibility of cadmium to the cells; however, in this study cadmium uptake by the cells was confirmed by analytical quantification. As for UVC irradiation, the medium with phenol-red slightly reduces the total dose due to some absorption (Brunborg, personal communication), but this “sun screen” effect is not large and the absolute dosimetry of UVC is not important. Our goal was not to measure the effects of a given cadmium concentration or UVC dose, but to evaluate their combined effects.

5.3. No differences were observed in cell-cycle progression

Flow cytometry showed no consistent differences between treatment groups indicative of cell cycle arrest in G1, S or G2 phase. However, our flow data is limited and consists of results from different experiments and separate passages of cells. Cell cycle arrest at any of the cell cycle checkpoints could very well be masked by normal variation between cultures at different cell passages. In a study of chick embryos, cadmium treatment did not attenuate cell growth and proliferation (Thompson *et al.*, 2005). On the other hand, a study performed on Chinese hamster ovary cells (CHO K1), knocked out for metallothionein (MT) and thus very sensitive to cadmium, demonstrated cell cycle arrest in response to low concentrations of cadmium (Yang *et al.*, 2004). TGCT cells exhibit genetic abnormalities which stimulate cell transition through the G1 phase of the cell cycle (reviewed by von Eyben, 2004). Consequently, cell cycle arrest in G1 may be expected to be difficult to observe in 833K cells. With respect to UVC (ignoring cadmium pre-treatment), we do believe that some of the cell cultures, especially the ones treated with the highest doses (3, 5 and 10 J/m²) were detained in their cell cycle progress. In fact, the PARP-cleavage seen in UVC (3 J/m²) treated samples (Figure 4.13f) is an indication of S-phase arrest and early apoptosis. However, due to the limited incubation after UVC exposure, differences in cell-cycle progress between the treatments are just not seen. In conclusion, further testing is needed to establish the cell-cycle profiles elicited by the different treatments used in our study.

5.4. DNA repair assays

As previously described, UVC-induced DNA lesions are mainly handled by NER. Following exposure to various doses of UVC, 833K cells were incubated in the presence of RI and assayed for DNA single strand breaks using alkaline elution. Only the efficiency of the initial steps (damage recognition, incision and excision), of NER is assayed with this approach. Alternatively, we combined alkaline elution with the enzyme T4-pdg treatment, which specifically cleaves at UV induced lesions (Lloyd, 2005).

5.4.1. Use of inhibitors revealed no apparent effects of cadmium on 833K repair efficiency

The use of inhibitors revealed no significant difference in incision of DNA lesions in 833K cells pre-treated with cadmium compared to untreated cells. There was however a great deal of variation within the treatment groups, indicating unknown variations in experimental conditions. Both of the inhibitors used are highly toxic anti-cancer drugs which can inflict additional harm, possibly obscuring potential differences between cadmium treated and

untreated cells. Furthermore, the cells were only incubated for 1 hour after UVC exposure, which may be too short to establish a difference between the samples by measuring SSBs. Finally, a higher number of both sample and experimental replicates would have been preferable.

5.4.2. Alkaline elution combined with enzyme treatment showed that cadmium inhibits NER

When using the T4-pdg enzyme, we did find cadmium to significantly attenuate DNA repair of UVC induced lesions. Our findings coincide with the results of a similar study (Fatur *et al.*, 2003), which demonstrated effects of cadmium on NER of UVC lesions in CHO cells. Moreover, their findings implied that both the initial and the later steps of NER were affected by cadmium. This was based on a slow formation of DNA strand breaks in cadmium treated cells, and that the level of strand breaks did not decline after a 1 h recovery period. As mentioned in the introduction, studies have shown cadmium to inhibit the NER protein XPA, which plays an essential, multifunctional role in NER (Asmuss *et al.*, 2000b); reviewed in (Hartwig *et al.*, 2002b). In addition to binding damaged DNA, XPA recruits and interacts with other NER proteins such as ERCC1, TFIIH, RPA, and XPC. It is proposed by Hartwig and co-workers that the inhibitory effects of cadmium on XPA are due to displacement of zinc in the protein's minimal DNA binding domain (MBD), resulting in conformational changes that disrupt DNA binding properties. However, it has also been shown that displacement of zinc with cadmium in XPA, only leads to negligible conformational changes of the protein (Buchko *et al.*, 2000). The latter authors proposed that the effects of cadmium on XPA must be attributed to various modes of action. For instance, cadmium might bind to negatively charged surface areas on XPA, thereby interrupting binding to DNA, or the binding of cadmium to DNA itself could be the cause (Buchko *et al.*, 2000). In general, moderate changes in the amount of a protein are often sufficient to cause serious biological effects. Cadmium inhibition of DNA repair is undoubtedly complex, and is most likely a consequence of several effects.

5.5. Western blot analysis

The mechanisms of cadmium-induced genotoxicity are diverse and several cellular functions, are affected some of which were described in the introduction. With this in mind, the induction levels of some proteins involved in DNA repair, cell cycle and apoptosis were studied.

833K cells are cancer cells, and thus their response to treatments might deviate from those of normal cells. Furthermore, protein turnover is variable; some proteins might be peaking while others are being degraded at a given time after exposure. The time at which the protein-induction levels are measured may very well affect the results. The protein samples used in this study were all extracted after 24 hours of incubation (including those treated with UVC at 20 hours and then further incubated for the remaining 4 hours).

5.5.1. UVC treatment (but not cadmium) reduced the protein levels of XPC, whereas DDB2 was not affected.

XPC and DDB2 (UV-DDB) are two of the proteins needed for initial recognition of DNA damage in the GGR pathway of NER. GGR removes the bulk of lesions in the overall genome. We therefore expected to find higher induction levels for both XPC and DDB2 in the samples treated with UVC, with or without cadmium pre-treatment. However, the levels of XPC were lower in the UVC treated samples (regardless of cadmium) compared to unexposed controls. The levels of DDB2, on the other hand, were unchanged after UVC, but showed a minor induction in cells treated with cadmium alone.

5.5.2. P53 is involved in DNA repair and is stabilised by low levels of cadmium

A study by Adimoolam (Adimoolam *et al.*, 2002) on human colorectal cancer cells (HCT116) and human fibroblasts (WI38), demonstrated that functional p53 protein is required for proficient induction of XPC in response to UVC irradiation, and that p53 regulates the basal expression of XPC. (Hwang *et al.*, 1999) demonstrated that the gene *p48* which codes for UV-DDB also requires p53 for proper induction in response to UVC. Interestingly, we found that the levels of p53 were high in our UVC-exposed cells, and thus we expected these samples to show induced levels of XPC and DDB2 as well. Other studies have shown cadmium disrupts proper DNA binding ability of p53 in human breast cancer cells (MCF7) (Meplan *et al.*, 1999). Similar to XPA, inactivation of p53 by cadmium is believed to be a result of zinc displacement by cadmium in the protein, causing conformational changes. However the effects of cadmium are diverse also on p53 and its mode of action is likely to be dependent on concentration. Micromolar concentrations of cadmium are typically thought to activate p53 proteins (by both transcription and stabilisation of p53) via oxidative stress induction, while higher doses inactivate p53 activity. We found that phospho-p53 was strongly induced by cadmium independent of UVC irradiation, which supports this hypothesis. One way in which cadmium can inactivate a p53 response in cells is by inhibiting

p53 translocation to the nucleus (Godon *et al.*, 2005). This inhibition is also believed to be brought about by conformational changes in p53 due to the displacement of zinc, leading to reduced transcription of p53 responsive genes. However, our data are still too limited to decide whether such a mechanism can explain the low levels of XPC and DDB2. Further measurements of protein levels, at different times after exposure, should be useful.

Protein induction levels of XPA and ERCC1 displayed minor differences between treatment groups and the results are too limited for conclusions about their role in cadmium and UVC treated cells. With respect to PCNA, which is involved in NER and BER, it was evenly distributed in all samples, confirming that this protein is constitutively expressed; its presence is associated with its role as a co-factor in polymerase δ required for chromosomal DNA replication (Majka *et al.*, 2004).

5.5.3. UVC induced PARP cleavage

The enzymes called Poly ADP-ribose polymerases, or PARPs, are zinc dependent enzymes involved in several cellular functions including DNA repair and apoptosis (Burkle, 2005). PARP was evenly represented in all our samples. Both UVC and cadmium are shown to induce PARP cleavage in apoptosis (Oh *et al.*, 2004; Nocentini, 2003), but we observed this induction after UVC whereas cadmium had no such effect. Correspondingly, our viability tests showed a ~ 15 % decline in viability of 833K cells in response to 3 J/m² UVC.

5.5.4. Cadmium induction of ERK and Cdk2 is counteracted by UVC

It was mentioned in the introduction that cadmium has been shown to induce the MAP-kinases; ERK1, ERK2, and p38 (Chao *et al.*, 2001; Chuang *et al.*, 2000; Lag *et al.*, 2005). ERK is typically associated with cell survival and proliferation, whereas p38 is a promoter of apoptosis. Induced levels of these proteins may, however, vary in response to different cadmium concentrations, as can the effects of the kinases, depending on cell type, duration of exposure etc. Our results showed that phospho-ERK, which is the activated form of the kinase, was induced in all treated samples compared to control. The levels were high in cells treated with cadmium alone, in correspondence with the observations (Chao *et al.*, 2001; Chuang *et al.*, 2000; Lag *et al.*, 2005). The protein levels in co-exposed cells were lower compared to those seen for cadmium alone, but slightly higher than in cells exposed to UVC alone. The levels seen in co-exposed cells suggest that UVC counteracts the effects of cadmium induction of phospho-ERK. UVC treatment of cells pre-incubated with cadmium could for instance lead to dephosphorylation of active ERKs. As previously described, Cdk2

is involved in cell transition through G1/S and S/G2 of the cell cycle. We found that cadmium treated cells displayed the highest levels of Cdk2, while UVC and especially co-exposed cells had the lowest levels. The apparent induction by cadmium seems therefore to be counteracted by UVC similarly to what was seen for phospho-ERK. UVC has been shown to reduce Cdk2 levels in Chinese hamster ovary cells (CHO k1), as a prelude to apoptosis (Liao *et al.*, 2005). In the case of p38 and phospho-p38 we found that all samples, including controls, displayed uniform protein levels of both forms.

Finally, we measured the levels of the “suicide protein” MGMT. The co-exposed cells displayed a reduction in MGMT protein levels, compared to the other samples. We did not expect to find any increased induction of MGMT due to the fact that neither UVC nor cadmium is an alkylating agent.

We once more emphasise that these results are preliminary and do not justify further conclusions to be drawn.

5.6. Microarray analysis

In order to conduct a successful microarray analysis it is vital that the isolated total RNA is pure and of good quality. The RNA used in our study fulfilled these criteria, as shown in Results. Microarrays are cost effective in the sense that the output of biological information is exceptionally high, but each experiment is expensive. This has limited the number of arrays that could be used in the present study. Out of twelve microarrays, one complete set of parallels (i.e. four arrays) was discarded due to poor hybridisation. As a result we ended up with three merged biological replicates hybridised onto eight arrays comprising two (instead of three (or preferable more) technical parallels for each treatment and control. After scanning the arrays, the raw data was processed through the steps as previously described. The resulting normalised data (K-means and Hierarchical clusters, and the gene functional-category table) show the trends in gene expression for each of the cell treatments. The K-mean clusters for instance are visually very informative and depict appreciable differences in gene-expression profiles between the treatment groups. The next step in processing the data would be to conduct more detailed investigations of the genes represented in the various categories depicted in Table 4.3. Relevant genes are those involved in regulation of cell cycle, DNA repair, transcription, signal transduction, apoptosis etc. Such investigations might help to further elucidate the genes involved in cadmium co-genotoxicity. At first such a task might

seem daunting, considering that even our relatively modest results comprise over 600 differentially expressed genes in response to the treatments.

5.6.1. Large toxicogenomic databases may be used in future toxicological evaluation of new compounds

Microarray data such as those generated by us for cadmium treated 833K cells may form part of large databases, accessible for anyone assessing the properties of new chemicals with potential use by consumers, in industry or in medicine. Large toxicogenomic databases can provide information on biological interactions by comparing expression patterns in new chemicals with those already present for existing chemicals. In this way, suspected environmental pollutants could be compared and classified. Toxicogenomics still represent a relatively young discipline; improvements, new products and databases in this field are rapidly being developed. While microarrays can hardly replace traditional investigative methods in the near future, they are already of great importance for the toxicologists. Comparing microarray results across laboratories still represents a great challenge. Universal standardisation of experimental procedures, data processing, gene annotation etc comprises major obstacles that must be resolved before the full potential of toxicogenomics can be put into use.

In addition to reduced costs and more rapid evaluation, the development of this field is driven by a strong pressure to reduce the use of animals in chemicals testing. Animal experiments are the cause of much controversy, especially among the public, but increasingly also within the scientific community. Good alternative methods are therefore being developed, but again extensive validation is needed. A further aspect is the risk always involved when extrapolating toxicological effects found in animals to humans. Microarrays provide a means of studying transcriptional responses elicited by a chemical or other treatment, in both human and animals.

6. Concluding remarks

The aim of our study was to elucidate the complex nature of co-exposures by integrating traditional tests with toxicogenomics, i.e. microarray technology. Cadmium and UVC have served as model agents, and their separate and combined effects have been investigated with emphasis on DNA-damage repair, and gene expression profiles. We found that pre-incubation of 833K cells to a low cytotoxic level of cadmium significantly altered the cells' response to ultraviolet radiation. Appreciable differences in DNA damage using alkaline elution were

observed between treatments, and several protein activities were altered as measured with Western analysis although these are preliminary data. The results from flow cytometry were inconsistent and may also be considered as preliminary. The microarray data suggest global changes in gene expression in the treated cells. These data should however be further analysed using other methods e.g. real-time PCR, but this has not been feasible within the scope of this thesis

Based on the collective results of our investigations, we consider this approach as especially useful when studying effects of co-exposures to environmental toxicants.

7. Future studies

In this thesis we used UVC irradiation as a model for genotoxic agents producing DNA lesions that are repaired via NER. An extension of this work would be to explore combined effects of cadmium with other known substances in stead of UVC, e.g. the highly carcinogenic benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE). Furthermore, other heavy metals interacting with repair enzymes or other proteins should be studied. The analysis of the microarray data was in this study limited to the identification of gene classes; a continuation of this analysis could be to identify single genes and their role in specific pathways.

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9. Appendix A

9.1. Materials

9.1.1. Chemicals, antibodies and commercial kits

<u>Product</u>	<u>Manufacturer</u>
β -Mercaptoethanol	Sigma
Acetic acid	Merck
Acrylamide/bis solution (30 %)	BioRad
Ammonium persulphate (APS)	BioRad
Anti-goat HRP-conjugate	Zymed
Anti-mouse HRP-conjugate	Jackson Laboratories
Anti-rabbit HRP- conjugate	Jackson Laboratories
Cytosine Arabinoside	Sigma
Bio-Rad CD Protein Assay Kit	BioRad
Bovine Serum Albumin (BSA)	Sigma
Bromophenol Blue	Sigma
Cdk2 (rabbit)	Santa Cruz
Citric acid monohydrate	Jungbunzlauer Ges. m.b.h
Complete mini	Roche
Coomassie [®] Brilliant Blue	BioRad
DDB2 (Goat)	Santa Cruz
Developer (LX 24)	Kodak
diSodium hydrogen phosphate-dihydrate	Merck
Dithiothreitol	Sigma
Dry milk (Blotting Grade Blocker)	BioRad
EDTA	Sigma
Na ₂ EDTA•2H ₂ O	Sigma
ERCC1 (Mouse)	Neomarkers
Ethanol (Absolute)	Arcus
Fairplay Microarray Labeling Kit	Stratagene
Fixer (AL 4)	Kodak
GAPDH (mouse)	Biogenesis
GenElute [™] Total RNA Kit	Sigma
Glycerol	Sigma
Hoechst 33258	Calbiochem
Hoechst 33342	Calbiochem
Hydrochloric acid	Merck
Hydroxyurea	Sigma
Medical X-ray film, Super RX	FUJI

Methanol	Merck
MGMT (mouse)	Neomarkers
n-Butanol	Merck
Nitro-cellulose membrane	BioRad
p38 (rabbit)	Cell Signalling tech
p53 (rabbit)	Cell Signalling tech
PARP (mouse)	Clontech
PBS	Dulbecco
PCNA (mouse)	Abcam
Penicillin/Streptomycin (P/S)	Cambrex
phospho-Erk (Thr 202, Tyr 204) (mouse)	Cell Signalling tech.
phospho-p38 (Thr 180, Tyr 204) (rabbit)	Cell Signalling tech.
phospho-p53 (Ser 15) (rabbit)	Cell Signalling tech.
Pierce Supersignal West Dura Extended Duration substrate	PerBio
Potassium-dihydrogen phosphate-dihydrate	Merck
Potassium-dihydrogen phosphate	Merck
Potassium Hydroxide	Merck
Precision Plus Protein™ Standards, Kaleidoscope	BioRad
Propidium Iodide	Sigma
Proteinase K	Merck
RNA 6000 Nano LabChip Assay	Agilent Technologies
RPMI-medium 1640 with HEPES and L-glutamine	Bio Whittaker
Sodium Chloride	Merck
Sodium dodecyl sulphate (SDS)	KEBO Lab
Sodium dodecyl sulphate (10 %)	Sigma
Sodium Hydroxide	Merck
Temed	BioRad
Thimerosal	Sigma
Trizma®base	Sigma
Trizma®Hydrochloride, Tris-HCl	Sigma
Trypan blue	Biowhittaker
Trypsin-EDTA	Sigma
Tween 20	BioRad
XPA (mouse)	Santa Cruz
XPC (mouse)	Abcam

9.2. Solutions

9.2.1. DNA alkaline elution

10X Merchant buffer

1000 ml:

81.8 g NaCl

2.0 g KH_2PO_4

2.0 g KCl

14.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

Distilled water up to 1000 ml

1X Merchant buffer (1.4 M NaCl, 15 mM KH_2PO_4 , 27 mM KCl, 81mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)

2000 ml:

200 ml 10X Merchant buffer

7.44 g Na_2EDTA

Distilled water up to 2000 ml

pH was adjusted to 7.4 with 4 M NaOH.

20 mM Na_2EDTA

1000 ml:

7.44 g Na_2EDTA

Distilled water up to 1000 ml

pH was adjusted to 9.6 with 10 M NaOH.

BE1 buffer

1000 ml:

1 mM Na_2EDTA (0.37 g)

100 mM KCl (7.46 g)

20 mM Tris-HCl (50 ml 1 M Tris-HCl)

Distilled water up to 1000 ml

pH was adjusted to 7.5 with 5 M KOH, and the solution was boiled to reduce air bubbles.

BE1 buffer with 0.1 mg/ml BSA

0.5 ml BSA stock (40 mg/ml)

200 ml BE1 buffer

50 $\mu\text{g/ml}$ T4-pdg

1 ml T4-pdg stock (6 mg/ml)

120 ml BE1 buffer with 0.1 mg/ml BSA

10X Elution buffer

1000 ml:

74.4 g Na₂EDTA

14 g NaOH

Distilled water up to 1000 ml

1X Elution buffer (20 mM Na₂EDTA)

4000 ml:

400 ml 10X Elution buffer

3600 ml Distilled water

pH was adjusted to 12.5 or 12.25 (If DNA was treated with T4-pdg), with 10 M NaOH.

Lysis solution

1000 ml:

25 mM Na₂EDTA (9.3 g)

100 mM Glycine (7.5 g)

1 % SDS (20 g)

Distilled water up to 1000 ml

pH was adjusted to 9.6 with 10 M NaOH, and the solution was boiled to reduce air bubbles.

9.2.2. Microarray analysis

Total RNA isolation

All reagents provided in the GenElute™ Total RNA Kit.

RNA Integrity control (Bioanalyzer)

All reagents provided in the RNA 6000 Nano LabChip Assay Kit.

cDNA generation and Dye-coupling reaction

All reagents provided in the Fairplay Microarray Labeling Kit.

Microarray Pre-hybridisation solution

0.1 % Bovine Serum Albumin (BSA) Fraction V (100 mg)

5X SSC (25 ml 20X SSC: 3M NaCl, 0.3 M sodium citrate, pH 7.0)

0.1 % SDS (1 ml 10 % SDS)

Sterile water (~ 74 ml)

First the BSA Fraction V was dissolved in 70 ml sterile water by gentle stirring, and then 20X SSC and SDS were added. Sterile water was added so that the final volume was 100 ml.

Manuel hybridisation solution

Labelled cDNA (~90 µl)

50 % of total volume 2X SDS buffer (130 µl)

0.1 mg/ml sonicated herring sperm (2.6 μ l of 10 mg/ml stock)
RNase free water (~40 μ l)
Total volume of 260 μ l per hybridization.

Post hybridisation solutions

All solutions were sterile filtered.

Wash 1

2X SSC (100 ml 20X SSC)
0.1 % SDS (10 ml 10 % SDS)
5 mM DTT (0.1 g)
Sterile water (898 ml)

Wash 2

0.1X SSC (5 ml 20X SSC)
0.1 % SDS (10 ml 10 % SDS)
Sterile water (985ml)

Wash 3

0.1X SSC (5 ml 20X SSC)
Sterile water (995 ml)

9.2.3. Western blot analysis

1X Lysis solution (for preparation of protein extract)

60 mM Tris-HCL (50 ml 0.5 M Tris-HCL pH 6.8)
10 % glycerol (40 ml 100 % glyserol)
2 % SDS (80 ml 10 % SDS)
1 mM EDTA (4 ml 0.1 M EDTA)
pH is adjusted to 6.8 with NaOH and dH₂O is added up to 400ml.
Protease inhibitor, Complete mini (14X): 70 μ l/ml lysis solution.
Phosphatase inhibitors:
Sodium fluoride (NaF) (50 mM stock): 50 μ l/ml lysis solution
B-glycerolphosphate (Stock 100X): 10 μ l/ ml lysis solution,
Na₃VO₄ (stock 200mM): 5 μ l/ml lysis solution.

Bio-Rad DC Protein Assay

Reagent A, an alkaline copper tartrate solution
Reagent B, a dilute Folin Reagent
Reagent S, (SDS)
A' = 1 ml solution A + 20 μ l S.

All reagents were provided in the kit.

10X Electrophoresis buffer

2000 ml:

60g TRIS Base (Tris-hydroxymethyl-aminomethane)

88g Glycine

20g SDS (Sodium Dodecyl Sulphate)

Distilled water up to 2000 ml

1X Electrophoresis buffer

1000 ml:

100 ml of 10X Electrophoresis buffer

900 ml distilled water

10X Transfer buffer

2000 ml:

60 g, TRIS base

288 g Glycine

Distilled water up to 2000 ml

1X Transfer buffer

1000 ml:

100 ml of 10X Transfer buffer

700 ml distilled water

200 ml methanol

The methanol was added just before use to avoid evaporation.

10X TBS-T (Tris buffer with Tween 20) – wash buffer

5000 ml:

10 mM Trizma base (60,5g)

137 mM NaCl (400g)

0.1% Tween 20 (50ml)

350 ml of 1M HCl

Distilled water was added up to 5000 ml and pH adjusted to 7.6. The solution was then diluted 1 : 10 before use.

Tween makes the removal of excess antibodies more efficient and reduces unspecified binding to the membrane. The risk is however that some proteins and peptides may be lost, in which case one should only wash with TBS (especially when measuring small proteins or peptides). The membranes should be stored in TBS not TBST.

10X TBS (Tris buffer)

5000 ml:

10 mM Trizma base (60,5g)

137 mM NaCl (400g)

350 ml of 1M HCl

Distilled water was added up to 5000 ml and pH adjusted to 7.6. The solution was then diluted 1 : 10 before use.

Acrylamide separating and stacking gel

For optimal separation of proteins the solutions should be de-gassed under vacuum.

Separating gel	6 %	10 %	12 %
Distilled water	3.95 ml	2.95 ml	2.45 ml
Acrylamide	1,5 ml	2.5 ml	3.0 ml
Buffer, pH 8,8	1.9 ml	1.9 ml	1.9 ml
10 % SDS	75 µl	75 µl	75 µl
APS	75 µl	75 µl	75 µl
TEMED	6 µl	3 µl	3 µl

Stacking gel	4 %
Distilled water	2.03 ml
Acrylamide	425 µl
Buffer, pH 6,8	825 µl
10 % SDS	33.5 µl
APS	17 µl
TEMED	3.5µl

Coomassie Brilliant Blue staining solution

0.1 % Coomassie Brilliant Blue

50 % Methanol

10 % Acetic acid

0.1 g of Coomassie Brilliant Blue G was dissolved in 500 ml of methanol. 100 ml of acetic acid was added to the solution, and the volume was adjusted to 1000 ml with distilled water.

Destain solution I

40 % Methanol

7 % Acetic acid

400 ml methanol was mixed with 70 ml acetic acid and 530 ml distilled water.

Destain solution II

5 % Methanol

7 % Acetic acid

50 ml methanol was mixed with 70 ml acetic acid and 880 ml distilled water.